A Photoreversible 39 kDalton Fragment from the P_{fr} Form of 124 kDalton Oat Phytochrome

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Dedicated to Professor Dr. H. Merxmüller *on the Occasion of His 65th Birthday*

Avena sativa L., Low Temperature Spectroscopy, Bleached Phytochrome, Dark Reversion

Limited proteolysis of 124 kdalton oat phytochrome in the P_{fr} form is described which leads to a photoreversible 39 kdalton fragment. Whereas the absorption maximum of the P_r form is only slightly shifted (from 667 to 660 nm) no long-wavelength band is observed in the P_{fr} form. A "bleached" (*i.e.* low absorbing) form appears instead with a broad absorption maximum at 640 nm. This property corresponds with that of a 40.3 kdalton fragment obtained from pea phytochrome by limited proteolysis (Yamamoto and Furuya, Plant and Cell Physiol. **24,** 713, 1983). Dark reversion of the bleached form to the P_r form is faster ($t_{1/2}$ = 101 min) than dark reversion of 118 kdalton or 60 kdalton P_{fr}. Low temperature spectroscopy of the 39 kdalton fragment showed that the intermediates lumi-R and meta-Ra are formed like in intact phytochrome or 60 kdalton or 114/118 kdalton fragments. It is discussed that limited proteolysis removed that part of the peptide chain which is responsible for the interaction with the P_{fr} chromophore but that the site for interaction with the chromophore of P_r and the intermediates lumi-R and meta-Ra is still intact.

Introduction

Phytochrome, the photoreceptor for many photomorphoses in higher plants, occurs in two forms which are mainly characterized by their absorption spectra [1]. The P_r form which is biosynthesized in the dark and is believed to be physiologically inactive has its absorption maximum at 667 nm. The physiologically active P_{fr} form which is formed by irradiation from the P_r form has its absorption maximum at about 730 nm. The position of the P_{fr} absorption maximum depends on the intactness of the protein: λ_{max} = 730–735 nm was found for native (124 kd) phytochrome whereas λ_{max} = 720–725 nm was found for partially degraded (114/118 kd and 60 kd) phytochrome [2–4]. Interestingly, limited proteolysis of P_{fr} can also lead to a partially degraded phytochrome form with λ_{max} = 733 nm [5] the size of which is 74 kd [6]. Further proteolysis of pea phytochrome yielded photoreversible fragments (40 kdalton, 39 kdalton and 33 kdalton) with nearly normal absorption spectra of the P_r form but with very low absorption (λ_{max} at about 650 nm) after red irradiation [7]. These bleached forms (P_{bl}) which appear instead of P_{fr} resemble the bleached intermediate meta-Rb on the pathway from P_r to P_{fr} [8–10]. We have demonstrated that the chromophore in this bleached intermediate and in chemically bleached forms is the 15(E) = (P_{fr}) chromophore without specific interaction with the protein [11–13]. It is possible that the specific interaction between P_{fr} chromophore and protein was lost in the 40 kdalton fragment by cleavage of the corresponding protein part. Whereas former attempts to prepare photoreversible fragments smaller than 60 kdalton from oat phytochrome failed [14] we describe here preparation and properties of a photoreversible 39 kdalton fragment from oat phytochrome.

Abbreviations: ANS, 8-anilinonaphthalene-1-sulfonate; EDTA, ethylenediaminotetraacetate; P_r, P_{fr}, red and far-red absorbing forms of phytochrome; P_{660}, red (= 660 nm) absorbing form of 39 kdalton phytochrome; P_{bl}, bleached form of 39 kdalton phytochrome; lumi-R, meta-Ra, meta-Rb, meta-Rc, phytochrome intermediates; SDS, sodium dodecylsulfate.

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Materials and Methods

Native phytochrome (124 kdalton) was isolated from 3 days old oat seedlings (*Avena sativa* L., cv. Pirol, Baywa, Munich) according to the method of

Vierstra and Quail [4] as modified by Eilfeld and Rüdiger [10]. The isolated phytochrome was, according to SDS gel electrophoresis [10] of at least 95% purity and had a molecular weight of 123 ± 2 kdalton. The A_{667}/A_{280} ratio was 0.65–0.7. This is less than the reported value of 0.92–0.94 for pure 124 kdalton phytochrome [4, 15] presumably because our preparation showed some aggregation which caused increased absorbance preferentially at short wavelengths.

The phytochrome sample (1.36 mg = 11 nmol) was dissolved in 1.7 ml 10 mM potassium phosphate buffer, pH 7.8, containing 1 mM EDTA and 7 mM mercaptoethanol, irradiated with 660 nm light (3 min, 64 Wm^2 [13]) and incubated with 25 µg trypsin (from pig pancreas, 8 units/mg, Merck) at 25 °C under green safelight for 30 min. At this time, the P_{fr} peak has nearly disappeared in the absorption spectrum (*cp.* Fig. 1). The proteolysis was then stopped by adding 400 µg trypsin inhibitor (from soybean, 70 units/mg, Boehringer, Mannheim). The mixture was incubated at 25 °C for 15 min in order to complete formation of the enzyme-inhibitor complex, the last 6 min thereof under far-red light in order to revert the P_{bl} completely to the P_r form. The mixture was then applied to a column (1.8 × 80 cm) filled with Sephadex G-100 (40–120 µ, Pharmacia Fine Chemicals). The column was washed with the incubation buffer (see above) at a flow-rate of 2.2 ml/h. Fractions of 2 ml were collected and characterized by absorption spectroscopy and SDS-polyacrylamide disk gel electrophoresis according to [10] but with 12% acrylamide. Glycerol (10% v/v) was added to the sample used for low temperature spectroscopy. The sample was then concentrated by lyophylization to a final glycerol concentration of 66% (v/v).

60 kdalton phytochrome for low temperature spectroscopy was isolated according to the procedure of Rüdiger *et al.* [16]. 114/118 kdalton phytochrome was obtained from 124 kdalton phytochrome: crude hydroxyapatite eluate ($A_{667}/A_{280} \sim 0.03$, still containing endogeneous proteases) was stored in the P_r form at 4 °C for 24 h. This material was applied to a Bio-gel A – 1.5 m column, yielding 114/118 kdalton phytochrome (λ_{max} (P_{fr}) = 722 nm; further spectral properties see Table I) eluting at approximately the same volume as 124 kdalton phytochrome. Irradiation at 660 or 730 nm, respectively for low temperature spectroscopy was performed as previously described [10, 13].

Results and Discussion

The time course of proteolysis of P_{fr} (124 kdalton) can easily be followed by spectroscopy. This contrasts to proteolysis of P_r which is accompanied by only minor changes in the absorption spectrum [3, 7]. In Fig. 1, a typical example of the spectral changes during digest of P_{fr} is given. These spectral changes shall be correlated with the formation of definite species in the following section.

First, the absorption maximum of P_{fr} is shifted from 730 nm to 720 nm (Fig. 1, a → b, c). This corresponds to the formation of a chromophore containing fragment with mol. weight 58 ± 2 kdalton, which can be purified by gel filtration and characterized by SDS gel electrophoresis (not shown). Spectral prop-

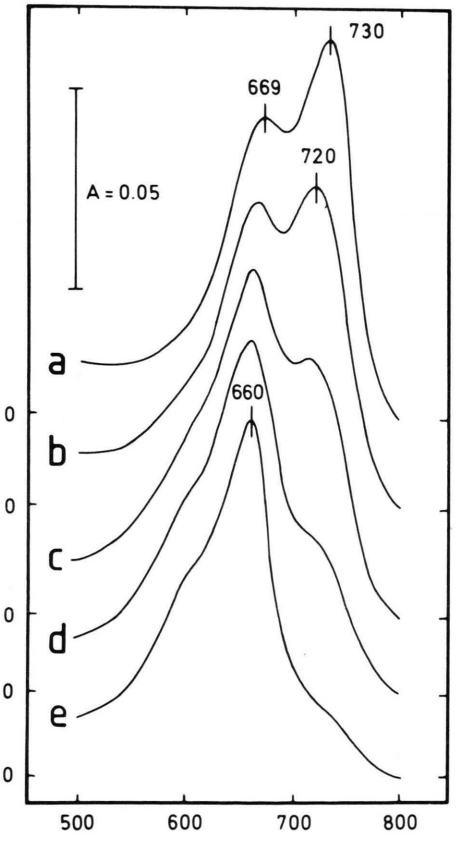

Fig. 1. Changes of the absorption spectrum (5 cm cuvettes) of red irradiated phytochrome during digestion with trypsin. 500 µg phytochrome (124 kdalton) in 15 ml of 10 mM potassium phosphate buffer, pH 7.8, were irradiated with red light (660 nm, 64 Wm^2) for 3 min and then incubated with 80 µg (0.64 units) of trypsin at 22 °C. Absorption spectra were recorded at $t = 0$ (a), $t = 6$ min (b), $t = 14$ min (c), $t = 35$ min (d) and $t = 70$ min (e).

erties are identical with the data reported for "small phytochrome" (60 kdalton, λ_{max} = 720 nm in the P_{fr} form [3, 7]). No 114/118 kdalton phytochrome could be detected under the conditions applied. This is in agreement with the report on short tryptic digest by Lagarias and Mercurio [17], who obtained the 114 kdalton fragment from P_r but predominately smaller fragments from P_{fr}.

Second, prolonged incubation of P_{fr} with trypsin leads to a decrease of the absorption above 700 nm (Fig. 1, curve c → e). In this degradation step a chromophore containing fragment with a mol. weight of 39 ± 2 kdalton is the main product. For isolation of this product, further proteolysis was stopped by the addition of excess trypsin inhibitor. Various isolation procedures were tested [18]. The most effective procedure proved to be gel chromatography on Sephadex G-100. Fractions with the highest A_{660} were collected and investigated by SDS gel electrophoresis.

The SDS gel electrophoresis of the purest fraction obtained by using only small amounts of trypsin and inhibitor is shown in Fig. 2. Its absorption spectrum (Fig. 3) is identical in the visible range with the spectrum of a 40.3 kdalton fragment reported in [7] despite the different starting material ([7]: 114 kdalton pea phytochrome P_r form; present work: 124 kdalton oat phytochrome P_{fr} form and different reaction conditions). The absorption maximum is found at 660 nm after far-red irradiation. The spectrum of this form (P_{660} [7]) differs from that of native P_r (λ_{max} = 667 nm) but is similar to that of P_r after binding of ANS (λ_{max} = 660 nm [13] or partial denaturation with urea [19]). We assume that the non-covalent binding sites of the chromophore are similar in all cases but that the rigidity of native P_r protein is decreased by either limited proteolysis, ANS binding or partial urea denaturation. This should lead to weaker chromophore-protein interaction, detectable by the shift from 667 to 660 nm.

The absorbance at 660 nm increased at prolonged incubation (2–7 h) in the dark due to dark reversion of the P_{bl} form (see below; curve a in Fig. 3). Short irradiation with far-red light (730 nm) decreased the absorption by 15% (curve b in Fig. 3) because a photoequilibrium between P_{660} and some bleached form (P_{bl}) was established. Most P_{bl} was formed by short red irradiation (660 nm, curve c in Fig. 3). Starting with this P_{bl}, the photoequilibrium of curve b was reestablished by 30 min irradiation at 730 nm.

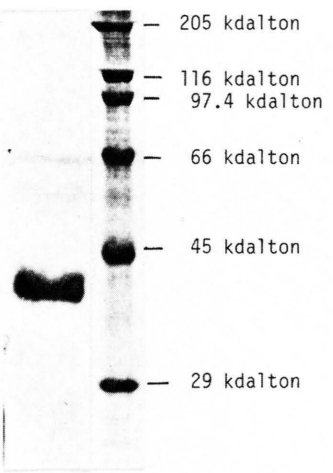

Fig. 2. SDS gel electrophoresis (12% acrylamide) of proteolytically degraded phytochrome (18 µg trypsin per 1 mg phytochrome). Digestion was stopped by addition of trypsin inhibitor (290 µg), the mixture was then applied to a Sephadex G-100 column. lane a: purest fraction of 39 kdalton phytochrome. lane b: marker proteins (from SIGMA).

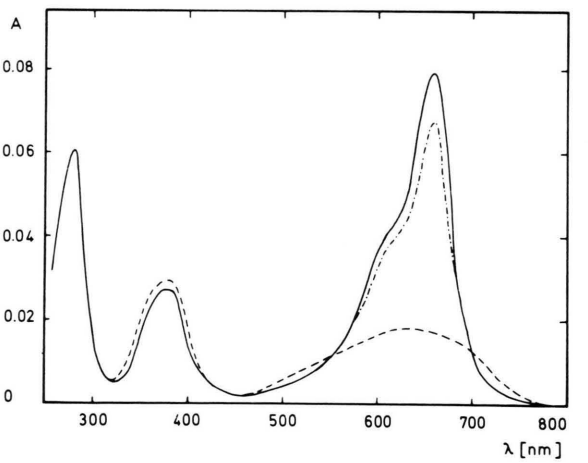

Fig. 3. Absorption spectrum of purified 39 kdalton phytochrome fragment (see Fig. 2).
a: (———) P_r form after completed dark reversion (incubation for 48 h at 4 °C in the dark).
b: (–.–.–) photoequilibrium obtained from the P_r form by irradiation at 730 nm (31 Wm^2) for 3 min. The same spectrum was also obtained from the P_{bl} form by irradiation at 730 nm for 30 min.
c: (– – – –) P_{bl} form obtained by irradiation at 660 nm for 2 min.

We conclude from these observations that full photoreversibility exists between P_{660} and P_{bl}. This agrees with the properties of the 40.3 kdalton fragment from pea phytochrome [7].

The P_{bl} form showed rapid dark reversion to P_{660} (Fig. 4). This is responsible for the increase of A_{660} observed during proteolysis (Fig. 1, curve b–e). Prolonged incubation in the dark gives quantitative yield of P_{660} from P_{bl} (Fig. 4). On the contrary dark reversion for 60 kdalton and 114/118 kdalton phytochrome stops when a certain value of P_{fr} is reached [20]. Only one kinetic component was found for dark reversion of 39 kdalton P_{bl} to P_{660} with a half time of 101 ± 1.5 min at 25 °C. At least two kinetic components had been reported for 60 kdalton and 114/118 kdalton phytochrome [21–23]. It had been suggested that only the slower component ($t_{1/2}$ = 300–600 min) is true dark reversion of P_{fr} whereas the faster component ($t_{1/2}$ = 13–28 min) is dark reversion of an intermediate to P_r [24]. It seems that dark reversion is accelerated by stepwise degradation of phytochrome; in small chromopeptides [11] it is even faster than in the 39 kdalton fragment described here. This corresponds to increased stabilization of the P_{fr} chromophore with increasing length, *i.e.* intactness of the protein chain.

More detailed information about the chromophore-protein interaction can be obtained by studying the photoconversion at low temperature. The absorption difference spectra (Fig. 5) clearly show that the first two intermediates, lumi-R and meta-Ra, occur upon red irradiation of P_{660} in the 39 kdalton fragment. At higher temperatures, *i.e.* above −35 °C, only P_{bl} is formed, no species with absorption maximum above 700 nm can be observed.

In Table I, spectral data of the intermediates of several phytochrome species (39, 60, 114/118 and 124 kdalton) are compared with each other. The intermediates lumi-R and meta-Ra are formed in all species in about the same amounts except for the 39 kdalton species which forms less lumi-R. At higher temperature, all degraded phytochrome species form P_{bl} as intermediate whereas native phytochrome forms meta-Rc instead [10]. P_{bl} is the final product in the 39 kdalton species.

Thus we conclude that the 39 kdalton fragment still contains all amino acids which are responsible for the spectral properties of P_{660}, lumi-R and meta-Ra whereas the chromophore-protein interactions

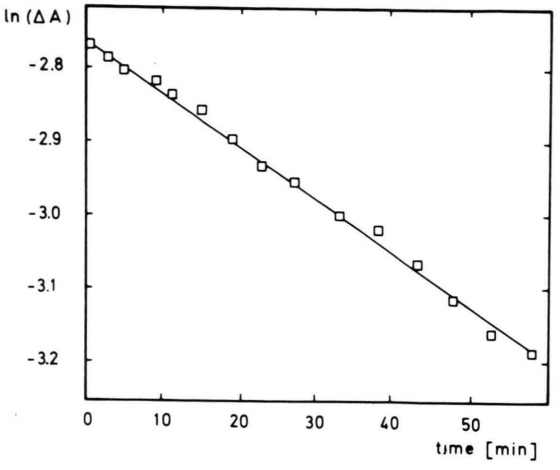

Fig. 4. Dark reversion of the P_{bl} form of the 39 kdalton phytochrome fragment. The P_{bl} form (see Fig. 3c) was incubated in the dark at 25 °C. The increase of absorbance was determined and plotted as ln (ΔA) with $\Delta A = A_{660}(\infty) - A_{660}(t)$. The value $A_{660}(\infty)$ is given in Fig. 3a.

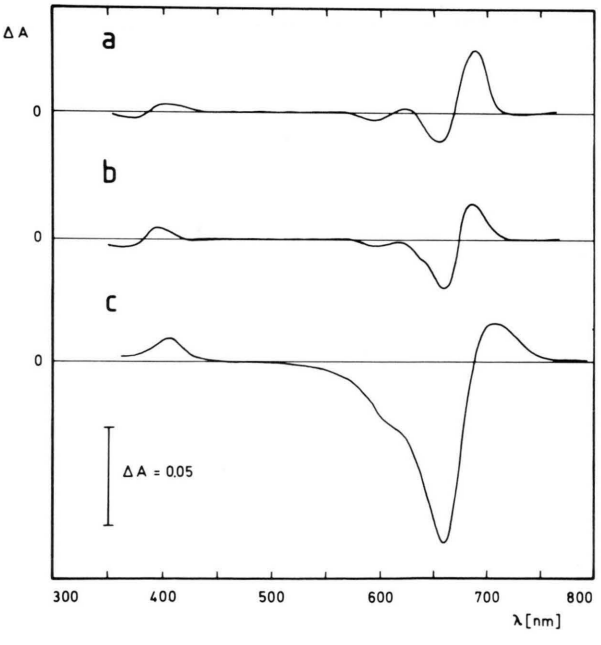

Fig. 5. Absorption difference spectra of red irradiated P_{660} minus unirradiated P_{660} at different temperatures for 39 kdalton phytochrome (buffer, irradiation lamp see Materials and Methods, saturating irradiation, A_{660} of the sample ~ 0.15 at 20 °C, spectra not corrected for temperature dependence of the absorption spectra).
a) Formation of lumi-R at −136 °C, b) formation of Meta-R_a at −73 °C, c) formation of Meta-R_b (= P_{bl}) at −37 °C.

Table I. Formation of intermediates determined by absorption difference spectra of various phytochrome species at low temperatures. Solvent: 100 mM potassium phosphate buffer, pH 7.8, diluted with glycerol (66% v/v).

	39 kdalton	60 kdalton	114/118 kdalton	124 kdalton
I) Lumi-R[a]				
T (°C)	−136	−117	−120	−120
$\lambda_1(\Delta A_1)$[b]	690 (0.14)	693 (0.38)	693 (0.40)	694 (0.40)
$\lambda_2(\Delta A_2)$	657 (−0.06)	660 (−0.11)	661 (−0.14)	663 (−0.14)
II) Meta-R_a				
T (°C)	−73	−60	−70	−65
$\lambda_1(\Delta A_1)$	694[c] (0.10)	710[d] (0.05)	695[c,d] (0.06)	700[d] (0.04)
$\lambda_2(\Delta A_2)$	660 (−0.14)	662 (−0.15)	663 (−0.17)	666 (−0.16)
III) Meta-R_b/Meta-R_c[e]				
T (°C)	−23	−26	−23	−35
$\lambda_1(\Delta A_1)$	710 (0.09)	703 (0.04)	710 (0.08)	716 (0.29)
$\lambda_2(\Delta A_2)$	660 (−0.64)	662 (−0.58)	663 (−0.61)	666 (−0.62)
IV) P_{fr}				
T (°C)	−	> −10	> −10	> −25
$\lambda_1(\Delta A_1)$	−[f]	720 (0.46)	722 (0.46)	730 (0.64)
$\lambda_2(\Delta A_2)$	−[f]	662 (−0.53)	664 (−0.53)	666 (−0.61)

[a] Formation for lumi-R is photoreversible for all species.
[b] λ_1, λ_2 = peaks of absorbance difference spectrum; ΔA_1 = normalized absorbance difference at λ_1, defined as absorbance at λ_1 after saturating red irradiation minus absorbance at λ_1 before irradiation, normalized to absorbance at 667 nm (P_r) or 660 nm (P_{660}), respectively.
[c] Increase of absorption partially arising from residual lumi-R.
[d] Broad absorption peak.
[e] Meta-R_c is found for 124 kdalton only.
[f] No P_{fr} formed with 39 kdalton phytochrome at any temperatures.

for the formation of meta-Rc [10] and P_{fr} are destroyed. This could mean either removal of that part of the polypeptide chain which exerts this interaction or a change of the tertiary structure such that no interaction occurs any more. This result supports the assumption [13, 25] that the non-covalent chromophore binding site of the protein is different in P_{fr} from that in P_r (or P_{660}), lumi-R and meta-Ra.

The chromophore-protein interaction in P_{fr} leading to the long-wavelength absorption (730 nm in 124 kdalton phytochrome, 720 nm in the 114/118 and 60 kdalton fragments) also facilitates photoconversion. Far-red irradiation yields P_r above −45 °C but intermediates below this temperature [10]. Starting from P_{bl}, far-red irradiation yields P_{660} only above −25 °C but does not change the absorption spectrum below this temperature. No intermediate can be detected at any temperature.

In summary, P_{fr}-specific chromophore-protein interaction seems to have two functions: facilitating P_{fr} formation and its backward photoconversion. Further experiments, especially sequence analysis, shall reveal which part of the polypeptide chain is responsible for the P_{fr}-specific chromophore-protein interaction.

Acknowledgements

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