Investigation of the Peptide Chain of 124 kDa Phytochrome: Localization of Proteolytic Fragments and Epitopes for Monoclonal Antibodies

Rudolf Grimm*, Friedrich Lottspeich**, Hansjörg A. W. Schneider***, and Wolfhart Rüdiger*

* Botanisches Institut der Universität München, Menzinger Straße 67, D-8000 München 19
** Max-Planck-Institut für Biochemie-Genzentrum, Am Klopferspitz 18a, 8033 Martinsried bei München
*** Botanisches Institut der Universität Köln, Gyrhofstraße 15, D-5000 Köln 41

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Dedicated to Professor Dr. H. Zuber on the occasion of his 60th birthday

Avena sativa L., *Avena* Proteases, Trypsin, Microsequencing, Western Blot

Isolated oat phytochrome (124 kDa) was digested with endogenous proteases from oat and with trypsin. Fragments were isolated by SDS polyacrylamide gel electrophoresis (PAGE) blotted onto an activated glass fiber sheet and applied to microsequencing. Comparison of the amino terminal sequences of the fragments with the complete amino acid sequence derived from DNA sequence studies (Hershey *et al.*, Nucleic Acid Res. **13,** 8543–8560, [1985]) allowed the exact localization of each fragment. The reaction of the various fragments with monoclonal antibodies raised against 114/116 or 124 kDa oat or maize phytochrome were tested by means of Western blotting. Differential reactivity of the localized fragments allowed the localization of epitopes for several antibodies.

Introduction

The chromoprotein phytochrome is the main light receptor for photomorphogenesis in higher plants [1]. Proteolysis of phytochrome during extraction and purification by endogenous proteases [2, 3] has hampered investigation of phytochrome *in vitro* for a long time. Preparation of native (124 kDa) phytochrome was first described in 1983 [4, 5]. Subsequently, partial proteolysis of native phytochrome under controlled conditions was used to define separate domains in the structure of phytochrome with regard to monoclonal antibodies [6], to spectral properties [7] and to the quaternary structure [8]. Differences between the Pr and the Pfr form of phytochrome were also detected by partial proteolysis [9, 10]. Fragments of partial proteolysis were characterized so far only by their size. A cleavage at 6 and 10 kDa apart from the amino terminus was assumed mainly [6] because 124 kDa phytochrome has a blocked amino terminus whereas in a previous study amino terminal sequences KAL and ALV were reported for presumptively 118/114 kDa phytochrome [11]. However, the amino acid sequence of phytochrome predicted from cDNA and genomic DNA sequence analysis [12] did not contain the sequence ALV at all. The sequence KAL was not found in the expected region. The only directly determined amino acid sequence of a phytochrome fragment is that of an undecapeptide in the chromophore region [13, 14]. This sequence is in accordance with the sequence derived form DNA analysis [12].

We found it desirable to determine amino acid sequences near the amino and near the carboxyl terminus in order to confirm the sequence predicted from the DNA sequence. Furthermore, partial sequences of the large fragments obtained by partial proteolysis of phytochrome would be enable us to exactly localize these fragments within the entire phytochrome sequence. Consequently, also epitopes for monoclonal antibodies could be localized.

Abbreviations: EDTA, ethylenediaminotetraacetate; HPLC, high performance liquid chromatography; MOPS, morpholinopropanesulfonic acid; M_r, relative molecular mass; PAGE, polyacrylamide gel electrophoresis; Pr, Pfr, red, far red absorbing form of phytochrome; TFA, trifluoroacetic acid; Tris, tris(hydroxymethyl)aminomethane.

Reprint requests to Prof. Dr. W. Rüdiger.

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Materials and Methods

Phytochrome (124 kDa) was isolated from 3.5 days old etiolated oat seedlings (*Avena sativa* L.,

c.v. Pirol, Baywa, Munich) as previously described [15]. The specific absorbance ratio $A_{667}^{Pr}/A_{280}^{Pr}$ of the preparations used in the present study was between 0.90 and 0.99.

Isolated phytochrome in 10 mM phosphate buffer, pH 7.8, containing 5 mM EDTA and 5% glycerol ($A_{667}^{Pr}=0.70$) was incubated with endogenous proteases from oat. As a source for endogenous proteases, etiolated oat seedlings (15 g) were extracted with 30 ml of a buffer containing 25 mM MOPS, 25 mM Tris, and 14 mM mercaptoethanol (pH 7.5) and centrifuged at $30{,}000 \times g$ as described by Grombein and Rüdiger [16]. Of this "crude extract", 0.1 ml were added to 1.0 ml of the above described phytochrome solution. After addition of mercaptoethanol (final concentration 400 mM), the mixture was incubated at 20 °C for several periods of time. Aliquots were applied to SDS PAGE after 31 h and 99 h (see Fig. 1).

Complete proteolysis of phytochrome with trypsin (from pig, crystallized, 8.0 units/mg, Merck, Darmstadt) was achieved as follows:

The solution of purified phytochrome (Pr form) in 10 mM phosphate buffer (see above) was incubated with 5% trypsin for 66 h at 20 °C. The reaction was then stopped by acidification with formic acid to pH 2.6. The solution was evaporated. The residue was dissolved in 0.1% trifluoroacetic acid. Separation of peptides was achieved by HPLC on a Vydac column (Chrompack, Müllheim) with a gradient of 5 to 40% 0.1% aqueous trifluoroacetic acid in acetonitrile. Isolated fractions of this first column were rechromatographed on a ODS-2 column (Bischoff, Leonberg) using the same solvents but gradients from 5 to 50% or 5 to 65% TFA/acetonitrile.

Partial digestion of phytochrome with trypsin (from pig, crystallized, 8.0 units/mg, Merck) was performed under the following conditions:

The solution of purified phytochrome (Pr form) in 10 mM phosphate buffer (see above) was incubated in the dark with 0.5% (w:w) trypsin for 1 min at 20 °C for preparation of the 113, 59, 55 and 52 kDa fragments. These fragments proved to be heterogenous with regard to their amino terminus (see Table I). Incubation with 0.1% trypsin for 20 h at 4 °C yielded the 59 kDa fragment in accordance with [17]. This proved to be homogenous, starting with V-66 (see Table I). The 39 kDa fragment was obtained by incubation of Pr with 1% trypsin for 3–4 h at 20 °C; under these conditions, also 31 kDa and 23.5 kDa fragments were obtained. The same 39 kDa fragment was obtained by incubation of Pfr with 1% trypsin for 1 h at 20 °C [see 17].

Table I. Products of partial proteolysis of phytochrome with trypsin. 124 kDa oat phytochrome was digested with trypsin. The products were then separated by SDS gel electrophoresis. Bands were blotted onto an activated glass fiber sheet and partially sequenced.

M_r determined by SDS PAGE [kDa]	Sequence determined	Amino terminal amino acid	Carboxyl terminal amino acid*	Calculated M_r** [kDa]
113a***	DGPP	D-54	R-1093	114.3
113b***	SEKV	S-63	R-1093	113.3
113c***	VIAY	V-66	R-1093	113.0
59a***	SEKVIA	S-63	K-595/R-596	58.5
59b***	VIAYLQ	V-66	K-595/R-596	58.2
55a***	REASL	R-596	R-1093	54.7
55b***	EASLD	E-597	R-1093	54.6
52a***	SEKVI	S-63	K-537	52.1
52b***	VIAYL	V-66	K-537	51.8
52c***	LMETA	L-625	R-1093	51.6
39	VIAY	V-66	R-426	39.6
31	ALG	A-143	R-426	31.1
23.5a***	VIA	V-66	R-278 (K-283)	23.3 (23.9)
23.5b***	IQS	I-210	R-426	23.7

* Nearest trypsin cleavage site according to M_r determined by SDS PAGE.
** Calculated from distance amino terminal-carboxyl terminal amino acid.
*** SDS PAGE band proved by sequencing to be heterogenous.

The SDS PAGE was carried out according to Laemmli [18]. High pH electroblotting, essentially according to Aebersold *et al.* [19], was performed using a semi-dry electroblotter from Janos, Denmark and GF/C glass fiber sheets (Whatman) derivatized with N-trimethoxysilylpropyl-N,N,N-trimethylammonium chloride (Petrarch). Some minor modifications in the acid etching of the glass fiber sheets, the blotting buffer composition, and the staining procedure were introduced (C. Eckerskorn and F. Lottspeich, to be published). N-terminal amino acid sequence analyses were performed on a gas phase sequencer 470 A from Applied Biosystems. The phenylthiohydantoin amino acid derivatives were analysed by a HPLC system which separates all components isocratically [20].

Monoclonal antibodies were raised, selected and characterized as described elsewhere [21].

Results an Discussion

Trypsin digestion

Trypsin was used as a protease which cleaves the peptide chain at defined sites, namely at Arg and Lys residues. In order to check the agreement of the amino acid sequence predicted from DNA-sequences studies [12] and the actual amino acid sequence, we prepared a total tryptic digest of phytochrome. Two small peptides were isolated from the digest by HPLC and sequenced by the automatic microsequence method. The sequences determined by this method, EVFDLTGYDR (peptide 1) and FSPVGGSVEISS (peptide 2), agree with the predicted sequence. They constitute the amino acid residues 228* to 237 (peptide 1) and 1028 to 1040 (peptide 2), respectively, of the entire phytochrome sequence. The cleavage had apparently occured at Lys-227 and Arg-237 (for peptide 1) and at Lys-1027 and Lys-1040 (for peptide 2), in accordance with the enzyme specificity of trypsin. The agreement of the sequences of these small peptides with predicted sequences in amino terminal and carboxyl terminal parts of the entire peptide chain can be taken as confirmation of the correct assignment of DNA with amino acid sequences.

* The numbering used here included the initiator methionine in accordance with Fig. 4 in Hershey *et al.* [12] although it is not known whether or not this amino acid residue has been removed in the mature peptide chain. The tetrapyrrole chromophore is linked to Cys-322 in this notation.

Partial proteolysis with trypsin resulted in peptide fragment patterns (Fig. 1A) similar to those described by Lagarias and Mercurio [10] and by Jones and Quail [8]. Differences in the relative amounts of the various fragments may be due to somewhat different conditions of proteolysis: We used a 1:100 ratio, Lagarias and Mercurio [10] a 1:500 ratio of soluble trypsin: phytochrome; Jones and Quail [8] used immobilized trypsin. Both laboratories performed the digestion at 4–5 °C whereas we applied 20 °C for digestion.

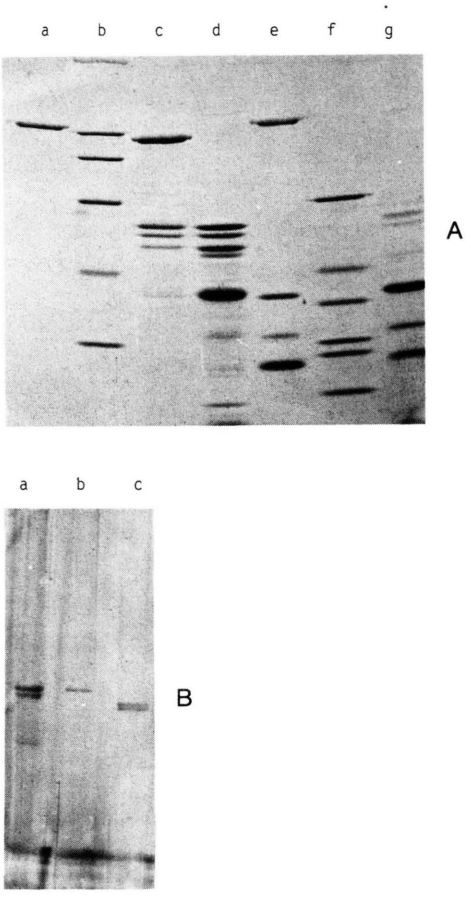

Fig. 1. SDS gel of native phytochrome and products of proteolysis. The Pr form was used if not otherwise stated. A. Degradation by trypsin. a: 124 kDa phytochrome; b: marker proteins 205, 116, 97.4, 66, 45 and 29 kDa; c: digestion with 0.5% trypsin at 20 °C for 1 min; d: 0.1% trypsin at 4 °C for 20 h; e: 1% trypsin at 20 °C for 3.5 h, 124 kDa phytochrome added to lane e; f: marker proteins 66, 45, 36, 29, 24 and 20 kDa; g: Pfr form digested with 1% trypsin at 20 °C for 1 h.
B. Degradation with endogenous proteases. a: incubation for 31 h; b: 124 kDa phytochrome; c: incubation for 99 h.

Several fragments were blotted from SDS gels onto activated glass fiber sheets and applied to microsequencing. The results (Fig. 1 A and Table I) show that all fragments can unequivocally be localized within the peptide chain of phytochrome. Three to six Edman degradation steps were applied to each investigated fragment. This was sufficient to localize the amino terminus of each fragment (see Table I). A preferred tryptic cleavage site is at K-65 which yields fragments with V-66 as amino terminal residue. This is true for the main fraction of a 113 kDa fragment; the 113 kDa fragments starting with S-63 and D-54 are minor components. After short incubation with trypsin, also the 59 and 52 kDa bands contain components starting with either S-63 or V-66. V-66 is amino terminal after prolonged incubation in 59, 39 and 23.5 kDa fragments. This means that the next few basic amino acids, namely K-75, K-77, K-91, R-121 and R-129, must be protected in these fragments. The next cleavage site (among major fragments) is at K-142 found in the 31 kDa fragment.

The position of the carboxyl terminal amino acids of the fragments can approximately be calculated, using M_r from SDS PAGE, as distance from the amino terminus of the respective fragment. Since the carboxyl terminus must be either K or R due to specificity of trypsin cleavage, the K or R residue next to the calculated carboxyl terminus is considered the most likely terminus.

The probability of such a consideration can be explained for a 23.5 kDa fragment. Starting with I-210, candidates for the carboxyl terminus are K-415, R-426 and K-445. The corresponding calculated M_r values are 22.5, 23.7 and 25.9 kDa. Nearest to the experimental M_r value of 23.5 kDa is the value calculated for R-426. This amino acid seems to be a preferred cleavage site for trypsin since calculated M_r values fit also for the 31 and 39 kDa fragments assuming R-426 as carboxyl terminus. The other 23.5 kDa fragment which starts at V-66 ends either at R-278 (calculated M_r 23.3 kDa) or at K-283 (calculated M_r 23.9 kDa). The experimental data do not allow to distinguish between these alternatives. We consider the next basic residues K-285 and R-287 as improbable candidates because the calculated M_r values are 24.1 and 24.3 kDa. According to the position in the gel, the fragment must be smaller than the 24.0 kDa marker protein. The most probable carboxyl terminus of the 59 kDa fragment is K-595/R-596 (calculated M_r 58.3 kDa). The nearest other candidates are K-608 (calculated M_r 59.6 kDa) and K-593 (calculated M_r 58.1 kDa). We assume that the position 595/596 is the preferred cleavage site because also the 55 kDa fragment results from fragmentation at this site (see Table I). The carboxyl terminus cannot be localized with high probability for the 55 kDa fragment which starts at R-596/E-597 and the 113 kDa fragment which mainly starts at V-66. The residues R-1093 and R-1097 would exactly fit with the determined M_r values of both fragments but other residues (R-1104, R-1107) would fit with the determined M_r value still within limits of error. The same is true for the 52 kDa fragment starting with L-625 (marked c in Table I). Also the carboxyl terminus of the other 52 kDa fragments (marked a and b in Table I) cannot exactly be determined. It could be K-537 (see Table I), R-532 (calculated M_r 51.3 kDa) or R-524 (calculated M_r 50.4 kDa).

A general conclusion from these digestion experiments is that certain regions of the peptide chain must be exposed, *i.e.* accessible for trypsin or become accessible after initial partial proteolysis. The regions around residues 54–66, around residue 426, around residues 595/596, and around residues 1093–1097 belong to this category. On the other hand, R and K containing residues which are still present in small fragments must be in the interior part of the protein, *i.e.* protected from trypsin attack. The remarkable sequence KKKK (residues 361–364) seems to belong to this category as well as about 30 further basic residues between position 66 and 426 including K-91 which was considered as a candidate for preferred protease attack by Hershey *et al.* [12].

Although partial proteolysis of 124 kDa phytochrome with trypsin and other defined proteases [10] yields different patterns of large fragments for Pr and Pfr, we obtained the identical pattern for Pfr and Pr in the region of 39, 31 and 23.5 kDa fragments (see Fig. 1 A). The 39 kDa fragment was applied to microsequencing. The determined sequence VIAY proved that the sites of proteolytic attack are identical for Pr and Pfr in this case. The same 39 kDa fragment can therefore be obtained from Pr (this paper) or (somewhat faster) from Pfr [17]. One has to assume that the first protease attack which is different for Pr and Pfr [10] leads to the exposure of further cleavage sites which then are identical for both phytochrome forms. Further R and K residues must

be somewhat protected even after initial proteolysis because further degradation of the 39 kDa fragment by trypsin is slow.

All investigated fragments, except the 55 kDa fragment starting with R-596/E-597, the 52 kDa fragment starting with L-625, and the 23.5 kDa fragment starting with V-66 contain the phytochromobilin chromophore (see Fig. 2). The spectral properties of the 113 kDa and 59 kDa fragments are known since a long time [16], those of the 39 kDa fragment have recently been described [17]. Both 39 and 59 kDa fragments have a "normal" Pr absorption spectrum. In the Pfr form, the 59 kDa fragment exhibits the "normal" 720 nm peak whereas this peak is missing in the 39 kDa fragment. Although the latter has only a broad band of low absorption ("bleached" phytochrome), it is fully photoreversible. The appearance of the 720 nm peak must be due to that part of the peptide chain which is present in the 59 kDa fragment but not in the 39 kDa fragment, namely residues 427 to 608. Whether the postulated point charge [22] is localized in this region or whether some other, eventually indirect, influence of this part of the peptide chain upon phytochromobilin induces this spectral difference, has still to be investigated.

The interaction of the peptide chain with the chromophore is small but still detectable in the 23.5 kDa fragment starting with I-210. After tryptic digestion of the Pr form, the Pr form of this fragment has an absorption peak at 667 nm with lower molar absorption than the parent 124 kDa Pr (Fig. 3). Irradiation with either red or far-red light induces bleaching of this peak which is irreversible. We conclude that there is only some residual stabilization of the "native" chromophore conformation in the Pr form but no stabilization whatsoever in the Pfr form.

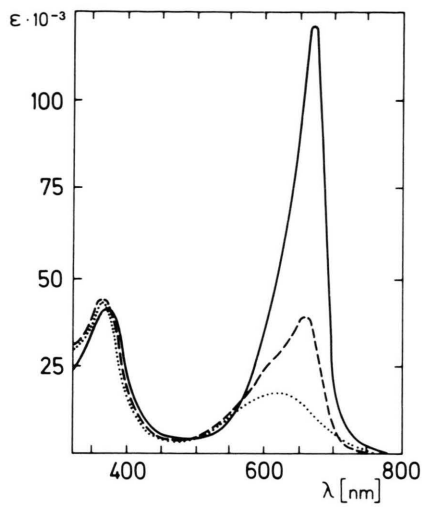

Fig. 3. Absorption spectrum of the 23.5 kDa fragment (I-210 to R-426) of phytochrome (Pr form) in comparison with the absorption spectrum of native (124 kDa) phytochrome. a: (———) 124 kDa phytochrome, Pr form; b: (---) the same after digestion with 10% trypsin in the dark at 20 °C for 23 h = 23.5 kDa fragment; c: (...) = b after saturating irradiation with HR.

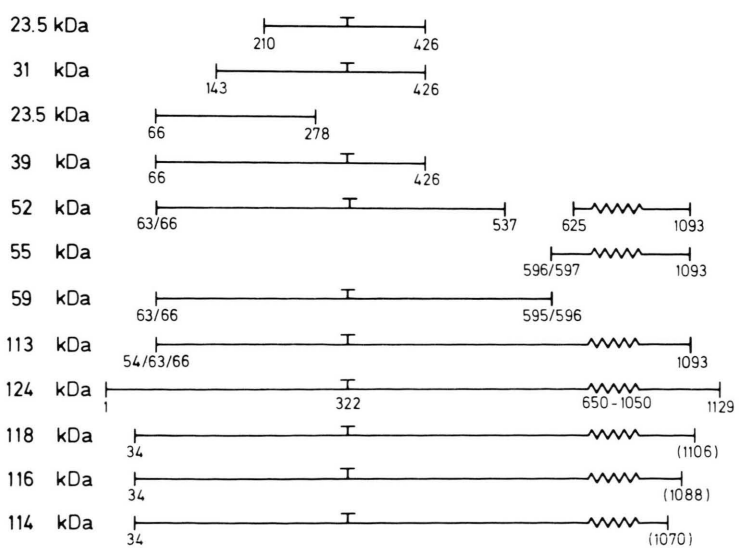

Fig. 2. Localization of proteolytic fragments within the complete amino acid sequence of phytochrome. Amino terminal sequences of fragments (see Table I) were used to localize the fragments within the sequence reported by Hershey *et al.* [12]. 124 kDa = native phytochrome. Upper part: fragments obtained with trypsin. Lower part: fragments obtained with endogenous proteases.

Endogenous proteases

Whereas most previous investigations [7, 9, 16] used the crude extract from oat shoots as a source for phytochrome as well as for proteases, we incubated isolated 124 kDa phytochrome with a small volume of crude extract in accordance with [6]. However, we used a much higher phytochrome concentration on a crude extract (*i.e.* proteases) base in our experiments as compared to previous works *e.g.* 10-fold higher than in [6]. Proteolysis was accordingly slow in our experiments; we obtained a 118 kDa fragment besides undigested 124 kDa phytochrome after 31 h and a double band at 116/114 kDa after 99 h (Fig. 1B). The double bands of both experiments were eluted and investigated by microsequence analysis. Surprisingly, all fragments (*i.e.* 114, 116 and 118 kDa bands) had a uniform amino terminal sequence, namely AEYE. The endogenous proteases must have cleaved between N-33 and A-34, *i.e.* all fragments start with residue 34 of the entire phytochrome sequence. Differences in the size of the fragments must therefore be due to differences in the carboxyl terminus. According to the fragment size, the carboxyl termini should be approximately at residues 1070, 1090 and 1110 for the 114, 116 and 118 kDa fragments, respectively.

Investigation of fragments with monoclonal antibodies

The exact localization of proteolytic fragments within the phytochrome amino acid sequence encouraged us to try to localize epitopes for monoclonal antibodies. For this purpose, Western blot analysis of the localized fragments was performed with a number of monoclonal antibodies raised against 114 to 124 kDa phytochrome from oat or maize [21]. The positive reaction of an antibody indicated that the corresponding epitope was present in the investigated fragment, the negative reaction indicated its absence (see Table II). All investigated antibodies react with 124 kDa phytochrome (not shown) and the largest fragment, namely the 118 kDa fragment obtained by endogenous proteases. Because

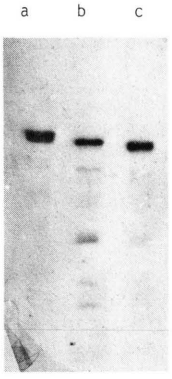

Fig. 4: Immunoblot of phytochrome fragments produced by endogenous oat proteases. Monoclonal antibodies [21] were used for the primary immunoreaction. a: digestion for 31 h, reaction with Z_2A_3. Staining of 124 and 118 kDa bands; b: digestion for 99 h, reaction with Z_3A_6. Only 116 kDa fragment is stained; c: digestion for 99 h, reaction with A_1B_3. 116 and 114 kDa fragments are stained.

Table II. Reaction of proteolytic fragments with several monoclonal antibodies. A or Z, at the beginning of the antibody numbering indicates antibodies raised against *Avena* or *Zea* phytochrome, respectively. Proteolytic fragments of isolated oat phytochrome were blotted from SDS-gels onto nitrocellulose and incubated with monoclonal mouse antibodies, then with polyclonal rabbit antimouse antibodies linked to alkaline phosphatase [23, 24]. The positive enzyme reaction indicated interaction of the monoclonal antibody with the respective fragment and hence the localization of the specific epitope on this fragment. Fragments were obtained either by endogenous proteases (A) or trypsin (B).

Fragment Size [kDa]	Source	Reaction with antibody							
		A_2A_3	Z_2D_2	Z_2B_4	Z_4A_5	Z_2A_3	Z_3A_6	Z_3B_1	Z_1C_4
118	A	+	+	+	+	+	+	+	+
116	A	−	−	−	−	+	+	+	+
114	A	−	−	−	−	−	−	+	+
59	B	−	−	−	−	−	−	+	+
39	B	−	−	−	−	−	−	+	+
31	B	−	−	−	−	−	−	+	−
23.5	B	−	−	−	−	−	−	+	+

this fragment has lost 33 amino acid residues of the amino terminus and approximately 20 residues of the carboxyl terminus of 124 kDa phytochrome (see above), we conclude that no epitope of the investigated antibodies is localized within this amino or carboxyl terminal site of phytochrome. Those antibodies, which react only with the 118 kDa fragment but not with the 116 kDa fragment must be specific for epitopes localized within the 2 kDa piece which makes up the difference between the 116 and 118 kDa fragments. According to the sequence analysis given above, this must be approximately between residues 1090 and 1110. Likewise, those antibodies which react with the 116 kDa fragment but not with the 114 kDa fragment, must be specific for epitopes localized approximately between residues 1070 and 1090. The localization of these epitopes is remarkable because Daniels and Quail [6] defined antibodies directed against 124 kDa phytochrome which do not recognize large fragments (in their case 118 and 114 kDa) as type 1 antibodies. They assume that type 1 epitopes are localized within a 6 kDa piece of the amino terminus of the entire sequence. This conclusion has now to be reconsidered because the preferred cleavage site of endogenous proteases is at residue 33. This means that only a 3.6 kDa piece will be cleaved from the amino terminal end; further fragmentation occurs from the carboxyl terminal site. Since we found a number of antibodies which are directed against these cleaved portions of the carboxyl terminal sequence it has to be asked again whether all antibodies previously identified as type 1 [6, 9] by this method are directed against epitopes in the amino terminal end. Localization of antibodies Z_3B_1 and Z_1C_4 was achieved in a similar manner (see Table II). It has to be recalled that the 23.5 kDa band obtained by SDS PAGE is heterogenous (see Table I). The epitope of Z_3B_1 must be in the sequence which is common to fragments 31 and 23.5 kDa, namely residues 210 to 426. This is the chromophore containing sequence. The epitope of Z_1C_4, on the other hand, must be localized in the sequence which is present in the (other) 23.5 kDa fragment but not in the 31 kDa fragment. From inspection of the data of Table I, this must be between residues 66 and approximately 143. The epitope could eventually reach somewhat into the 31 kDa fragment (*e.g.* to residue 144, 145 or 146) but only insofar that the epitope were not complete within the sequence of the 31 kDa fragment.

In conclusion, the method employed here enables us to localize epitopes for monoclonal antibodies with some precision in the peptide chain of phytochrome. With this knowledge of the particular epitopes, the antibodies can be used for detailed studies of structure-function relationships of phytochrome.

Acknowledgements

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Note added in proof: According to a recent report (A. M. Jones, Yamada Conference of Phytochrome and Photomorphogenesis, Okazaki, Oct. 1986), fragmentation of phytochrome with subtilisin yielded a 16 kDa chromopeptide with spectral properties similar to our 39 kDa fragment but different from our 23.5 kDa fragment (see Fig. 3). We conclude therefore that the chromophore-protein interaction does not depend on the fragment size but on the particular peptide sequence. The exact localization of the 16 kDa fragment within the phytochrome sequence is still pending.

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