Topography of the Phytochrome Molecule as Determined from Chemical Modification of SH-Groups

Petra H. Eilfeld, Gabriele Widerer, Heike Malinowski, Wolfhart Rüdiger, and Peter G. Eilfeld

Botanisches Institut der Universität München, Menzinger Straße 67, D-8000 München, Bundesrepublik Deutschland

Z. Naturforsch. **43c,** 63–73 (1988); received August 31, 1987

Oat, *Avena sativa* L., Mercurials, 124 kDa Phytochrome, Reporter Group

124 kDa phytochrome has been isolated from etiolated oat seedlings. Specific chemical modification of SH-groups has been performed using several agents, especially mercurials. These procedures allowed covalent fluorescence labelling of phytochrome. Bleaching of the tetrapyrrole chromophore and reduced photoreversibility has been investigated and analyzed in terms of steric effects, *i.e.* size of the reagents and number of modified residues. The Pr form was found to exhibit two highly reactive SH-groups, the Pfr form three, however. Two highly reactive groups have been localized on the 55 kDa C-terminal half of the protein. The 3rd group predominantly reacting in the Pfr form has been localized on a 39 kDa chromopeptide, *i.e.* on the N-terminal half. The covalently attached fluoresceine chromophores were used as reporter groups. The environment of the fluoresceine chromophore is more hydrophobic in the Pfr form than in the Pr form, indicating that conformational changes take place during photoconversion.

Introduction

Phytochrome is an important information processing photoreceptor in plants. It shows its maximal spectral sensitivity in the red spectral region. The chromophore of this chromoprotein was found to be a linear tetrapyrrole, which undergoes *Z-E* isomerization upon irradiation [1]. The primary structure of the polypeptide chain has been recently derived from the polynucleotide sequence [2]. Analysis of the protein's sequence, however, has given no information concerning the molecular mechanism of the action of Pfr, which has been found to be the physiologically active form. Some evidence has been presented that conformational changes take place on the protein surface as probed by monoclonal antibodies [3, 4] and circular dichroism measurements in the ultraviolet spectral region [5]. These conformational changes, however, should be limited to small areas, as the overall conformation has been shown to be the same after photoconversion of the chromoprotein [6]. Although conformational changes on the protein surface should be detectable by chemical agents, no reports about chemical modification of the 124 kDa phytochrome chromoprotein have been published so far.

The following discussion will deal with the reactivity of SH groups towards specific reagents. Both reactivity and the properties of the products formed shall be investigated especially regarding biochemical and physicochemical differences between Pr and Pfr form and chromophore-protein interactions.

Furthermore, reactive nucleophilic groups (Cys) will be attributed to specific domains. These reactive groups may indicate yet unknown functional sites as high chemical reactivity should accompany exposed sites.

Abbreviations: ANS, 8-Anilino-1-naphthalenesulfonic acid; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); FITC, fluoresceinisothiocyanate; FMA, fluoresceinemercuricacetate; 1.8-IAEDANS, N-iodoacetyl-N′-(8-sulfo-1-naphthyl)ethylenediamine; KPB, potassium phosphate buffer; NTCB, 2-nitro-5-thiocyanobenzoic acid; Pbl, bleached form of phytochrome; PCMB, p-chloromercuribenzoic acid; Pfr, far-red absorbing form of phytochrome; PMBS, p-chloromercuribenzene sulfonic acid; PMI, N-(1-pyrenyl)-maleimide; Pr, red absorbing form of phytochrome; SDS, sodium dodecyl sulfate.

Reprint requests to Dr. Peter G. Eilfeld.

Verlag der Zeitschrift für Naturforschung, D-7400 Tübingen
0341–0382/88/0100–0063 $ 01.30/0

Materials and Methods

Phytochrome

124 kDa oat phytochrome was isolated according to [7]. For this purpose etiolated oat shoots were extracted as described by Vierstra and Quail [8] and submitted to hydroxyapatite chromatography according to Datta and Roux [9]. The hydroxyapatite eluate was precipitated with ammonium sulfate and

the pellet was washed with 100 mM KPB, pH 7.8 (0.5 ml per mg of phytochrome); the residual pellet was dissolved in 20 mM KPB, pH 7.8, yielding a concentration of 1 to 2 mg/ml. This solution was purified on Biogel A-1.5 m in order to remove low molecular weight contaminations, *e.g.* mercaptanes, and contaminating proteins of about 60 kDa and 160 to 200 kDa molecular weight. Phytochrome could be eluted from Biogel with 20 mM KPB, pH 7.8, yielding material showing a specific absorbance ratio of A(667, Pr) / A(280) = 0.90–0.95. Other spectral properties are described in detail in [7]. Short term storage could be achieved in the same buffer, containing 3–4% (vol/vol) glycerol at 250 K without affecting any spectral or biochemical properties. SDS gel chromatography on 7.5 or 10% acryl amide gels according to Laemmli [10] showed only one band at 124 kDa at concentrations of 0.2 mg/ml (not shown).

Spectroscopy

All spectra were run on a Hewlett-Packard diode array spectrophotometer HP 8451A with disc storage and processing capability. Spectra were run at 298 K if not noted otherwise. Irradiation of the samples was achieved by the aid of a fiber optic system and interference filters [7, 11].

Kinetics were run on a dual-wavelength spectrophotometer Sigma-ZWS II (Sigma, Berlin, FRG) with connected Bascom-Turner 8110 calculator and plotter. Cooling of the samples was done with a Haake cooling device (Haake, Berlin, FRG). Fluorescence spectra were recorded with a Zeiss DMR 22 photometer and fluorescence attachment ZFM 4 (Zeiss, Oberkochen, Germany).

Phytochrome concentrations were calculated based on a molar absorbance value of 132000 l mol^{-1} cm^{-1} at 667 nm for the monomer of Pr [12].

Chemicals

Reagents for modification were obtained from Sigma (Sigma, FRG). Solutions were prepared freshly in 100 mM KPB, pH 7.80, with the exception of PMI, which had to be dissolved in ethylene glycole (see below).

Modification experiments and quantification of modified residues

Phytochrome was used either freshly prepared in 20 mM KPB, pH 7.8 or in the same buffer, containing glycerol (see above) after storage at 250 K. No differences could be detected after one freezing-thawing cycle. Modifications with PMBS or PCMB were performed by adding 1 to 25 moles of reagent per mole of phytochrome by applying definite amounts of stock solutions in 100 mM KPB (2.0 mM for PMBS, 0.16 mM for PCMB, respectively). Both reagents show the same molar absorbance change of 7600 l mol^{-1} cm^{-1} at 250 nm after reaction with SH-groups [13].

The modification by FMA was done essentially in the same way, but the course of reaction was followed by absorbance changes at about 500 nm [14]. Excess FMA binds noncovalently to phytochrome giving similar spectral changes, however. In order to determine the amount of FMA covalently bound, samples were dialyzed against 20 mM KPB, pH 7.8. Molar extinction values were taken from Heitz and Anderson [14]. However, due to spectral alterations of the fluoresceine chromophore during dialysis, the number of covalently bound FMA molecules could not be determined precisely. Chemical removal of covalently bound FMA could be achieved by adding 20 mM 2-mercaptoethanol and dialysis of the samples. This reaction could be followed by a shift of the fluorescein absorption band from 510 to 502 nm and an increase of extinction.

Reactions with PMI were done by addition of 1 to 25 equivalents PMI, dissolved in ethylene glycole (stock solution 0.2 mM). Excess reagent was removed by dialysis against 20 mM KPB, pH 7.8, as PMI as well as FMA binds noncovalently to phytochrome. Removal of excess PMI was shown to be complete on SDS gels, as only the 124 kDa band showed the blue pyrene fluorescence. The amount of PMI was determined using $\varepsilon(348) = 18000$ [15, 16].

Modification with DTNB was performed as described by Hermann and Müller [17], modification with NTCB in an analogous way. For reactions at 298 K due to side reactions [17] an extrapolation to time zero was performed.

Reactions with IAEDANS derivatives and FITC were done by incubation with a large excess (up to 1 mM) of reagens (stock solution 10 mM in 100 mM KPB, pH 7.8). The reaction was stopped by addition of 2-mercaptoethanol (30 mM) and gel-filtration on Bio-Gel-A 1.5 m or dialysis against 20 mM KPB, pH 7.8. Amount of bound IAEDANS was determined using $\varepsilon(360) = 6800$ l mol^{-1} cm^{-1} [16]. For FITC $\varepsilon(495) = 42000$ l mol^{-1} cm^{-1} was used instead

[16]. Higher concentrations of IAEDANS had to be avoided, as it shows a similar behaviour as ANS at concentrations > 3 mM, *i.e.* bleaching due to intercalation of the ANS-like chromophore (*cf.* [11]).

Tryptic digest of FMA-labelled phytochrome

Phytochrome at concentrations of 15 to 20 μM was incubated either as Pr or Pfr with definite amounts of FMA for 45 min at 298 K as Pr, or 15 min at 298 K as Pfr, respectively. After this pretreatment, the solutions were incubated with trypsin (Sigma Co., T8253) at concentrations of 1% w/w for 2 min (yielding the 59 kDa chromophore containing fragment) or 30 min (39 kDa fragment) at 298 K after transformation into Pfr. The course of digest was followed by monitoring phytochrome absorption in the spectral region above 500 nm according to Reiff *et al.* [18]. Trypsin digest was stopped by addition of soybean–trypsin inhibitor (Sigma Co., T9003, 400% w/w based upon trypsin amount) and incubation for 15 min at 298 K. Peptides were separated by gel filtration on Bio-gel-A 1.5 m (Bio-rad, Uppsala, Sweden, column 15 × 900 mm) at flow rates of 10 ml per h. Fractions of 2.5 ml were collected and analyzed by absorption spectra and SDS gel electrophoresis (see below).

Phytochrome labelled by 1.8-IAEDANS or FITC

The same procedure as described for FMA was employed, but incubations with 1.8-IAEDANS and FITC were performed at higher concentrations of the reagent (2 mM), and the reaction was stopped by addition of 14 mM 2-mercaptoethanol followed by incubation at 298 K for 15 min. After this treatment, tryptic digest and separation of peptides were performed in the same manner as described above.

Additionally, analysis of the fractions was done by fluorescence spectroscopy using wavelength's of 365 nm (1.8-IAEDANS) or 480 nm (FITC) for excitation and of 480 nm or 512 nm for detection, respectively.

SDS-polyacrylamide electrophoresis

SDS-polyacrylamide electrophoresis was performed according to [10] using polyacrylamide concentrations of 7.5 or 10%. Gels were run on a LKB-Midget apparatus.

Staining was either performed with Coomassie Brillant Blue R (Sigma B 0630) or by applying the silver staining method according to [19]. Fluorescence was visualized by a mercury lamp (Hanau, Germany) combined with interference filters passing 313 or 365 nm light or by a Bachhofer illuminator TL-350-M (Bachhofer, Reutlingen, Germany) irradiating at 302 nm. Photography of fluorescence was performed by the aid of the latter irradiation device through a filter BG7, 2 mm (Schott, Germany, highest transmission at 480 nm) using KODAK Ektachrome 100 film.

Results

The effects of chemical modification of 124 kDa oat phytochrome are summarized in Table I in terms of number of modified residues and of chromophore absorption and photochemistry.

The results depend upon the structure of the agent, its concentration, the temperature and irradiation conditions.

Mercurials like PCMB, PMBS, or FMA have been reported to react with SH groups in a quite specific way [20]. With partially degraded phytochrome, strong bleaching has been observed after incubation with mercurials [17, 21].

124 kDa oat phytochrome was incubated with PCMB, PMBS and FMA under various conditions (Table Ia). PMBS has some advantages as compared to PCMB, for example a much higher solubility in buffered aqueous solutions and a lower absorbance at 250 nm. In any case, the reaction is very fast even at 277 K, *i.e.* 2–3 SH-residues of phytochrome react within seconds. This reactivity is comparable with that of free mercaptanes.

The reaction with a large excess of SH-modifying agents is associated with alterations of the phytochrome chromophore spectrum. The bleaching of the longwavelength absorption band upon excessive modification is shown in Table Ib. In general, bleaching increases as follows:

NTCB < PCMB < DTNB < PMBS < PMI < FMA.

This series is correlated with the size of the residue introduced by chemical modification. Similar steric effects have already been observed with inactivation of enzymes by NTCB *vs.* DTNB [22].

Generally, upon modification of Pr stronger bleaching of the Pfr form takes place as compared to Pfr. The formation of Pbl-like species is more likely to happen upon modification of Pr, too (Table I).

Table Ia: Number of modified SH-residues in 124 kDa oat phytochrome[a,b].

Agent	Immediate modification			Saturating modification		
	Conditions	Number of mod. Res. Pr	Pfr	Conditions	Number of mod. Residues Pr	Pfr
PCMB	20 μM, 30 s 277 K	2	3	25 μM, 30 min 277 K	11	11
PMBS						
FMA						
PMI	25 μM, 2 h 277 K	2	3	25 μM, 20 min 300 K	9	9
DTNB	1 mM, 1 min 298 K	2	3	1 mM, 2 h 298 K	11	11
NTCB	1 mM, 1 min 298 K	1	1	1 mM, 2 h 298 K	15	15
IAEDANS	1 mM, 5 min 277 K	1	1	1 mM, 24 h 277 K	3	3

[a] FITC is not included due to low selectivity, see text and [29].
[b] Phytochrome concentration 1 μM.

Table Ib: Bleaching of phytochrome upon modification with SH-specific agents, as expressed by residual absorbance in % of maximum absorption band. (Saturating modification achieved, conditions see Table Ia.)

Reagent	Modification of Pr Residual absorbance of Pr	Pfr	Modification of Pfr Residual absorbance of Pr	Pfr
PCMB	84	84	93	86
PMBS	50	Pbl[a]	46	Pbl
FMA	<10	–[b]	<10	–
PMI	23	Pbl	23	Pbl
DTNB	68	Pbl	62	48
NTCB	94	67	85	82
IAEDANS	91	67	89	79

[a] Bleached phytochrome, *cf.* Fig. 2b.
[b] Completely bleached phytochrome showing no photoreversibility, see Fig. 3.

The bleaching effects [17, 21] can be correlated with the number of modified residues by titrating with stoichiometric amounts of mercurials. Upon modification of less than 3 SH groups, no considerable spectral changes can be observed (Fig. 1). Modification of further SH groups, however, causes strong bleaching and affects the Pfr spectrum, especially the long wavelength absorption band is shifted from 730 nm to 720 nm, the short wavelength absorption band from 400 nm to 390 nm (Fig. 2a). Upon excessive modification (*i.e.* > 9 SH groups), bleached species (Pbl) are formed instead of Pfr showing no absorption maximum above 700 nm and a blue absorption band at 380 nm (Fig. 2a). Comparable spectra have been found for a 39 kDa fragment obtained by proteolysis from 124 kDa phytochrome [18]. In that case, the lack of specific interaction between Pfr chromophore and part of the peptide chain has been discussed to be the reason for disappearance of the Pfr absorption band. Nevertheless, upon even excessive modification of native phytochrome its mass proves to be still 124 kDa on SDS gels (data not shown). The chemically bleached species (*cf.* Fig. 2b) show photoreversibility but no dark reversion as reported for 39 kDa phytochrome [18] or ANS-bleached phytochrome [11].

Upon prolonged incubation (*e.g.* 2 h at 298 K with 30 μM FMA), extremely bleached species are formed with $\varepsilon(660) \sim 10{,}000\ l\ mol^{-1}\ cm^{-1}$ showing no photoreversibility at all. These species significantly differ from previously reported bleached forms with higher molar extinction values (at about 20,000 $l\ mol^{-1}$

cm^{-1}, [18, 23, 24]). The alterations of the chromophore spectrum introduced by modification of SH groups with excessive FMA are irreversible, as shown by curve 3 in Fig. 3. FMA can be cleaved off from the protein with typical spectral changes, *i.e.* a hypsochromic shift and an increase of absorbance of the fluorescein chromophore, but without effect upon the (bleached) phytochrome chromophore. Especially, photochromic behaviour of the tetrapyrrole chromophore is not restored even after excessive dialysis and nearly complete (> 90%) removal of bound FMA.

With NTCB, DTNB or PMI, similar results can be obtained, but no completely bleached phytochrome as with FMA is formed. With PMI (25 μM, 30 min), strongly bleached species can be obtained at higher temperature (300 K), still being photoreversible. With NTCB, introducing the smallest residue, maximum numbers of cysteins can be modified while causing smallest spectral alterations (see Table I). In this case, no Pbl can be obtained.

In order to test if intactness of the phytochrome protein affects its sensitivity towards SH-modifying reagents, 59 kDa phytochrome was treated with FMA and NTCB, too. Incubation with FMA results in complete bleaching of the chromophore after addition of 6 equivalents of FMA to one mole of phytochrome; with one equivalent, respectively, the long wavelength absorption of Pfr is slightly bleached (90% residual absorbance). With NTCB, about 7–8 SH groups are accessible, yielding still photoreversible phytochrome (absorption maximum of Pfr shifted from 722 to 718 nm) with small extent of bleaching. In general, SH-modification of 59 kDa-phytochrome results in similar effects as that of 124 kDa-phytochrome, only the number of available SH groups being smaller.

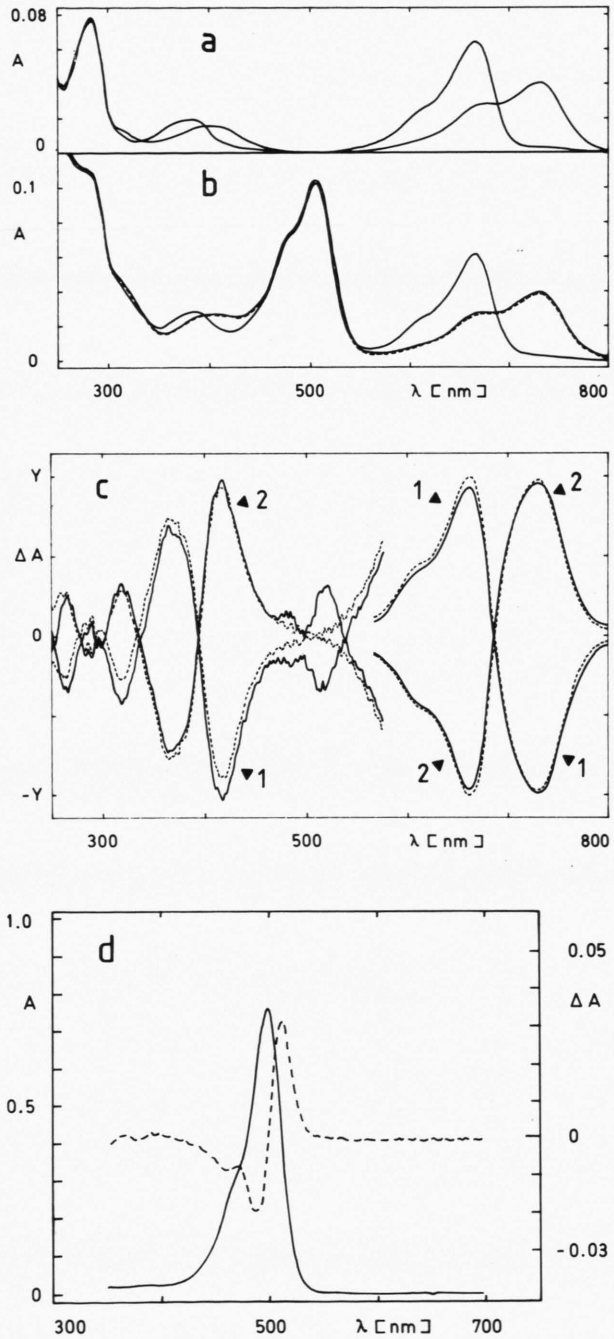


Fig. 1. FMA as reporter group.
a) Absorption spectra of unmodified 124 kDa oat phytochrome in 20 mM KPB, pH 7.8.
b) Absorption spectra of modified phytochrome (3 equivalents FMA bound to Pfr) passing an irradiation cycle: Pfr (——) → Pr (——) → Pfr (---).
c) Absorption difference spectra of modified phytochrome (——, *cf.* Fig. 1b) and unmodified phytochrome for comparison (----, *cf.* Fig. 1a). Absorbance scale is $Y = 0.01$ for wavelengthes below 580 nm, and $Y = 0.04$ for wavelengthes above that value.
(1) Difference spectrum Pr minus Pfr for phototransformation Pfr → Pr,
(2) Difference spectrum Pfr minus Pr for phototransformation Pr → Pfr.
d) Solvens perturbation spectrum of the fluorescein chromophore upon transformation from KPB buffer into KPB buffer containing 6% (vol/vol) glycerol. (fluorescein as FMA, concentration 10 μM, ——; difference spectrum FMA in buffer containing glycerol minus FMA in pure buffer: ----).

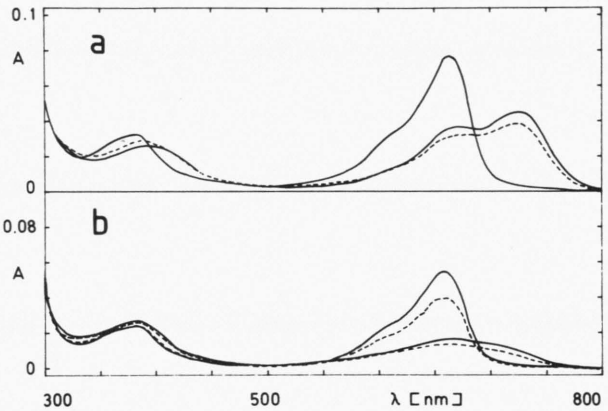


Fig. 2. Formation of spectrally altered phytochrome upon treatment with PMBS.
a) Pfr (0.84 μM) was incubated with PMBS (12.6 μM) for 500 s at 298 K. As calculated from A (250), 7 SH-residues had been modified. Afterwards, an irradiation cycle Pfr (——) → Pr (——) → Pfr(722) (---) was performed.
b) Pr (0.84 μM) was incubated with PMBS (25 μM) for 24 h at 277 K, or 60 min at 298 K. As calculated from A(250), 10–11 SH groups had been modified. In this case, a repeatedly photoreversible system P(660) ⇌ Pbl is found (first irradiation cycle: ——, second irradiation cycle: ——).

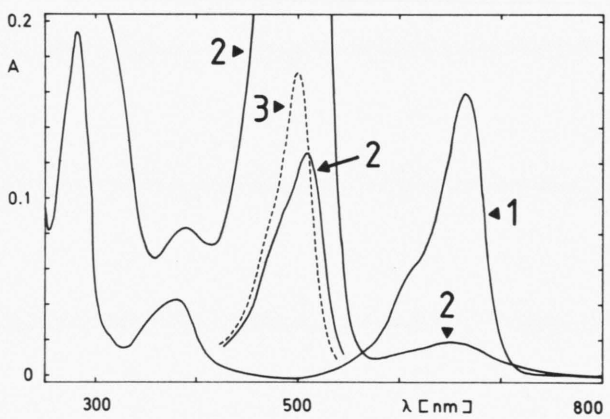


Fig. 3. Formation of completely bleached species, which are no longer photoreversible, upon incubation with FMA. Curve (1) shows unmodified phytochrome (Pr form, 1.45 μM), curve (2) same phytochrome incubated with 14.5 μM FMA for 48 h at 277 K. Complete binding of FMA is indicated by its absorption maximum at 510 nm (middle part of the spectrum, absorbance scale 0–1). This binding can be cleaved by addition of 30 mM 2-mercaptoethanol and incubation for 15 min at 298 K: curve (3) with maximum FMA absorption at 502 nm (---, absorbance scale 0–1). No changes of phytochrome chromophore could be detected upon this treatment, *i.e.* denaturation induced by FMA binding is irreversible.

Covalently attached chromophores like the fluoresceine chromophore in FMA or the pyrene chromophore in PMI are useful tools in detecting environmental changes. They may act as reporter groups like other compounds with characteristic absorption spectra [16] as shown in Fig. 1b and 1c. So small photoreversible changes of the FMA absorption can easily be detected (Fig. 1c), *i.e.* especially an increase in the long wavelength edge of the absorption band of FMA. With this respect, these difference spectra are similar to solvens perturbation spectra of FMA as shown in Fig. 1d for transfer of FMA from pure aqueous buffer in to buffer containing 6% (vol/vol) glycerol. This transfer corresponds to an increased hydrophobicity. So, the reporter group detects an exposure of a more hydrophobic environment in Pfr. As by labelling of phytochrome with FMA, the tetrapyrrolic chromophore is not disturbed at all (*cf.* Fig. 1a *vs.* 1b); these effects appear to be an intrinsic property of the native protein.

Tryptic digests of modified phytochrome

FMA labelling

For localization of highly reactive SH groups, the following procedure was employed. Phytochrome was labelled with 1 or 3 equivalents of FMA. Then labelled phytochrome was digested with trypsin, because tryptic peptides recently have been localized on the primary structure [25]. Tryptic digest maps have turned out to be the same for modified and unmodified phytochrome (data not shown). This easily allows localization of modified peptides after their isolation.

Fig. 4 a shows spectral changes of FMA labelled phytochrome upon proteolysis. Conditions were chosen such that 124 kDa phytochrome was digested up to formation of the 39 kDa chromopeptide. This digest is accompanied by absorption changes not only of the tetrapyrrole, but also of the fluoresceine chromophore. The absorption peak of the latter is shifted from 510 to 502 nm and its height increased by about 10%. Similar, but larger effects take place by addition of mercaptanes to FMA bound to proteins thus cleaving the S-Hg bond (see curve 3 in Fig. 3). Obviously, the fluoresceine chromophore changes its environment upon desintegration of the protein structure during proteolysis.

Separation of tryptic peptides by gel chromatography is shown in Fig. 4b. It becomes obvious that

FMA labelled peptides can be separated from chromophore containing peptides by gel chromatography. Some lines of evidence indicate that predominantly labelled peptides belong to the C-terminal half of the primary structure, especially to a 55 kDa peptide (amino acid 596/597 to 1093, *cf.* [25]):

a) In spite of a molecular weight of ≤ 55 kDa on denaturating SDS gels (see Fig. 4c), these peptides migrate at a lower elution volume than chromophore containing 59 kDa peptides. This finding points to aggregation of the 55 kDa fragment, which is in coincidence with the previously reported dimerization site at the C-terminal half [26].

b) Absorption spectra as well as fluorescence visualization of tetrapyrrolic chromophores [27] indicate the absence of any tetrapyrrolic chromophore from the peptides that are predominantly labelled with FMA.

c) In many cases, even upon short incubation with trypsin, contamination with smaller fragments (especially 42 kDa) can be seen according to the high reactivity towards proteases, found for the C-terminal half [28].

More precise localization of groups reactive towards FMA is still not possible due to rapid further fragmentation of the C-terminal half.

Upon increased labelling with FMA (3 equivalents applied), chromophore containing peptides, *i.e.* the 59 kDa and 39 kDa peptides (amino acid 63/66 to 596/597, 63/66 to 426) become labelled, too. From

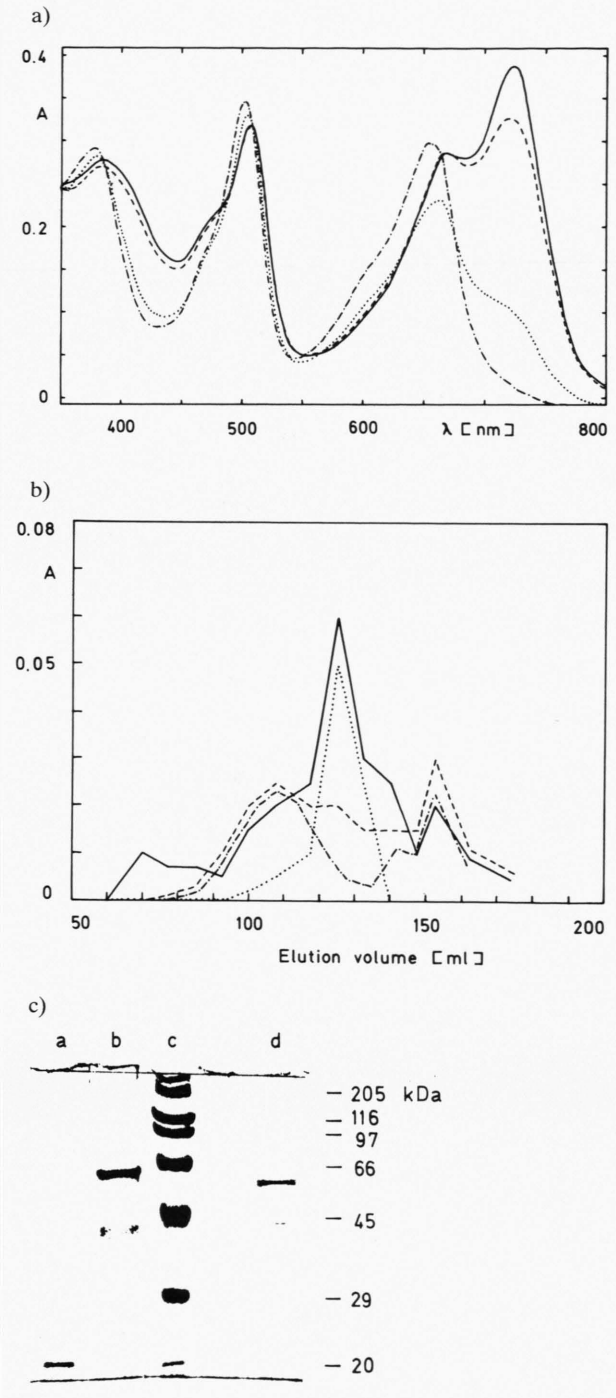


Fig. 4. Tryptic digest of FMA labelled 124 kDa phytochrome.
a) Spectral changes of 124 kDa phytochrome, labelled with 1.0 equivalents FMA in Pfr, upon protelysis with trypsin (1% w/w): (——) is before addition of trypsin, (----) is after 1 min, (·····) is after 30 min, and (-·-·-·) is after 2 h of incubation with trypsin at 298 K.
b) Separation of tryptic peptides by gel chromatography. Pfr was incubated with 3 equivalents of FMA and digested until formation of the 59 kDa chromophore containing peptide. Absorbancies were recorded at 280 nm (——), 504 nm (----) and 664 nm (····). Additionally, the scan at 504 nm (-·-·-) for the digest of phytochrome labelled in the Pr form is shown for comparison. (Conditions of chromatography see Materials and Methods.)
c) Analysis of main fractions of gel chromatography by SDS gel electrophoresis from the above (Fig. 4b) scan on 10% polyacrylamide gels.
Lane a: fraction with 140 ml elution volume (trypsin inhibitor);
lane b: fraction with 125 ml elution volume (59 kDa chromophore containing peptide with contaminants at 52 and 39 kDa, which have been recognized to be chromophore bearing too; see [25]);
lane c: Marker proteins, positions are given right by arrows;
lane d: fraction with 110 ml elution volume (55 kDa non-chromophore containing peptide).

Fig. 4b it becomes obvious, that this labelling is significantly less upon incubation of the Pr form. So this is presumably the site, which is responsible for different reactivity of Pr and Pfr (*cf.* Table I). This indicates a SH group located in the neighborhood of the phytochrome chromophore, which is accessible only in the Pfr form (data see Table II).

From Table II further it becomes obvious, that this less reactive SH-residue located on a 39 kDa peptide from amino acid 66 to 426 is exposed in the Pfr but not in the Pr form. Upon incubation of both Pr and Pfr with 1 equivalent FMA, FMA reacts preferently with available residues on the C-terminal site thus showing no difference between Pr and Pfr. However, upon incubation with 3 equivalents FMA, only in the Pfr form strong labelling of the 39 kDa fragment can be found. More precise localization of this Cys is not possible because S-Hg-C bonds are easily cleaved upon conditions of sequencing and smaller tryptic peptides are difficult to obtain in a pure form, which would be a prerequisite for precise ascribing FMA labelling to a specific peptide.

Fluorescence labelling with IAEDANS

FMA used as a labelling agent suffers from two disadvantages, namely its low fluorescence quantum yield [14] and its easy removal by mercaptanes. Both effects disable any visualization of labelled peptides on SDS-polyacrylamide gels, as 2-mercaptoethanol has to be used in high concentrations for unfolding the peptide chain. Using 1.8-IAEDANS, both shortcomings can be overcome. However, labelling is not as specific as with FMA (see Table I), especially this reagent is not able to discriminate between Pr and Pfr. Besides this, a similar reactivity pattern expressed as number of moles IAEDANS bound per protein-molecule is obtained after tryptic digest of 1.8-IAEDANS labelled phytochrome (Fig. 5). The non-chromophore containing peptide with 55 kDa mass contains most numbers of IAEDANS. This is supported by the characteristic fluorescence of 1.8-IAEDANS bound to proteins (Fig. 5a). 1.8-IAEDANS is incorporated into chromopeptides, too, as can be derived from evaluation of absorption spectra (see values in Fig. 5a). However, these labelled peptides don't show any strong fluorescence in the native state but do so after denaturation, *e.g.* on SDS-gels (Fig. 5b). This indicates some structural differences between N-terminal and C-terminal site of phytochrome, *e.g.* exposure of SH groups. Further digest of 1.8-IAEDANS labelled phytochrome leads to a labelled 39 kDa chromopeptide, too (Fig. 5b), as in the case of FMA modification. With

Table II: FMA labelling pattern of tryptic peptides[a]. (Numbers are molar ratios of bound FMA per peptide[b]).

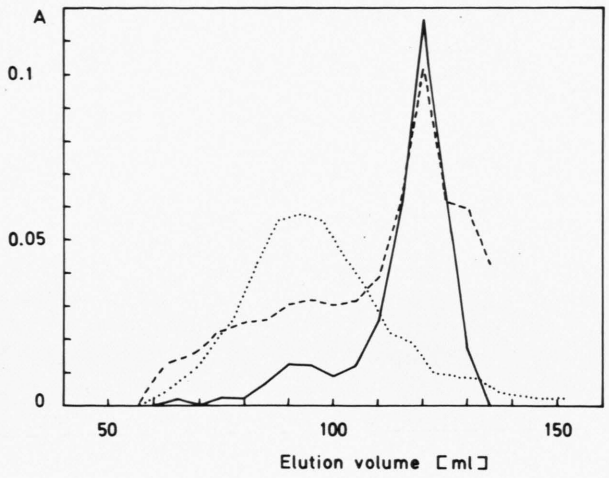
Incubation conditions[a]	Peptide		
	55 kDa C-terminal 596–1093[c]	59 kDa N-terminal 63/66–596	39 kDa N-terminal 63/66–426
Pr + 1 FMA	0.5	<0.15	<0.15
Pfr + 1 FMA	0.6	<0.15	<0.15
Pr + 3 FMA	1.1	0.15[d]	0.25
Pfr + 3 FMA	1.1	0.45[d]	0.55

[a] Incubation conditions and separation see Materials and Methods, for an example see Fig. 4b.

[b] Evaluation of absorption spectra was performed only for pure fractions showing only one protein band and calculated with $\varepsilon(508) = 48000$ and $\varepsilon(280) = 9000$ for bound FMA (*cf.* [14]); protein concentrations were determined from absorbance at 280 nm by subtraction of FMA absorption and taking 1 mg/ml for $A(280) = 1$ [16]. Molar absorbance at 280 nm was calculated from this value and the molecular weight determined from SDS gels (*e.g.* Fig. 4c) and performed only for pure fractions showing predominantly one protein band. Due to overlapping of elution with other protein species, these values are wrong by about 0.1 units (*cf.* [a]).

[c] Numbers are numbers of amino acids, *cf.* Fig. 6.

[d] See also Fig. 4b, in which this difference becomes immediately evident. Values given here are for a pair of identical samples.



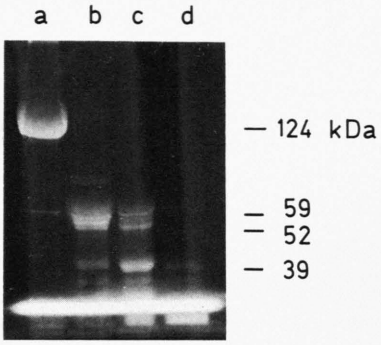


Fig. 5. Tryptic digest of 1.8-IAEDANS labelled phytochrome. Labelling was performed as described in Materials and Methods.
a) Separation of tryptic peptides by gel-chromatography; digest was performed until formation of the 59 kDa chromopeptide. Absorbancies were recorded at 664 nm (——) and 280 nm (---). Additionally, fluorescence in relative units at 480 nm (excitation at 365 nm) is shown, too (·····). Incorporation of 1.8-IAEDANS are 2.4 molecules dye per protein for peakfraction at 90–95 ml (55 kDa peptide according to SDS electrophoresis) and 1.3 molecules dye per protein for fraction at 120 ml elution volume (59 kDa chromopeptide according to SDS electrophoresis), calculated according to molar extinction values in [16] and the procedure used in Table II.
b) Fluorescence visualization of 1.8-IAEDANS labelled on SDS-polyacrylamide gel (7.5% polyacrylamide; cp. Materials and Methods).
Lane a: 30 μg 124 kDa phytochrome, labelled with 3 equivalents of 1.8-IAEDANS;
lane b: same as a, digested with 1.5% trypsin (w/w) for 1 min: formation of 52, 55, 59 kDa peptides;
lane c: same as b, but digested for 6 min: formation of the 39 kDa chromopeptide;
lane d: same as b, but digested for 45 min: no higher molecular weight (> 25 kDa) proteins labelled.
Arrows with numbers denote molecular weights as determined from Coomassie staining.

FITC similar results as with IAEDANS were obtained, but incorporation of labels was higher (1.5 molecules into 55 kDa peptide, 2–2.5 molecules into 59 kDa peptide). So FITC, which is a common fluorescence labelling agent, seems to be much less specific than the previous reported chemicals. This is in accordance with recent literature, as FITC reacts both with Cys and Lys in quite an unspecific way [29].

Discussion

Results from Fig. 1 to 3 and Table I clearly indicate that SH groups, which easily react with many reagents, especially mercurials, are not directly involved in the photochemistry of the phytochrome chromophore. Alterations of chromophore absorbance and photochemistry, which are found after extensive modification of SH groups, seem to be introduced by steric effects of bulky residues. This effect may reduce chromophore- protein-interaction thus causing similar effects as upon proteolysis. This is in contrast to degraded rye phytochrome [17] for reasons presently difficult to analyze, as the primary structure of rye phytochrome is not yet known. Different accessability of SH groups due to proteolysis cannot be accounted for this effect, because 59 kDa oat phytochrome behaves similar to 124 kDa oat phytochrome.

Further, the observed difference between Pr and Pfr of 1 SH group reacting initially in the study of Hunt and Pratt [30] could be reproduced for 124 kDa phytochrome. So, this difference previously reported for degraded phytochrome, is not due to differential accessability resulting from proteolysis (*cf.* [11]), but rather differential reactivity of surface SH groups. This is in accordance with the previously reported observation, that SH groups are changing their environment during photoconversion as concluded from far-UV difference spectra [31].

Most reactive SH groups (Cys) are located on two entirely different domains (see Fig. 6). One domain is located on the C-terminal nonchromophore-bearing half of the peptide chain, which predominantly reacts with SH-modifying reagents. This domain seems not to be involved in phototransformation as photoreversible changes of reporter groups (as shown in Fig. 1c) are significant only upon modification of a SH group in the other domain. The other domain is located in the vicinity of the chromophore. A reactive Cys is located on the 39 kDa chromo-

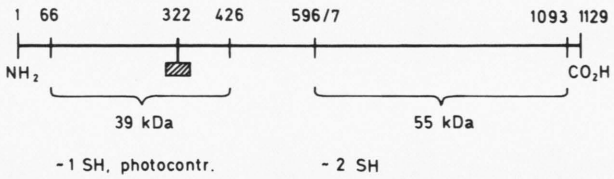


Fig. 6. One dimensional domain model for phytochrome (small marks denote tryptic cleavage sites according to [25]); the shadowed box at amino acid 322 denotes position of the tetrapyrrolic chromophore).

phore-bearing domain. Reactivity of this Cys is under photoreversible control, that is, it is predominantly reactive in Pfr form.

So, regions which are photoreversibly exposed, are residing near the chromophore. Recently, using monoclonal antibodies, two domains, one of these residing near the N-terminus, the other one in the middle of the amino-acid-sequence have been recognized [3, 4, 32, 33]. Both antigenic regions are in close vicinity to the 39 kDa chromopeptide.

These findings are in agreement with recent data concerning antigenic regions conserved throughout nearly the whole plant kingdom (Schneider-Poetsch *et al.*, to be published; [32]). Two regions, one of these being localized on the C-terminal half, the other near the chromophore, may be in close spatial relationship to the observed highly reactive SH groups reported in this work. This still speculative idea may ascribe a physiological function to these conserved antigenic domains more likely than identity just being merely formal.

Future research will deal with the function of the domains associated with high chemical reactivity. Recent results concerning *in vitro* control of transcription by addition of exogeneous phytochrome to isolated nuclei [34] indicate that even non-photoreversible, modified phytochrome is able to enhance transcription rate. This supports the idea that domains not directly involved in phototransformation may also play an important physiological role, *e.g.* by pre-binding to a receptor.

Acknowledgements

This work was supported by the Deutsche Forschungsgemeinschaft, Bonn-Bad Godesberg and the Fonds der Chemischen Industrie.

[1] W. Rüdiger, P. Eilfeld, and F. Thümmler, in: Optical Properties and Structure of Tetrapyrroles (G. Blauer and H. Sund, eds.), pp. 349–366, Walter de Gruyter & Co., Berlin, Heidelberg, New York 1985.
[2] H. P. Hershey, R. F. Barker, K. B. Idler, J. L. Lissemore, and P. H. Quail, Nucl. Acids Res. **13,** 8543–8559 (1985).
[3] M.-M. Cordonnier, H. Greppin, and L. H. Pratt, Biochemistry **24,** 3246–3253 (1985).
[4] B. Thomas and S. E. Penn, FEBS Lett. **195,** 174–178 (1986).
[5] R. D. Vierstra, P. H. Quail, T.-R. Hahn, and P.-S. Song, Photochem. Photobiol. **45,** 429–432 (1987).
[6] P.-S. Song, in: Optical Properties and Structures of Tetrapyrroles (G. Blauer and H. Sund, eds.), pp. 331–348, W. de Gruyter & Co., Berlin, Heidelberg, New York 1985.
[7] Peter Eilfeld, Petra Eilfeld, and W. Rüdiger, Photochem. Photobiol. **44,** 761–769 (1986).
[8] R. D. Vierstra and P. H. Quail, Biochemistry **22,** 2498–2505 (1983).
[9] N. Datta and S. J. Roux, Photochem. Photobiol. **41,** 229–232 (1985).
[10] U. K. Laemmli, Nature **227,** 680–684 (1970).
[11] P. Eilfeld and W. Rüdiger, Z. Naturforsch. **39c,** 742–745 (1984).
[12] J. C. Lagarias, J. M. Kelly, K. L. Cyr, and W. O. Smith Jr., Photochem. Photobiol. **46,** 5–13 (1987).
[13] J. F. Riordan and B. L. Vallee, Methods. Enzymol. **25,** 449–456 (1972).
[14] J. R. Heitz and B. M. Anderson, Arch. Biochem. Biophys. **127,** 637–644 (1968).
[15] J. H. Clarke and M. Martinez-Carrion, J. Biol. Chem. **261,** 10063–10072 (1986).
[16] C. R. Cantor and P. R. Schimmel, Biophysical Chemistry. Part II. Techniques for the study of biophysical structure and function. W. H. Freeman & Co., San Francisco 1980.
[17] G. Hermann and E. Müller, Biochem. Physiol. Pflanzen **175,** 755–763 (1980).
[18] U. Reiff, P. Eilfeld, and W. Rüdiger, Z. Naturforsch. **40c,** 693–698 (1985).
[19] C. R. Merril, D. Goldman, and M. L. Van Keuren, Methods Enzymol. **96,** 230–239 (1983).
[20] G. E. Means and R. E. Feeny, Chemical Modification of Proteins, Holden Day Inc., San Francisco, Cambridge, Amsterdam 1971.
[21] W. L. Butler, H. W. Siegelman, and C. O. Miller, Biochemistry **3,** 851–857 (1964).
[22] G. R. Stark, Methods Enzymol. **47,** 129–132 (1977).
[23] F. Thümmler and W. Rüdiger, Physiol. Plantarum **60,** 378–382 (1984).
[24] P. Eilfeld and W. Rüdiger, Z. Naturforsch. **40c,** 109–114 (1985).
[25] R. Grimm, F. Lottspeich, H. A. W. Schneider, and W. Rüdiger, Z. Naturforsch. **41c,** 993–1000 (1986).
[26] A. M. Jones and P. H. Quail, Biochemistry **25,** 2987–2995 (1986).
[27] T. R. Berkelman and J. C. Lagarias, Anal. Biochem. **156,** 194–201 (1986).

[28] P. H. Quail, J. T. Colbert, N. K. Peters, A. H. Christensen, R. A. Sharrock, and J. L. Lissemore, Phil. Trans. R. Soc. Lond. **B 314,** 469–480 (1986).
[29] G. Swoboda and W. Hasselbach, Z. Naturforsch. **40c,** 863–875 (1985).
[30] R. E. Hunt and L. H. Pratt, Biochemistry **20,** 941–945 (1981).
[31] E. M. Tobin and W. R. Briggs, Photochem. Photobiol. **18,** 487–495 (1973).
[32] M.-M. Cordonnier, H. Greppin, and L. H. Pratt, Plant Physiol. **80,** 982–987 (1986).
[33] Y. Shimazaki, M.-M. Cordonnier, and L. H. Pratt, Plant Physiol. **82,** 109–113 (1986).
[34] D. Ernst, F. Pfeiffer, Petra Eilfeld, Peter Eilfeld, W. Rüdiger, and D. Oesterhelt, Phytochrome regulation of mRNA levels of ribulose-1,5-bisphosphate carboxylase and light-harvesting chlorophyll a/b protein in etiolated rye seedlings (SECALE CEREALE), in: "Plant Molecular Biology", NATO ASI Series (von Wettstein and Chua, eds.), pp. 679–688, Plenum Publishing Corporation, New York 1987.