Preparation and Properties of Chromopeptides from the P_{fr} Form of Phytochrome

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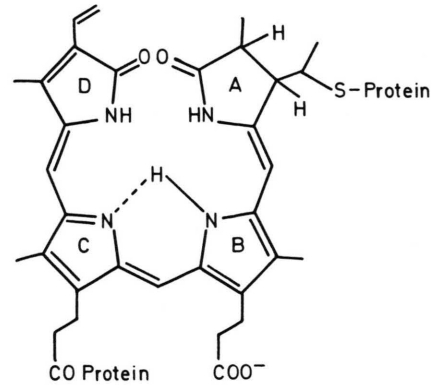
Dedicated to Professor Dr. H. H. Inhoffen *on the Occasion of His 75th Birthday*

Avena sativa L., Phytochrome, Pepsin Digestion, Phytochromobilin Peptide, P_{fr} Peptide

Chromopeptides were prepared from the P_{fr} form of phytochrome by pepsin digestion. After separation from colorless peptides and P_r peptides by column chromatography, spectral characteristics of this P_{fr} peptide were determined ($\lambda^1_{max} = 610$ nm, $\lambda^2_{max} = 380$ nm in acid methanol). Irradiation of P_{fr} peptide produces P_r peptide without liberation of a detectable compound. The P_{fr} peptide is more sensitive to oxidation and reduction than the P_r peptide. Oxidation with iodine and reduction with dithionite leads to partial chemoconversion of the P_{fr} peptide to the P_r peptide. The results favor the model of *cis-trans* isomerization for the $P_r \rightleftharpoons P_{fr}$ transformation.

Introduction

The preparation of chromopeptides from phytochrome in the P_r from proved to be useful for the elucidation of the structure of the peptide-bound P_r chromophore (**1**) via NMR spectroscopy [1] and via isolation of free phytochromobilin (**2**) [2]. The method of chromopeptide preparation was in both cases pepsin digestion of phytochrome at pH 1.5–2.0 according to the procedure of Fry and Mumford [3]. These authors had stated that "no difference in yield or composition of chromopeptide obtained was observed using phytochrome in the P_{fr} form as starting material (instead of the P_r form) indicating the photo-state of the original phytochrome is not a factor in the isolation." Red irradiation of the chromopeptide itself produced no change in the spectrum indicating that the product was not photochromic. The chromopeptide obtained from P_r has the same spectral properties (absorption, circular dichroism) as denaturated P_r [4]. Grombein *et al.* [5] had shown that acid-denaturated P_{fr} was spectroscopically different from acid-denaturated P_r presumed the reaction was carried out in the dark. Irradiation of denaturated P_{fr} irreversibly yielded a product with the absorption properties of denaturated P_r. We considered the possibility that Fry and Mumford [3] had not rigorously excluded light during their chromopeptide preparation from P_{fr}. We therefore carried out pepsin digestion of P_{fr} in the dark and obtained indeed a photo-sensitive chromopeptide. We describe here preparation and some properties of this P_{fr} peptide which give some information about the chemical structure of the P_{fr} chromophore.



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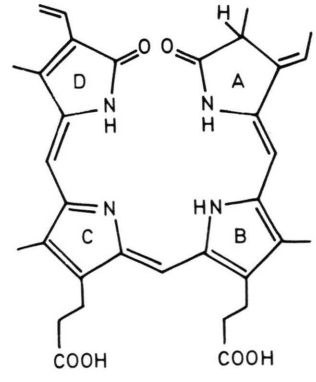

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Materials and Methods

Small phytochrome (60000 daltons) was isolated from 3.5 day old etiolated oat seedlings as previously described [2]. All procedures of the isolation were carried out either in the dark or under dim-green safety-light to keep the phytochrome in the P_r form. The final solution of P_r (purity index $A_{665}:A_{280} = 1:10$) in 10 mM Tris-HCl, pH 7.4, containing 10 mM KCl, 1 mM EDTA, and 1 mM NaN_3, was irradiated for 1 min at 660 nm with a fluence rate of 63 W m^{-2} (Leitz Prado projector and interference filter 660.3 nm, half width 12.8 nm, Searom, Argenteuil, France). This saturating irradiation resulted in about 80% P_{fr} [6]. The P_{fr} solution was acidified with formic acid to a final concentration of 5% and ascorbic acid to a final conc. of 50 mM was added (pH about 1.8). The clear solution which contained the P_{fr} chromophore cation and the denaturated protein [5] was stable in the dark at 4 °C for more than 1 day (dark reversion to the P_r cation: within 24 h 14%, within 62 h 24% of the original P_{fr}). At 37 °C, only 5% dark reversion to denatured P_r was found within 2 h. All subsequent procedures were carried out in the dark or under dim-green safety-light. Two mg lyophilized pepsin were added per 10 mg total protein. The solution was then incubated for 2 h at 37 °C (water bath). This resulted in complete digestion as monitored by complete extraction of the color (in form of chromopeptides) from the aqueous layer into *n*-butanol/0.05% trichloroacetic acid.

A Biogel P-10 column (1.6 × 16 cm) was equilibrated with 0.1% aqueous formic acid. The digest (from 50–100 mg total protein = 5–10 mg phytochrome) was placed onto the column. The column was washed with 0.1% formic acid at a flow rate of 0.6 ml min^{-1} until most of the colorless peptides were removed (control by absorption at 280 nm, see Fig. 2). The Biogel column was then connected with a silica gel column (1.6 × 5 cm) so that the eluate of the former was directly placed on top of the latter. The columns were then washed with 400 ml 0.1% formic acid at a flow rate of 2 ml min^{-1}. The P_{fr} peptides were eluted from the Biogel but strongly adsorbed at silica gel under these conditions. After complete elution of the P_{fr} peptide from Biogel (see Fig. 2), the columns were disconnected and the P_r peptide eluted from the Biogel column with 30% aqueous acetic acid. The P_{fr} peptide (adsorbed on top of the silica gel column) was washed with water to remove colorless peptides and then eluted either with 30% aqueous acetic acid or with methanol containing 0.1% trichloroacetic acid.

For detection of compounds which are eventually eliminated from the P_{fr} peptide by irradiation, the following experiment was performed. 45 mg phytochrome ($A_{665}:A_{280} = 1:12$) were irradiated at 660.3 nm and then incubated in 5% formic acid/50 mM ascorbic acid with 100 mg pepsin at 37 °C for 2 h. The P_{fr} chromopeptide was purified on Biogel P-10 with 0.1% aqueous formic acid. The fractions containing the P_{fr} peptide were combined and divided into two equal parts. Each part was placed onto a silica gel column (1.6 × 10 cm). The P_{fr} peptide was adsorbed at the top of each column in a sharp zone which appeared dark under dim-green safety-light. Both columns were washed with 500 ml 5% aqueous formic acid. One column was then irradiated with white light (slide projector) for 30 sec. The dark band of the P_{fr} peptide disappeared by this treatment, the P_r peptide is not visible under the safety-light. Both columns were eluted with 5% aqueous acetic acid (100 ml each). The eluates were lyophilized, and the residues dissolved in 400 µl methanol/acetic acid (3 : 1, v : v). Aliquots of 2 µl were applied to thin layer plates coated with silica gel, chromatograms were developed with water/acetic acid/*n*-butanol 1 : 1 : 4 (v : v : v) and stained with either ninhydrine, iodine, conc. sulfuric acid [7] or chlorine/tetramethylbenzidine [8]. The chromatograms of the eluates from both columns were similar, no significant differences could be detected.

Results and Discussion

Spectral investigations had previously demonstrated that denatured phytochrome (P_r form) and the P_r-chromopeptide have the same spectral properties [1, 4, 5]. For the coresponding investigation of phytochrome in the P_{fr} form, we compared the spectrum of an irradiated phytochrome sample after acidification with formic acid without and with pepsin (Fig. 1). We observed essentially the same shape of the long-wavelength absorption band of P_{fr} before and after proteolysis. The only difference is a small shift of the absorption band: the maximum of acid denaturated P_{fr} is found at 610–620 nm, that after short pepsin digestion at 600–610 nm (Fig. 1). This could be due to a residual chromophore-protein interaction in denatured P_{fr} which is destroyed by

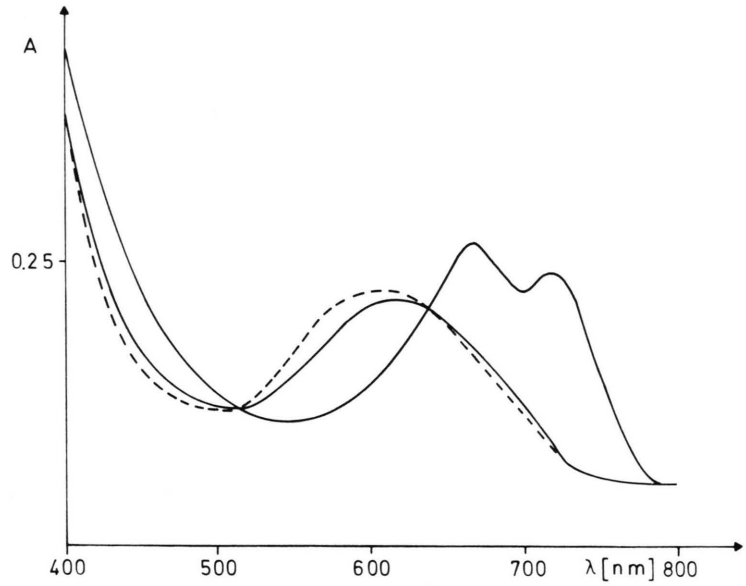


Fig. 1. Absorption spectra of native phytochrome after red irradiation (— double peak), the same in 5% aqueous formic acid (— one peak), the same after addition of pepsin in 5% aqueous formic acid (- - - -).

proteolysis. Photosensitivity is not destroyed by proteolysis: Irradiation produces the absorption of denatured P_r ($\lambda_{max} = 660$ nm) before and after proteolysis of the denatured P_{fr} solution. The product of proteolysis is as stable in the dark as denatured P_{fr}: dark reversion is very slow for both solutions (see Materials and Methods). This stability is the precondition for purification of the P_{fr}-peptide.

Lagarias and Rapoport [1] had purified P_r-peptides by adsorption on Biogel P-4 from 5% aqueous formic acid and elution with 25% acetic acid. In our hands, $\mathbf{P_r}$ peptides are adsorbed on Biogel P-4 or P-10 only if the concentration of formic acid was 1% or less. $\mathbf{P_{fr}}$ peptides are, however, eluted at these low formic acid concentrations. Migration of P_{fr} peptides is slower than that of colorless peptides. The best separation of P_{fr} peptides from colorless peptides is achieved with Biogel P-10 (Fig. 2). P_r-peptides can then be eluted with 30% aqueous acetic acid. The final purification is then achieved by chromatography on silica gel. The prepurified chromopeptides are adsorbed on top of the column as a sharp zone. Colorless peptides are eluted with water. The P_{fr} peptide can be eluted with 30% acetic acid, the P_r peptide with 50% acetic acid. Alternatively, both chromopeptides can be eluted with methanol containing 0.1% trichloroacetic acid. The products obtained by this procedure are presumably mixtures of chromopeptides which differ in the number of amino acids per chromophore as determined before with P_r peptides [1]. However, the chromophore is the same in all of these chromopeptides and, furthermore, the purity index ($A_{280} : A_{660}$) of our product is about the same as that of pure phytochromobilin-undecapeptide [1]. Therefore the product was used for spectral investigations of the chromophore without further purification.

The separation of P_{fr}-peptides from P_r-peptides is an important step for spectral investigations. Optimum irradiation of phytochrome yields about 80% P_{fr} and 20% P_r. Therefore proteolysis yields chromopeptides which consist of not more than 80% P_{fr} peptides and at least 20% P_r peptides. Because of the slow dark reversion of P_{fr} to P_r also in the denatured state or as peptide (see above), the percentage of P_r peptides is normally higher. Previous spectral investigations were only possible with this mixture. The separation described here enabled us for the first time to determine the absorption spectrum of the P_{fr}-chromophore (peptide-bound) free from any P_r-chromophore. The P_{fr}-chromophore has absorption maxima at about 610 and 380 nm (Fig. 3). The product is light-sensitive even in acid methanol. Irradiation leads to the known absorption spectrum of the P_r peptide with maxima at 660 and 380 nm (Fig. 3). The absorbance of the red band is about the same in P_{fr} peptide and P_r peptide whereas the blue band is about 20% more intense in the P_r peptide than in the P_{fr} peptide. The light-sensitivity of the P_{fr} peptide is also preserved in more apolar solvents like butanol

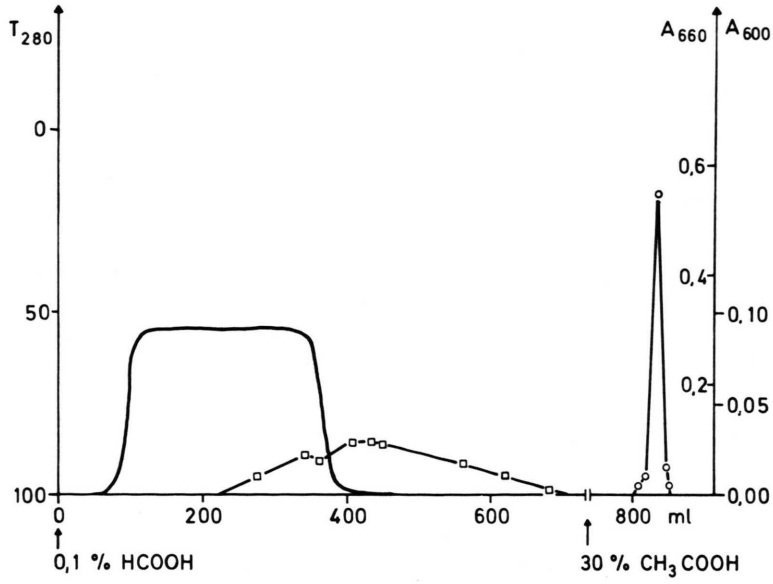


Fig. 2. Elution diagram of peptide separation on Biogel P-10. Scan for colorless peptides = transmission at 280 nm (—), for P_{fr} peptide = A_{600} (□-□-□), for P_r peptide = A_{660} (○-○-○).

as deduced from similar changes of the absorption spectrum after irradiation.

Previous spectral investigations of denatured P_{fr} (containing about 20% P_r) were restricted to the long wavelength band (λ^1_{max}) [5] whereas the position of the short wavelength band (λ^2_{max}) could not be determined with enough precision. Because λ^1_{max} was found for P_{fr} at 610–615 nm and for P_r at 675–680 nm it was concluded that the chromophoric system of P_{fr} is shorter than the P_r chromophoric system by the 4.5 double bond [5]. Two types of model compounds meet this requirement (review

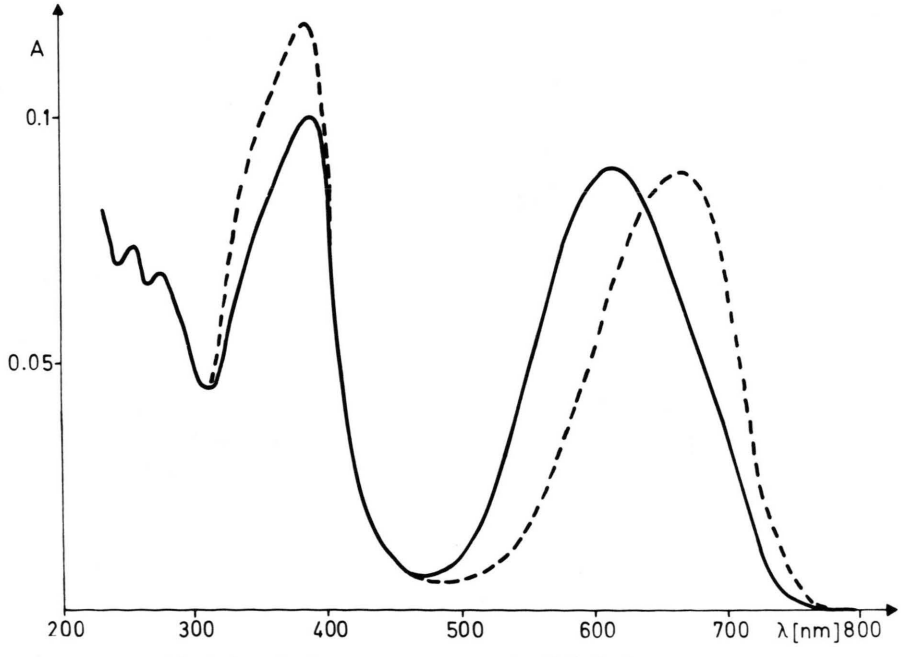
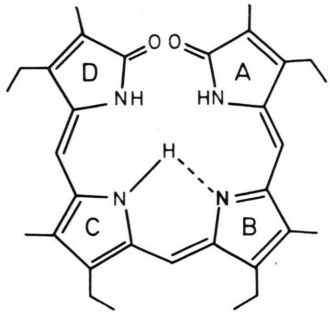
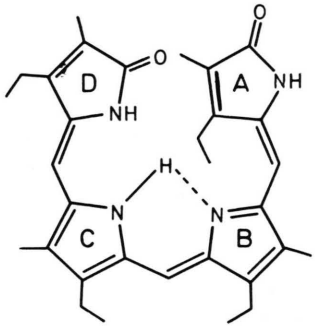


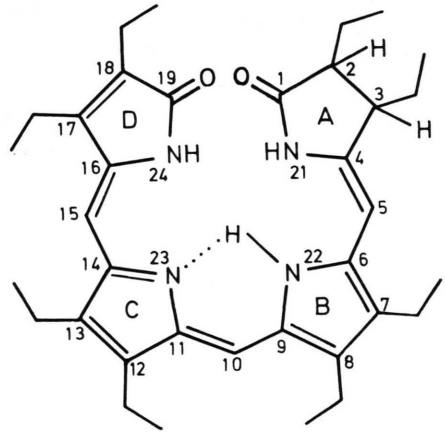
Fig. 3. Absorption spectra of purified P_{fr} peptide (—) and of the same solution after irradiation with white light for 10 s (- - -). Solvent: methanol/0.1% trichloroacetic acid.



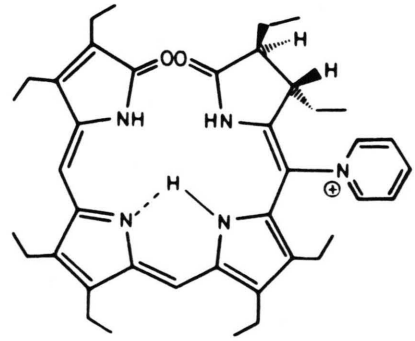
3a



3b



4a



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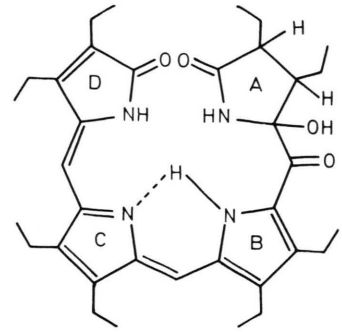
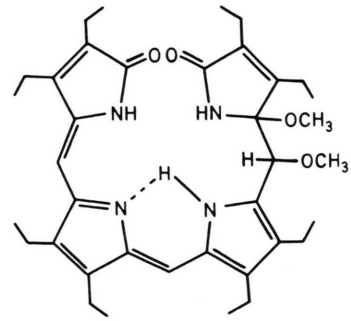


4c



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[9]), namely products of *Z-E*-isomerization at this double bond (*e.g.* **3b**) and products of photooxidation or photoaddition at this double bond (*e.g.* **4b**, **4c**, **5**). The purification of the P_{fr} peptide enabled us now to determine also the short wavelength band (λ^2_{max}) with precision. Comparison with model compounds (Table 1) revealed fundamental differences: λ^2_{max} of all model compounds obtained by photooxidation or photoaddition (**4b**, **4c**, **5**) is found at considerably shorter wavelengths (327–337 nm) than λ^2_{max} of the corresponding P_r model **4a** (351 nm). No spectral shift is, however, found between the P_{fr} peptide (λ^2_{max} = 380 nm) and the P_r peptide (λ^2_{max} = 380 nm). In the meantime, λ^2_{max} of denaturated P_{fr} was also found at 380 nm, the same position as λ^2_{max} of denatured P_r (Brandlmeier, unpublished result). A product with λ^2_{max} = 335 nm can be obtained from the P_{fr} peptide by oxidation with iodine (Table 1). This product is not photosensitive any more; it is spectrally identical with the product obtained from the P_r peptide with iodine.

The second type of model compound for the P_{fr} chromophore, namely *EZZ*-etiobiliverdin **3b**, has the same λ^2_{max} as the corresoponding *ZZZ*-compound **3a**, the model for the P_r chromophore. Spectral differences between **3a** and **3b** are the position of λ^1_{max}

Table I. Spectral data of chromopeptides from phytochrome in the P_r form and P_{fr} form and of model chromophores.

Compound	λ^1_{max}	[nm]	λ^2_{max}	[nm]	Solvent	Reference
P_r-chromopeptide (**1**)	660		380		methanol/H^+	this paper
P_{fr}-chromopeptide		610	380		methanol/H^+	this paper
ZZZ-Etiobiliverdin IV_γ (**3a**)	650		380		chloroform	[10]
EZZ-Etiobiliverdin IV_γ (**3b**)		600	380		chloroform	[10]
A-dihydrooctaethylbiliverdin (**4a**)	665		351		methanol/H^+	[11]
pyridinium adduct (**4b**)		603		327	methanol/H^+	[12]
purpurin (**4c**)		605		337	methanol/H^+	[13]
P_r- or P_{fr}-chromopeptide + J_2		600	(390)	335	methanol/H^+	this paper
Dimethoxyviolin (**5**)		585		330	methanol/H^+	[14]

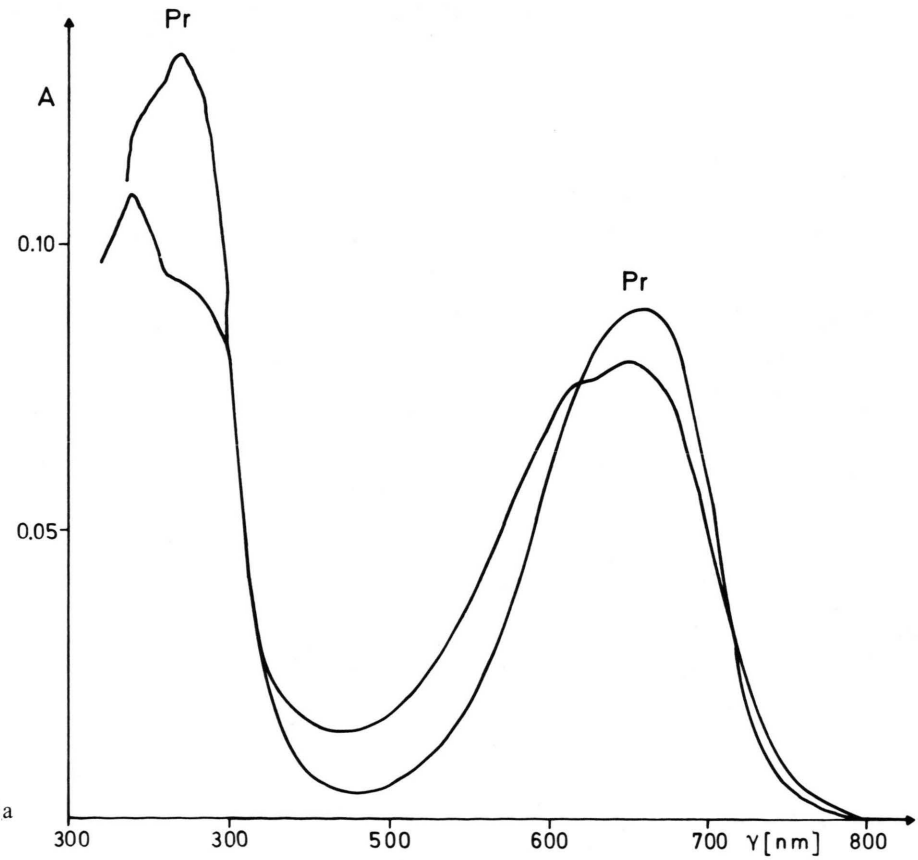


Fig. 4a

Fig. 4. Oxidation of phytochromobilin peptides with iodine. Solvent: *n*-butanol/0.1% trichloracetic acid. a: Absorption spectrum of P_r peptide before (P_r) and 20 h after addition of 0.2 mol iodine per mol chromophore under anaerobic conditions. b: Absorption spectrum of P_{fr} peptide. A before and B directly addition of 0.2 mol iodine per mol chromophore under anaerobic conditions, C: the same after 15 min, D: the same after 2 h.

(**3a** = 650 nm; **3b** = 600 nm) and the molar extinction coefficient at λ^2_{max} which is about 25% higher for **3a** than for **3b** (values taken from Fig. 8 in [10]). These data were determined in neutral chloroform whereas all other data were determined in acid methanol. Although a direct comparison is therefore problematic, the coincidence with the properties of the phytochromobilin peptides is striking: besides the above discussed position of λ^1_{max} we find the extinction at λ^2_{max} about 20% higher in the P_r peptide than in the P_{fr} peptide (see Fig. 3). A discrepancy concerns the pH stability: the P_{fr} peptide is irreversibly converted into the P_r peptide at pH values above 4–5 (exactly as denatured P_{fr} [5]) whereas the model compound **3b** is only stable in neutral or alcaline solution but converted into **3a** by acid (H. Falk, personal communication).

For a further consideration of the two types of model reactions for the $P_r \rightleftharpoons P_{fr}$ transformation we performed a number of experiments with the P_{fr} peptide. As described above, the P_{fr} peptide is more easily eluted from the silica gel column than the P_r peptide. Taking into consideration the photoaddition model, this could be due to addition of an unknown compound at the 4.5 double bond of the P_r chromophore. The compound should be very mobile itself and therefore increase the mobility of the P_{fr} peptide over that of the P_r peptide. Irradiation of the P_{fr} peptide which transforms this into the P_r peptide should lead to elimination of the unknown compound. The following experiment was designed for the possible detection of this compound. P_{fr} peptide from 45 mg phytochrome was prepurified on Biogel P-10. The P_{fr} peptide solution was divided into two equal parts which were each adsorbed on a silica gel column of the same size. Both silica gel columns were washed with 5% aqueous acetic acid (about 100 ml). One column was then irradiated with white light to phototransform the P_{fr} peptide into the P_r peptide whereas the control column was kept under dim-green safety-light. Both columns were further washed with 5% acetic acid (about 100 ml). These

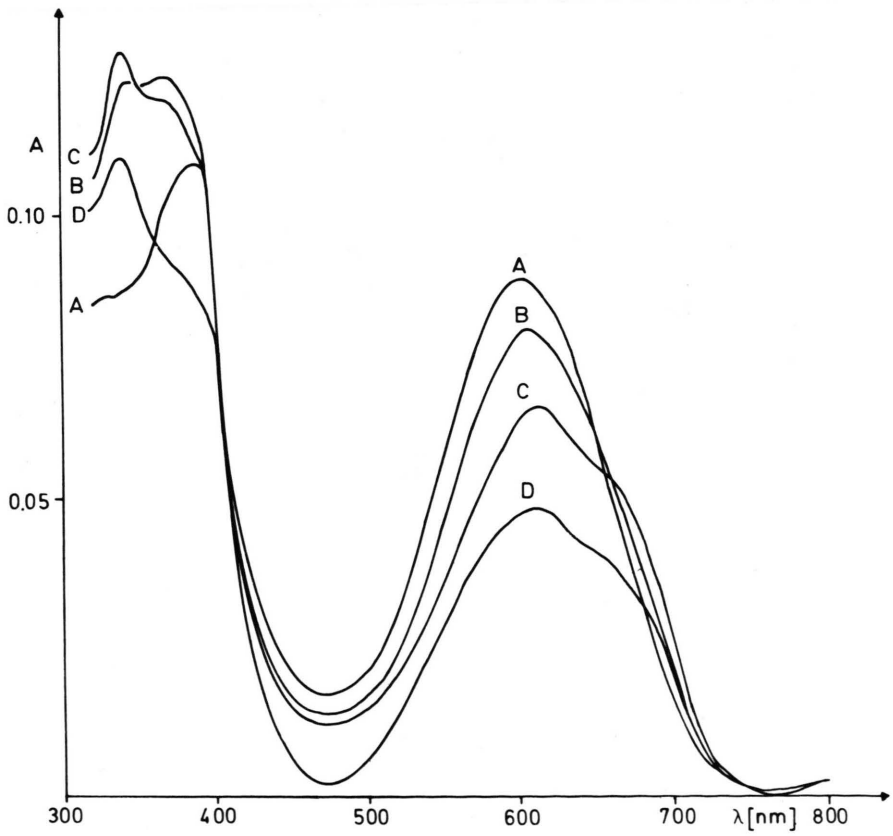


Fig. 4b

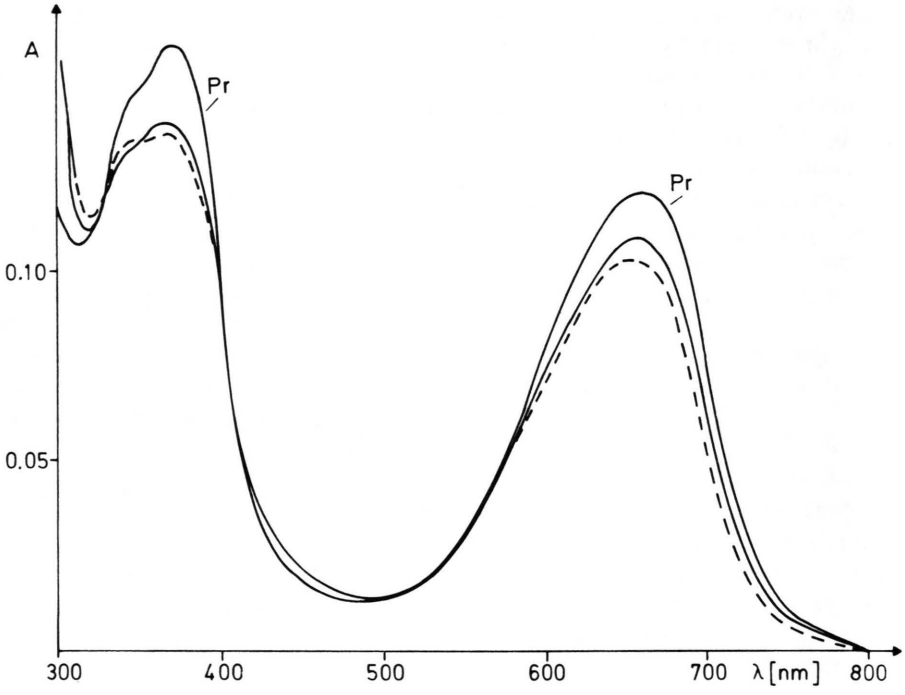


Fig. 5a

Fig. 5. Reduction of phytochromobilin peptides with sodium dithionite. Solvent: methanol/0.1% trichloroacetic acid. a: Absorption spectrum of P_r peptide before (P_r) and directly after addition of 0.5 mM sodium dithionite (—), the same after 15 min (- - -). b: Absorption spectrum of P_{fr} peptide. A before and B directly after addition of 0.1 mM sodium dithionite, C: after addition of further 0.1 mM sodium dithionite, D: after subsequent irradiation with white light for 10 s.

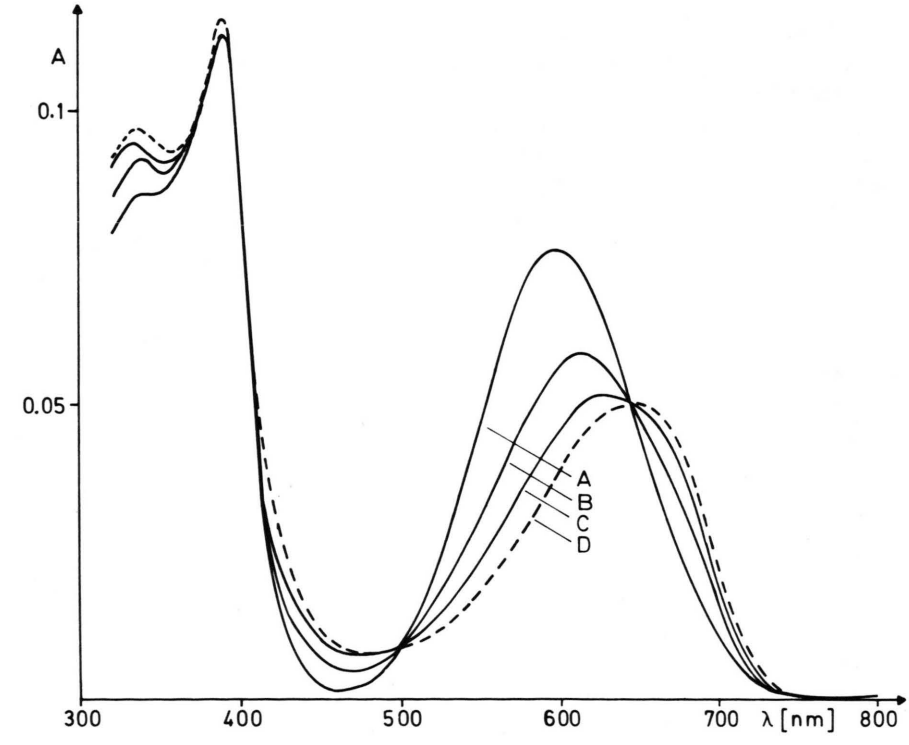
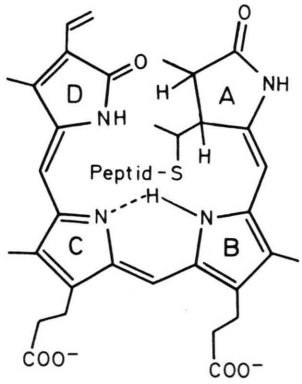


Fig. 5b

eluates were lyophilized and investigated by thin layer chromatography (see Materials and Methods). Traces of colorless peptides were detected in both eluates but no new compound could be detected in the eluate from the irradiated column with a number of staining reagents (ninhydrin, chlorine/tetramethylbenzidine, iodine, conc. sulfuric acid). This means either that the hypothetical compound is – contrary to our assumption – not eluted from the column or that the photoaddition model is wrong. The P_{fr} peptide was eluted from the control column with 30% aqueous acetic acid whereas the P_r peptide from the irradiated column was not eluted with this acid concentration but only with 50% acetic acid. This is an important observation: The photoconversion of denatured P_{fr} to denatured P_r had so far been monitored only by spectral properties. Irradiation of the P_{fr} peptide produced not only the spectral properties but also the elution characteristics of the P_r peptide.

Another series of experiments with the P_{fr} peptide concerned the oxidation with iodine in methanol/trichloroacetic acid. The first experiments of this type were performed under air. We found that P_r peptide and P_{fr} peptide reacted spontaneously and completely to a product with $\lambda^1_{max} = 600$ nm and $\lambda^2_{max} = 335$ nm (Table I) even with catalytical amounts of iodine (molar ratio chromophore: iodine = 10:1). Under these conditions, dihydrobiliverdin (**4a**) is oxidized to a product which has been identified by cochromatography as the dimethoxyviolin **5** (H. Scheer, personal communication). Because previously only λ^1_{max} was investigated [5] it was erroneously concluded that the spectrum of P_{fr} is not changed by oxidation. The change in the spectrum can best be detected at λ^2_{max} (P_{fr} peptide 380 nm, oxidation product 335 nm). When looking for milder conditions for the oxidation, we observed a relative stability of the P_r peptide against iodine under strictly anaerobic conditions in butanol/trichloroacetic acid (Fig. 4a). Under these conditions, the P_{fr} peptide is unstable (Fig. 4b). Interestingly, a long wavelength shoulder (at about 670 nm) is observed during oxidation of the P_{fr} peptide which indicates partial chemoconversion of the P_{fr} peptide to the P_r peptide. This observation is less compatible with the photoaddition-elimination model but very well with the *Z-E*-isomerization model: *cis-trans*-isomerization of C = C double bonds can be catalyzed by iodine. Longer incubation of the P_{fr} peptide with iodine leads mainly to bleaching.

Dark reversion of native P_{fr} to native P_r can be catalyzed by reductants [15]. The reaction of native and denatured phytochrome with dithionite has been investigated in detail [16]. A chemoreversion of native P_{fr} to native P_r (with only partial reduction of the chromophore) has been explained by the possible intermediate production of a bilirubin-like species [16]. Reversible chromophore reductions with dithionite have also been demonstrated with other biliproteins [17]. Such a reaction should also be possible with the phytochrome chromophore in the denatured state. We had to modify the conditions at first for the investigation of the P_{fr} peptide: 1) the reaction had to be carried out at low pH (in the stability range of the P_{fr} chromophore) and 2) the P_r chromophore should be as little affected as possible. We found suitable conditions with 0.5 mM dithionite which gives only a very low reduction of the P_r peptide (Fig. 5a). Under the same conditions, the P_{fr} peptide is drastically affected (Fig. 5b). Only part of the P_{fr} chromophore is reduced to a rubinoid



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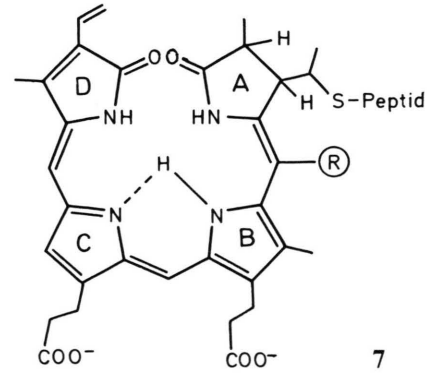

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chromophore under these conditions (increase in absorption at 430 nm). A considerable part of the P_{fr} chromophore is converted into the P_r chromophore ("chemoreversion"). This would again be compatible with the *Z-E*-isomerization model: *Z-E*-isomerization of model bilins is facilitated by reduction [10]. The conversion of the P_r chromophore can be completed by irradiation (Fig. 5 b). Because this phototransformation has the same isosbestic points as the chemoreversion the same products must be involved in both processes.

The results reported here confirm the conclusion [4, 5] that the P_{fr} chromophore is chemically different from the P_r chromophore. This difference survives not only acid denaturation of the protein but also proteolysis. In summary, our results on spectral properties of the P_{fr} peptide (Table I), lacking of detectable photoelimination, and last not least the partial chemoreversion of the P_{fr} peptide to the P_r peptide during reduction and oxidation reactions strongly favor the *Z-E*-isomerization model (**6**) but do not rigourously excluded the photoaddition model (**7**) for the P_{fr} peptide. Further experiments are needed to clarify this point.

Acknowledgements

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