Partial Proteolysis of Rice Phytochrome: Comparison with Oat Phytochrome

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Phytochrome was isolated from etiolated rice seedlings (*Oryza sativa* L.) by slight modification of the procedure for oat phytochrome. Spectral data of rice phytochrome are comparable with those of oat and rye phytochrome. Controlled proteolysis with endoproteinases Lys-C and Glu-C yielded defined fragments some of which were different for Pr and Pfr. The fragments were identified by comparison with the corresponding fragments of oat phytochrome and by comparison of the amino acid sequences of rice and oat phytochrome. Regions of the peptide chain which are differently exposed in Pr and Pfr were identified. A highly conserved sequence around residues 740–750 is discussed as candidate for an "active center" of signal transduction.

Introduction

The photoreceptor phytochrome is widely distributed in the plant kingdom [1–5]. Whereas physiological effects have been determined in many plants, isolation of the chromoprotein has been performed only from a few plant species, *e.g.* oat, rye, pea and zucchini (review: [1]). Most investigations on the isolated chromoprotein delt with phytochrome from etiolated oat seedlings which are a relatively rich source for phytochrome. Conformational changes of oat phytochrome due to photoconversion Pr ⇋ Pfr have been demonstrated by several methods, *e.g.* by circular dichroism [6, 7], by binding of monoclonal antibodies [8], and by differences in the fragment patterns after partial proteolysis of Pr and Pfr [9]. By microsequencing of fragments, we were able to map exposed and interior regions of the peptide chain of native oat phytochrome in the Pr and Pfr forms [10, 11]. This approach is extended here to phytochrome from etiolated rice seedlings. The sequence of the rice phytochrome gene has recently been published [12, 13]. By comparison of our experimental data with the amino acid sequence derived from the published nucleotide sequence, we were able to correlate the properties of rice phytochrome with its molecular structure.

Abbreviations: EDTA, ethylenediaminetetraacetic acid; HEPES, N-2-hydroxyethylpiperazine-N′-2-ethanesulfonic acid; kDa, kilodalton; PAGE, polyacrylamide gel electrophoresis; PMSF, phenylmethylsulfonylfluoride; Pr, Pfr, red, far red absorbing form of phytochrome; SDS, sodium dodecylsulfate.

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Materials and Methods

Rice seeds (*Oryza sativa* subsp. *japonica* var. Nongken 58) were immersed in water and kept under moist conditions for one day at 32 °C. The imbibed seeds were then placed onto moist vermiculite and germinated in the dark for 5 days at 27 °C. Harvesting of seedlings (about 1 kg fresh weight), extraction and isolation of phytochrome followed essentially the procedure of Grimm and Rüdiger [14] for phytochrome from oat seedlings. This procedure implied extraction with ethylene glycol containing Tris-buffer, precipitation of contaminants with polyethyleneimine, precipitation of phytochrome with ammonium sulfate, chromatography on a hydroxyapatite column, precipitation of the pooled phytochrome fractions with ammonium sulfate and washing with phosphate buffer. The following modifications were applied for rice phytochrome: Pre-irradiation of seedlings was omitted, the supernatant after polyethyleneimine precipitation was irradiated instead with red light for 10 min. The pH of the extraction buffer was 8.5 (instead of 8.3). The phytochrome pellet obtained by ammonium sulfate precipitation after hydroxyapatite chromatography was further purified by washing with 10 mM potassium phosphate buffer, pH 7.8 and then with 200 mM and finally with 100 mM phosphate buffer, pH 7.8 (0.8 ml of each buffer per unit of phytochrome). The purification

with polyvinylpyrrolidone was omitted. The final pellet was dissolved in 2 mM HEPES buffer containing 5 mM EDTA, 14 mM 2-mercaptoethanol and 5% (v/v) glycerol.

Oat phytochrome was isolated from 3.5 day old etiolated oat seedlings (*Avena sativa* L., cv. Priol, Baywa, Munich, F.R.G.) as previously described [14]. For digestion of phytochrome the following proteases were used: endoproteinase-Glu-C (from *Staphylococcus aureus* V 8, 20 $U \times mg^{-1}$), endoproteinase-Lys-C (from *Lysobacter enzymogenes*, 30 $U \times mg^{-1}$) and endoproteinase-Asp-N (from *Pseudomonas fragi*) purchased from Boehringer (Mannheim, F.R.G.). Phytochrome samples in either 2 mM HEPES or 10 mM phosphate buffer, pH 7.8, containing 5 mM EDTA, 14 mM 2-mercaptoethanol and 5% (v/v) glycerol were incubated with the first 2 proteinases (1% w/w) at 20 °C, with endoproteinase-Asp-N (0.1% w/w) at 4 °C without EDTA in the dark for the indicated time. The digestion was stopped by addition of sample buffer and heating for 5 min at 95 °C. The sample buffer contained 65 mM Tris, pH 6.8, 5% v/v 2-mercaptoethanol, 2% (w/v) SDS. 15% (v/v) glycerol, 0.5% (w/v) bromophenolblue and 4 mM PMSF. SDS-PAGE was carried out according to Laemmli, 1970 [15]. Discontinuous nondenaturing gel electrophoresis and isoelectric focusing in agarose gels was performed as previously described [16]. Phytochrome spectra were recorded with a Hewlett Packard HP 8451 diode array spectrophotometer.

Results and Discussion

Phytochrome of etiolated rice seedlings behaves similarly as phytochrome of etiolated oat seedlings with only some exemptions which have to be taken into account for isolation of the protein. We found that more phytochrome can be extracted from etiolated rice seedlings without preirradiation than from preirradiated seedlings. We did not yet check whether phytochrome undergoes a very rapid destruction in rice seedlings. It could as well be that the Pfr form becomes bound to membranes or other cellular structures and can therefore not be extracted as easily as the Pr form. The Pr and Pfr forms behave somewhat differently during chromatography on hydroxyapatite. In order to have comparable conditions for oat and rice phytochrome on the hydroxyapatite column, we transformed the Pr form of rice phytochrome into the Pfr form in the crude extract after polyethyleneimine precipitation of contaminants.

The absorption spectrum of isolated rice phytochrome is shown in Fig. 1. According to the absorption ratio A_{664}/A_{280} (rice phytochrome: 0.5; oat phytochrome: 0.8–0.9), we have to assume that the rice preparation contains still some contaminating proteins. However, only traces of other protein bands are detectable on SDS PAGE of rice phytochrome (see Fig. 2). The spectral data, summarized in Table I, are comparable with those of oat and rye phytochrome [17]. A blue shift of 2–4 nm is generally observed for rice phytochrome. This is not due to partial proteolysis as deduced from the ratios A(Pfr) $\lambda_{max}/A_{red\ shoulder}$ and A(Pfr) λ_{max} (Table I) and from SDS PAGE.

Rice phytochrome migrates in SDS PAGE (see Fig. 2) as a single, homogeneous band of the apparent size of 120 kDa. It looks therefore somewhat smaller than oat phytochrome which has an apparent size of 124 kDa (Fig. 2). This is surprising because both proteins contain about the same number of amino acid residues (rice: 1128; oat 1129) and about the same calculated molecular weight (rice: 125287 Da; oat 124987 Da, all values without tetrapyrrole chromophore). The same discrepancy has been found for zucchini phytochrome in SDS PAGE which migrates as 120 kDa band [18]: from the amino acid analysis, 124 950 Da were calculated [19]. A possible explanation could be differential binding of SDS. This is not directly evident, however, from the amino acid composition or sequence. The hydropathy plot (not shown) is nearly identical for rice and

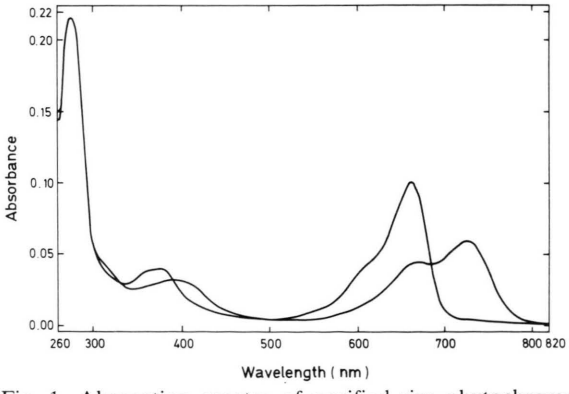


Fig. 1. Absorption spectra of purified rice phytochrome (A_{664}/A_{280} = 0.5) in 2 mM HEPES, pH 7.8, containing 14 mM 2-mercaptoethanol, 5 mM EDTA and 5% (v/v) glycerol. Spectra were recorded after saturating red (Pfr) or far-red (Pr) irradiations.

Table I. Comparison of spectral data of phytochrome from various plants.

Absorption spectra	Rice	Oat		Oat	Oat	Rye	Zucchini
Ref.		– this paper –			[17]	[17]	[18]
Buffer	A	A		B	C	C	D
Pr λ_{max} (red)	664	662	662	664	668	666	666
Pr λ_{max} (blue)	380	378	378	380	381	381	379
Pfr λ_{max} (red)	728	728	728	730	730	734	730
Pfr λ_{max} (blue)	394	402	402	402	402	402	400
A(Pfr) $\lambda_{max}/A_{red\ shoulder}$	1.38	1.5	1.5	1.45	1.33	1.46	1.47
A(Pfr) λ_{max}/A(Pr) λ_{max}	0.58	0.6	0.6	0.63	0.56	0.581	0.59
A(Pr) $\lambda_{max}/A(Pr)_{280}$	0.5	0.81	0.86	0.8	0.9–1.0	1.0–1.1	0.18

Buffers: A = 2 mM HEPES, pH 7.8; 5 mM EDTA, 14 mM 2-mercaptoethanol, 5% (v/v) glycerol.
B = 10 mM phosphate buffer, pH 7.8, 5 mM EDTA, 14 mM 2-mercaptoethanol, 5% (v/v) glycerol.
C = 50 mM N-methylmorpholine acetate buffer, pH 7.8
D = 100 mM phosphate buffer, pH 7.8, 1 mM EDTA, 14 mM 2-mercaptoethanol.

oat phytochrome; oat phytochrome contains only 5 more hydrophobic and 4 less hydrophilic amino acids than rice phytochrome. This consideration does not take into account any secondary modification of phytochrome. It should be recalled here, that oat phytochrome contains one phosphate risidue per peptide chain [20] whereas previously reported carbohydrate content is doubtful [21]. No corresponding data are available for rice phytochrome at the moment. If there were a difference between oat and rice phytochrome, this should reside in the C-terminal part of the peptide chain because the N-terminal fragments of both proteins correspond to each other.

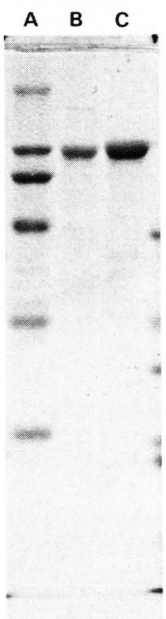
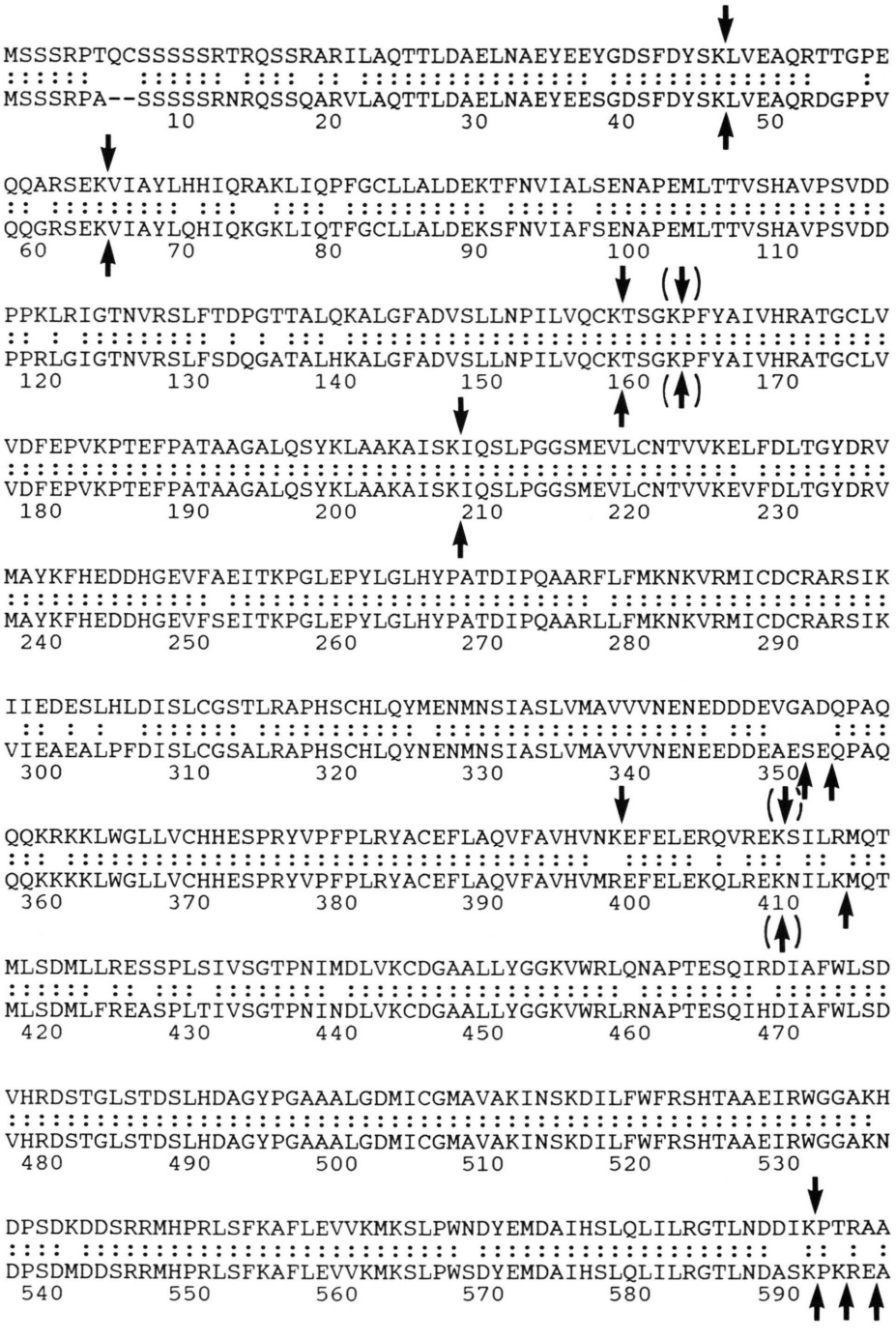


Fig. 2. SDS gel electrophoresis of rice and oat phytochrome. Gels containing 9% acrylamide were prepared according to Laemmli [15] and stained with Coomassie Blue R. Lane A: molecular weight markers, from above: 205, 116, 97, 66, 45, 36 kDa. Lane B: phytochrome from rice. Lane C: phytochrome from oat.

For proteolytic fragmentation of rice phytochrome we chose 2 enzymes which had been shown to produce distinctly different patterns with the Pr and Pfr forms of oat phytochrome, namely endoproteinase-Lys-C and endoproteinase-Glu-C. The comparison of rice and oat phytochrome was essential for these experiments because mapping of cleavage sites by microsequencing was performed only with oat phytochrome. Since about 90% of the amino acid sequence are identical (see Fig. 3) and the hydropathy plot (not shown) nearly superimposible, we assume that the same preferred cleavage sites exist in both phytochromes presumed that suitable amino acid residues are in the same positions. If the corresponding fragments of rice phytochrome have not the same size as those of oat phytochrome we looked for suitable amino acid residues in different positions predicted by the size of the fragments. The results are shown in Fig. 3 and 4 and – together with its evaluation – in Table II.

Several fragments in the digest obtained with endoproteinase Lys-C show identical sizes for oat and rice phytochrome. This is true for the Pfr specific 81 and 64 kDa fragments. The size of the former fragment had previously been determined as 83 kDa and the most probable cleavage site as K-753 [11]. According to the size of 81 kDa, the residue K-744 is a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MSSSRPA--SSSSSRNRQSSQARVLAQTTLDAELNAEYEESGDSFDYSKLVEAQRDGPPV
10 20 30 40 50
QQARSEKVIAYLHHIQRAKLIQPFGCLLALDEKTFNVIALSENAPEMLTTVSHAVPSVDD
QQGRSEKVIAYLQHIQKGKLIQTFGCLLALDEKSFNVIAFSENAPEMLTTVSHAVPSVDD
60 70 80 90 100 110
PPKLRIGTNVRSLFTDPGTTALQKALGFADVSLLNPILVQCKTSGKPFYAIVHRATGCLV
PPRLGIGTNVRSLFSDQGATALHKALGFADVSLLNPILVQCKTSGKPFYAIVHRATGCLV
120 130 140 150 160 170
VDFEPVKPTEFPATAAGALQSYKLAAKAISKIQSLPGGSMEVLCNTVVKELFDLTGYDRV
VDFEPVKPTEFPATAAGALQSYKLAAKAISKIQSLPGGSMEVLCNTVVKEVFDLTGYDRV
180 190 200 210 220 230
MAYKFHEDDHGEVFAEITKPGLEPYLGLHYPATDIPQAARFLFMKNKVRMICDCRARSIK
MAYKFHEDDHGEVFSEITKPGLEPYLGLHYPATDIPQAARLLFMKNKVRMICDCRARSIK
240 250 260 270 280 290
IIEDESLHLDISLCGSTLRAPHSCHLQYMENMNSIASLVMAVVVNENEDDDEVGADQPAQ
VIEAEALPFDISLCGSALRAPHSCHLQYNENMNSIASLVMAVVVNENEEDDEAESEQPAQ
300 310 320 330 340 350
QQKRKKLWGLLVCHHESPRYVPFPLRYACEFLAQVFAVHVNKEFELERQVREKSILRMQT
QQKKKKLWGLLVCHHESPRYVPFPLRYACEFLAQVFAVHVMREFELEKQLREKNILKMQT
360 370 380 390 400 410
MLSDMLLRESSPLSIVSGTPNIMDLVKCDGAALLYGGKVWRLQNAPTESQIRDIAFWLSD
MLSDMLFREASPLTIVSGTPNINDLVKCDGAALLYGGKVWRLRNAPTESQIHDIAFWLSD
420 430 440 450 460 470
VHRDSTGLSTDSLHDAGYPGAAALGDMICGMAVAKINSKDILFWFRSHTAAEIRWGGAKH
VHRDSTGLSTDSLHDAGYPGAAALGDMICGMAVAKINSKDILFWFRSHTAAEIRWGGAKN
480 490 500 510 520 530
DPSDKDDSRRMHPRLSFKAFLEVVKMKSLPWNDYEMDAIHSLQLILRGTLNDDIKPTRAA
DPSDMDDSRRMHPRLSFKAFLEVVKMKSLPWSDYEMDAIHSLQLILRGTLNDASKPKREA
540 550 560 570 580 590

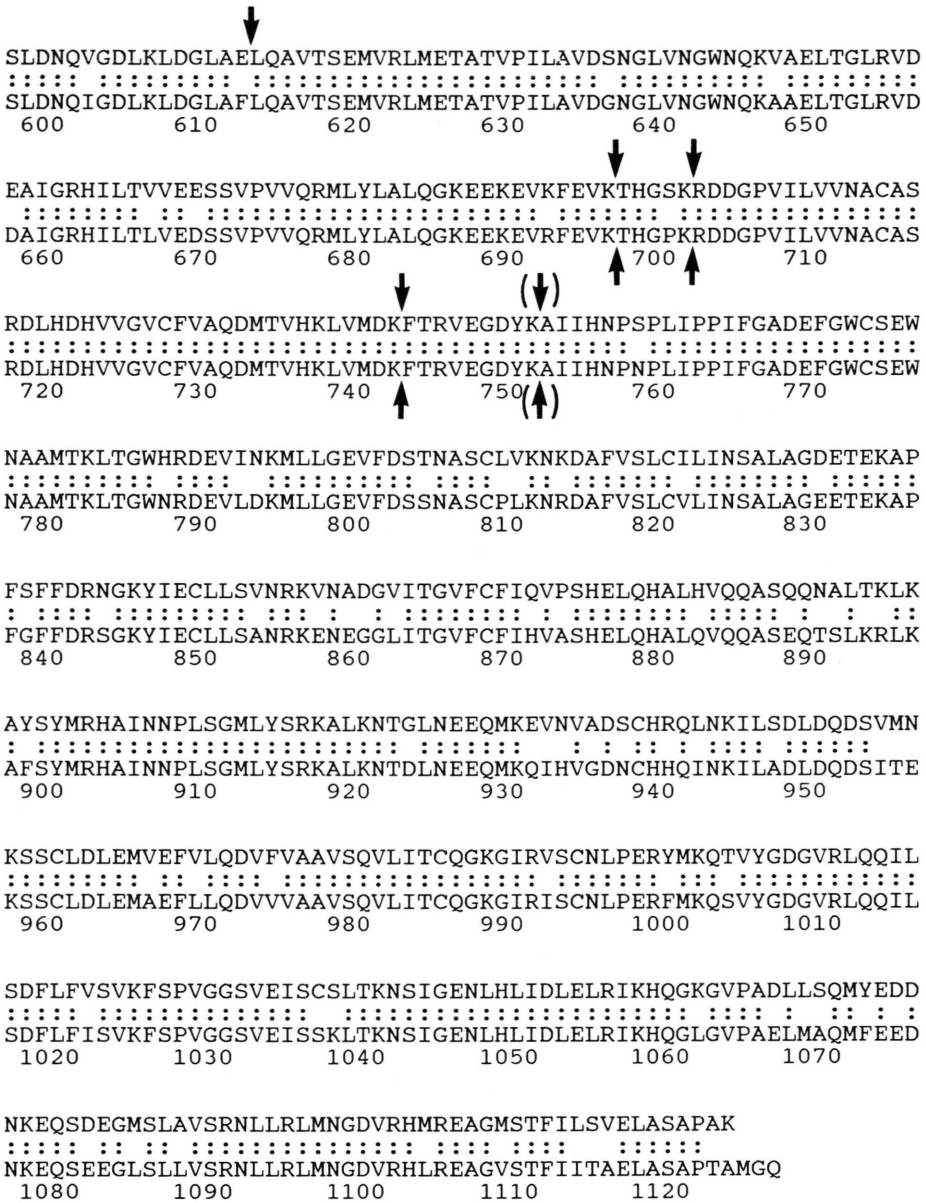
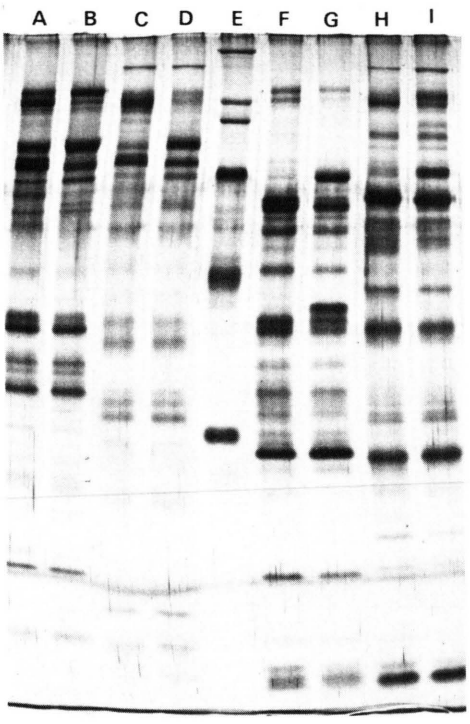


Fig. 3. Alignment of peptide sequences of rice phytochrome [12] (upper lanes) and oat phytochrome [22] (lower lanes). The rice phytochrome sequence was translated from the published nucleotide sequence [12] with the SPLICE program. Identical amino acid residues (997 out of 1129) are marked by double points. Enzymatic cleavage sites discussed in this paper are indicated by arrows. Less probable cleavage sites are indicated by arrows in brackets.



more probable candidate for the C-terminus of the fragment. Both K residues, K-744 and K-753, are present in both phytochromes. The 64 kDa fragment has not yet been investigated by microsequencing. If the Pfr specific cleavage site at K-744/K-753 is taken as C-terminus, the most probable other cleavage site would be at either K-160 or K-164 leading to either T-161 or P-165 as N-terminus of the fragment. P-165 is less likely because the bond K-P is not easily cleaved. Identical sizes are also found for the Pr specific 72 kDa fragment and the 70 kDa fragment which is formed from both phytochrome formes. The K residues before L-48 and V-66 and at positions 698 and 703, calculated as cleavage sites in oat phytochrome [11], are present in rice as well as in oat phytochrome.

Fig. 4. Enzymatic digestion of rice and oat phytochrome. SDS PAGE was performed with 10% acrylamide; the gel was silver stained. Lanes A–D: digestion with endoproteinase-Lys-C for 72 h, lanes F–I: digestion with endoproteinase-Glu-C for 27 h. A: oat, Pr; B: oat, Pfr; C: rice, Pr; D: rice, Pfr; E: molecular weight markers (see Fig. 2); F: oat, Pr; G: oat, Pfr; H: rice, Pr; I: rice, Pfr.

Table II. Comparison of proteolytic fragments from rice and oat phytochrome.

Mr determined by SDS PAGE			
Rice [kDa]	Oat [kDa]	Oat Sequence	Rice Probable sequence
Endoproteinase Lys-C			
81[a]	81[a,b]	N-terminus to K-753 or K-744	identical
72[c]	72[c]	L-48 to K-698/K-703	identical
70	70	V-66 to K-698/K-703	identical
64[a]	64[a,d]	n. d. eventually T-161/P-165 to K-744/753	identical
58	58	V-66 to K-593/K-595	– to K-593
38	39	V-66 to K-415	– to K-400/411
22	23	I-210 to K-415	– to K-400/411
Endoproteinase Glu-C			
66[a]	64[a]	N-terminus to E-597	– to E-614
62[c]	60[c]	A-51 to E-597	– to E-614
61	58	K-65 to E-597	– to E-614
–	41[a]	N-terminus to E-352/E-354	–

[a] Only or preferably for the Pfr form.
[b] Formerly determined as 83 kDa [11].
[c] Only for the Pr form.
[d] Formerly determined as 66 kDa [11].

The 58 kDa fragment formed from both Pr and Pfr has been described for oat phytochrome extending from V-66 to either K-593 or K-595 [11]. Since rice phytochrome does not contain K-595, this fragment must end at K-593 in this case. Instead of the 39 kDa and 23.5 kDa fragments derived from oat phytochrome, somewhat smaller fragments sized 38 kDa and 22 kDa are found with rice phytochrome. Since the most probable C-terminus of the oat phytochrome fragments, K-415, is exchanged by R in rice phytochrome, the next K residues have to be considered as cleavage sites in rice phytochrome. The best candidate is K-400; this would lead to fragments of smaller size than in oat phytochrome as observed. Oat phytochrome is not cleaved here by endoproteinase-Lys-C because it contains an R-400 residue instead. K-411 is a less probably candidate because it is present in oat and rice phytochrome; fragments terminated at K-411 could probably not be distinguished from those terminated at K-415 on SDS PAGE.

A general conclusion from these results is that either residue-415 is exposed in oat phytochrome and residue-400 in rice phytochrome or – more likely – both sites are exposed in both phytochromes. It is not yet clear, however, whether residue 411 is hidden in the interior of the peptide chain or whether it is not cleaved for another reason.

More differences between oat and rice phytochrome are found after digestion with endoproteinase-Glu-C. Several fragments are about 2 kDa larger in size with rice phytochrome than with oat phytochrome. The C-terminus for these fragments of oat phytochrome, namely the 64, 60 and 58 kDa fragment, has been determined as E-597 [11]. This residue is substituted in rice phytochrome by A-597 and is therefore no cleavage site for endoproteinase-Glu-C. On the other hand, a glutamic acid residue, E-614, is present in rice phytochrome instead of F-614 in oat phytochrome (see Fig. 3). This should lead to larger fragments by about 2 kDa after cleavage with endoproteinase-Glu-C in accordance with the experimental results. We assume therefore that the C-terminus of these fragments in rice phytochrome is E-614 (see Table II). No corresponding fragment exists in rice phytochrome for the Pfr-specific 41 kDa fragment of oat phytochrome. This fragment contains the blocked N-terminus of oat phytochrome and ends at either E-352 or E-354. Both of these residues are substituted in rice phytochrome, namely by G-352 and D-354. Control incubation with endoproteinase-Asp-N did not yield this fragment from rice phytochrome. The residue which is highly exposed in the Pfr form is therefore probably rather E-352 than E-354 in oat phytochrome.

The acid cluster of residues 344 to 350, ENEDDDE, is probably exposed in the Pfr form also in rice phytochrome. We can at least demonstrate by electrophoresis and isoelectric focusing, that the Pfr form of rice phytochrome carries more negative charges on the surface than the Pr form (Fig. 5 and 6). The same observation with oat phytochrome has been used as argument for preferential exposure of the same region, ENEEDDE, in the Pfr form of oat phytochrome [16]. It is not yet clear why no cleavage occurs in this region with endoproteinase-Glu-C. Enzymatic cleavage at clusters of charged amino acids seems to be unfavorable anyhow [23]. The isoelectric points of "native" rice phytochrome are about the same as those of "native" oat phytochrome in the corresponding forms, Pr and Pfr (see Fig. 6).

In summary, all results are in accordance with the assumption of the same or very similar native conformation of oat and rice phytochrome. Conformational changes during photoconversion of oat and rice phytochrome include the N-terminal 70 amino acid re-

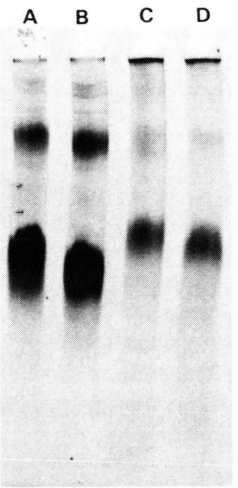


Fig. 5. Non-denaturing PAGE of oat and rice phytochrome on a 4% acrylamide gel, silver stained. Lane A: oat, Pr; B: oat, Pfr; C: rice, Pr; D: rice, Pfr. The Pfr form of phytochrome from both plants shows faster migration at pH 8.8 than the Pr form. Lower bands are presumably dimers, upper bands are higher aggregates of phytochrome.

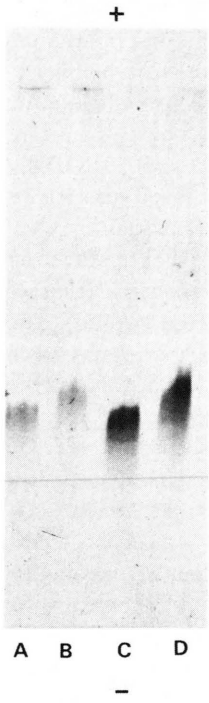


Fig. 6. Isoelectric focusing of rice and oat phytochrome according to [16]. Lane A: rice, Pfr; B: rice, Pr; C: oat, Pfr; D: oat, Pr.

sidues which are exposed in the Pr form, an "acid" cluster around residues 340–350 and another region around residues 740–750 which are both exposed in the Pfr form. Of all the highly conserved partial sequences of phytochrome from different plants, those which are differently exposed in Pr and Pfr are the best candidates for "active centers" of signal transduction. The region around residue 744 meets this requirement. More sequence variations are found in the other regions mentioned above (see Fig. 3 and [19]). It remains to be shown whether the sequence around residues 740 to 750 is responsible for physiological activity of the Pfr form.

Acknowledgements

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