Electrophoresis and Electrofocusing of Phytochrome from Etiolated *Avena sativa* L.

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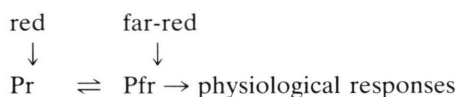
Microheterogeneity, Pest Sequence, Phytochrome Denaturation, Protein Conformation

Phytochrome from etiolated oat seedlings (*Avena sativa* L.) was investigated by "native" gel electrophoresis and by isoelectric focusing. At pH 8.8 the Pfr form migrated faster than the Pr form in electrophoresis. We assume a difference in the surface charge rather than a difference in shape for the phytochrome forms. This assumption was confirmed by isoelectric focusing which clearly showed relatively more negative charge in the Pfr form than in the Pr form. The role of the peptide region from residue 323 to 360 is discussed in this connection. It carries 9 negatively charged residues, it is exposed only in the Pfr form and it has already been described as a signal region for rapid protein degradation (PEST sequence, see Rogers *et al.*, Science **234**, 364−368, 1986). The experiments on electrofocusing revealed a microheterogeneity of phytochrome which was present in the native state as well as in the completely unfolded state. The most probable reason could be either posttranslational modification or genetic polymorphism of phytochrome in oat.

Introduction

Phytochrome is the main photoreceptor for light-dependent development and differentiation in higher plants [1, 2]. A characteristic feature is its photoreversibility: The Pr form is phototransformed into the Pfr form; optimum conversion occurs with red light. The back reaction, Pfr → Pr, can best be achieved with far-red light.

$$
\begin{array}{cc}
\text{red} & \text{far-red} \\
\downarrow & \downarrow \\
\text{Pr} \quad \rightleftharpoons & \text{Pfr} \rightarrow \text{physiological responses}
\end{array}
$$

Photoreversibility of phytochrome can be observed *in vivo* and *in vitro*. Since formation of Pfr *in vivo* is directly connected with light-dependent physiological responses, only Pfr is considered as physiologically active. Differences between Pr and Pfr, detectable *in vitro*, have been investigated in several laboratories [3−5]. We were able to demonstrate recently the specific exposure of glutamate-354 only in the Pfr form (not in the Pr form) of oat phytochrome [6]. The region around E-354 (L-324 to Q-360) has been estimated as PEST sequence, *i.e.* a signal sequence for rapid protein turnover which is rich in proline, glutamic and aspartic acid, serine and

threonine [7]. If not only E-354, but this whole region were exposed upon Pr → Pfr transformation, the high number of 9 acidic amino acids in this region should change the netto charge of phytochrome during photoconversion. We therefore investigated phytochrome in both forms by electrophoresis and electrofocusing.

Materials and Methods

Phytochrome (124 kDa) was isolated from 3.5 day-old etiolated oat seedlings (*Avena sativa* L., *cv.* Pirol, Baywa, Munich, F.R.G.) as previously described [8]. The specific absorbance ratio A_{660}/A_{280} of the preparations used in the present study was between 0.90 and 0.99.

Discontinuous undenaturing gel electrophoresis was performed with stacking gels of 2.5% (w/v) acrylamide, 125 mM Tris-HCl pH 7.8, 3% (v/v) glycerol and separating gels of 4% or 4−8% acrylamide (w/v) in a linear gradient, 375 mM Tris-HCl pH 8.8, 3% (v/v) glycerol. The electrode buffer was 25 mM Tris, 192 mM glycin, pH 8.3. Phytochrome samples were irradiated with red or far-red light to saturation; glycerol (final concentration 15%, v:v) and bromophenol blue (final conc. 0.002%, w/v) were added to the sample which was then applied to the gel. Separation was carried out in the dark at 4 °C. The gel was then stained with Coomassie brillant blue R 250 (0.2%, w/v) in destaining solution. This solution contained acetic acid/methanol/water (7:40:53, v:v:v).

Isoelectric focusing was performed in agarose gels. For preparation of the gel, 0.24 g agarose IEF (Pharmacia) and 2.88 g sorbite were dissolved in 21.6 ml boiling water. After cooling of the clear solution to 65 °C, 1.5 ml carrier ampholyte (either Servalyte or Pharmalyte, pH range as indicated in the single experiments) and DTT (final conc. 0.13 mM) were added under stirring. The warm solution was immediately filled into a casting frame consisting of two glass plates with a polyester sheet (Serva) mounted with water on one plate and a Gel-Fix for agarose sheet (Serva) similarly attached to the other plate. These were divided by a 0.75 mm thick, U-shaped silicone gasket. After cooling and hardening of the gel, the casting frame was removed and the gel was equilibrated with water vapor (humid, closed box containing a water saturated atmosphere) for about 12 h at 4 °C. Separation was performed using a Multiphor chamber II (LKB, Gräfelfing, F.R.G.) and a power supply with V × h integrator (Bachofer, Reutlingen, Typ E 532). The cooling plate was thermostated to 10 °C or 15 °C. The electrode solutions were 1 M sodium hydroxide as catholyte and 0.1 M acetic acid as anolyte. Whatman 3 MM filter paper was cut into strips and moistened with the electrode solutions. It was used in double layer as electrode wicks. Before focusing excess water was removed from the surface of the gel by placing one sheet of filter paper (No. 577 Schleicher and Schüll) on the gel surface for 30 sec. Kerosene was used between the support sheet and the cooling plate to facilitate heat transfer from the gel. The interelectrode distance was approximately 10 cm. Phytochrome samples in 10 mM potassium phosphate buffer, pH 7.8 were applied to the gel 1−2 cm from the cathode in slots of silicone applicator strips (Serva). At the beginning of the focusing run, the voltage was fixed at 300 V for 30 min in order to remove small ions. The power supply was then fixed at 10 W/2000 V for gels of the size 24 × 12 cm in dimension. Focusing was finished at about 4000−6000 V × h. If required, the pH gradient was measured at the end of the run using a pH surface electrode. Alternatively, the pH gradient was documented in the stained gel by pI- marker proteins (Pharmacia). Fixation and staining was performed according to Olsson and Låås [9].

For isoelectric focusing with urea, ampholyte and DTT (final conc. 1.3 mM) were added to agarose/sorbite solution as above. After cooling below 50 °C, 11.6 g urea were dissolved in the mixture according to [9]. The further procedure was as above but the gel was not cooled below +10 °C in order to avoid crystallization of urea. The samples were in this case dissolved in 8.9 M urea containing 1.3 mM DTT. In this case cooling temperature was set on 5 °C. Focusing was finished at about 4000−5000 V × h.

SDS polyacrylamide electrophoresis was performed according to Laemmli [10].

Results

For investigation of the Pr and Pfr forms of phytochrome, electrophoresis had to be carried out in the dark in order to preserve the respective form. As shown in Fig. 1, the Pfr form migrates faster at pH
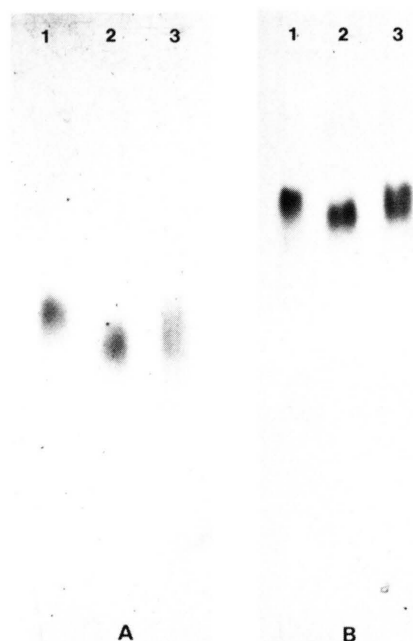

Fig. 1. Discontinuous polyacrylamide gel electrophoresis of undenatured, 124 kDa phytochrome. A: gel with 4% acrylamide; B: gradient gel, 4 to 8% acrylamide. Each lane contained 4.5 µg phytochrome; 1 = Pr form; 2 = Pfr form; 3 = mixture of 50% Pr and 50% Pfr.

8.8 than the Pr form. This property was found for all phytochrome preparations independent of the purity of the samples (not shown). Red irradiation of Pr yields about 85% Pfr and 15% Pr. Whereas the Pr band can be detected besides a Pfr band in a 1:1 mixture of Pr and Pfr either as a distinct band or a broad band extending over the whole range of Pr + Pfr (Fig. 1), addition of only 20% Pr does not change

the appearance and size of the Pfr band. One cannot expect therefore to detect 15% Pr besides 85% Pr. The phytochrome bands are anyhow not very sharp. One possible reason is microheterogeneity of phytochrome (see below). Another reason could be the dimeric nature of phytochrome. Van der Woude [11] has pointed out that the following species have to be expected: PrPr, PrPfr, PfrPfr. If we assume that our phytochrome bands contain only dimeric phytochrome, we would expect PrPr in lane 1 and mainly PfrPfr with some PrPfr in lane 2. This fact might contribute to the poor separation of Pr from Pfr.
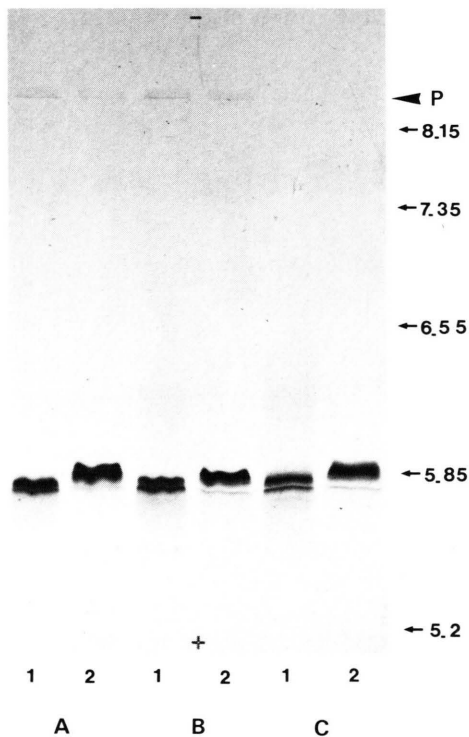

Fig. 3. Isoelectric focusing of undenatured, 124 kDa phytochrome on agarose with Pharmalyte, pH 5−8. Phytochrome (5 μg in 10 μl phosphate buffer per lane) was applied in Pr form (3) or Pfr form (2), 1 = marker proteins. Focusing was finished at 4200 V × h. P = point of application. Arrows indicate the isoelectric points of marker proteins.

Fig. 2. Isoelectric focusing of undenatured, 124 kDa phytochrome on agarose with Servalyte, pH 5−8. Phytochrome samples (6 μg in 10 μl phosphate buffer each) were from several independent preparations (A, B, C). 1 = Pfr form, 2 = Pr form. Focusing was finished at 6000 V × h. P = point of application. Arrows indicate the isoelectric points of marker proteins.

We then investigated phytochrome by isoelectric focusing. The results (Fig. 2 and 3) confirmed our hypothesis of different surface charge of both phytochrome forms: the isoelectric point for Pfr is in the range pH 5.80−5.85 whereas that of Pr is in the range pH 5.85−5.90 in all experiments. The ob-
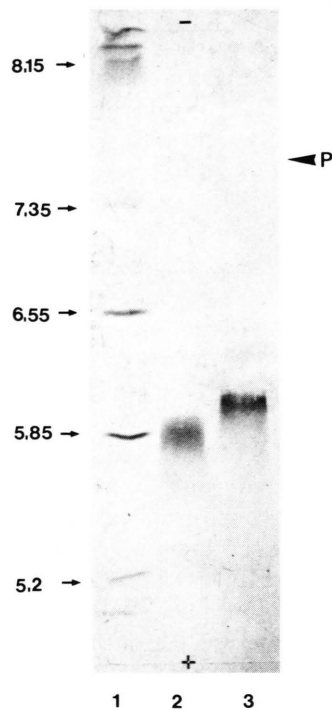
served difference furthermore confirmed our proposal that Pfr contains more acidic groups (or negative charges at neutral pH) than Pr at the surface. It should be mentioned here that the phytochrome sample has to be applied to the gel after pre-focusing at pH > 7; otherwise phytochrome seems to be denatured so that either no phytochrome protein or only part of it migrates to the correct position.

Surprisingly, the result was somewhat different for several carrier materials. As shown in Fig. 3, only diffuse bands were obtained with Pharmalyte whereas several sharp bands were observed with Servalyte (Fig. 2). This difference for which we have no explanation is reproducible.

In order to check the possible heterogeneity of phytochrome, we investigated urea-denatured phytochrome by isoelectric focusing next. Any heterogeneity caused by aggregation or different conformers should disappear after complete unfolding of the peptide chain. We expected therefore that
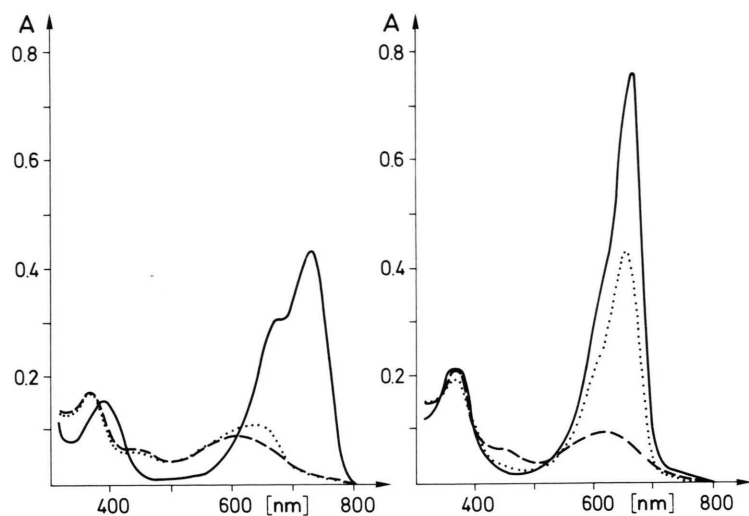
Fig. 4. Denaturation of Pfr (left) and Pr (right) with 8 M urea. Absorption spectra of a) native protein in 10 mM potassium phosphate buffer, pH 7.8, containing 14 mM mercaptoethanol (———); b) the same after quantitative precipitation with ammonium sulfate and resuspension in 8 M urea containing 1.3 mM DTT (....); and c) = solution b) after saturating red irradiation (− − − −).

the difference between Pr and Pfr should disappear under these conditions. Determination of the absorption spectra revealed that Pfr is completely denatured but the Pr form is not denatured in 8 M urea (see Fig. 4). Complete denaturation required irradiation of the solution of Pr in 8 M urea. Higher resistance of Pr than of Pfr against denaturation by medium urea concentrations had already been described by Butler *et al.* [12] for the 60 kDa fragment and by Thümmler [13] for the 114/118 kDa fragment of phytochrome; contrary to the present results with 124 kDa phytochrome, complete denaturation by 8 M urea had been achieved with each fragment even in the Pr form.

Completely unfolded phytochrome shows the same pattern of bands in isoelectric focusing for Pr and Pfr (see Fig. 5): Two bands of medium intensity (1 and 2) are followed with decreasing pH by two major band (3 and 4) and another strong band (5); two minor bands are close to (= 6) and somewhat distant from (= 7) band 5. Carbamylation which can easily occur in 8 M urea was avoided here by DTT; this was checked by independent control experiments (not shown). Isoelectric focusing with Pharmalyte yields also in urea containing gels only one diffuse band over the same pH range (not shown). In order to exclude any artefacts which could be formed during isoelectric focusing, the gel section with the phytochrome bands were cut out and applied to a refocusing gel. Fig. 5 clearly shows that the number of bands is not changed by refocusing; the single bands keep their position and orientation also in the second dimension. We excluded also the possibility that partial proteolysis could lead to the observed heterogeneity: SDS gel electrophoresis clearly showed that all bands have the same apparent size of 124 kDa (Fig. 6).

We checked furthermore also the possibility that heterogeneity was introduced during the purification procedure. For this purpose, urea was added to crude extracts from 2 varieties of oat (*Avena sativa* L. var. Garry and var. Pirol); the samples were then immediately applied to the focusing gel. As shown in Fig. 7, the same pattern of phytochrome-bands as for isolated phytochrome was detected in the immunoblot of the crude extract with monoclonal antibodies directed against phytochrome.

Discussion

Whereas previous data on electrophoresis and isoelectric focusing of phytochrome are scattered through the relevant literature, we report here on the systematic comparison of Pr and Pfr under non-denaturing conditions and on denatured *versus* native phytochrome. By this means, differences between Pr and Pfr could be documented for separation by electrophoresis and by isoelectric focusing.
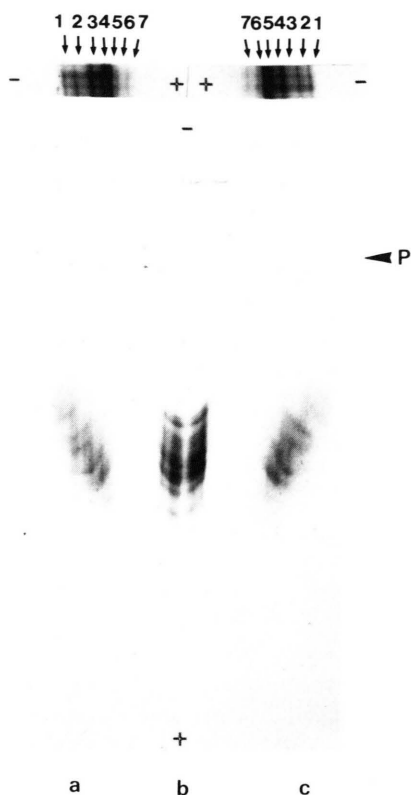
Fig. 5. Isoelectric focusing of 124 kDa phytochrome in 8 M urea. The phytochrome samples (10 μg each) were completely denatured in 8 M urea before application (see Fig. 4). Upper part: first focusing run with Servalyte, pH 6−7 (70%) and pH 3−10 (30%). The phytochrome bands are numbered 1−7. Lower part: refocusing of bands of the first run. Phytochrome containing gel pieces were placed on the surface of the second gel (P = point of application) and focused rectangular to the first run. Lane a: phytochrome bands 1 to 7 from left to right; lane b: direct focusing of phytochrome in the second gel; lane c: phytochrome bands 7 to 1 from left to right.
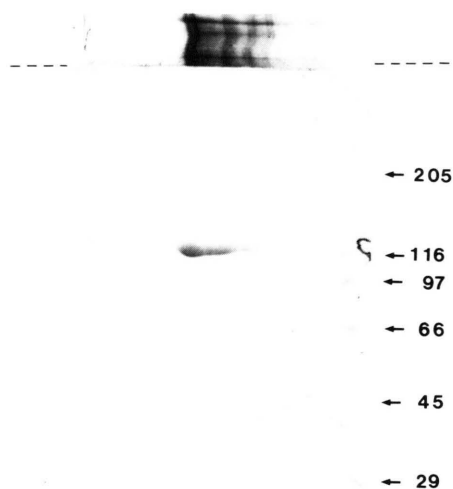

Fig. 6. Two-dimensional separation of 124 kDa phytochrome. SDS gel electrophoresis of phytochrome bands from isoelectric focusing was performed in the second dimension.
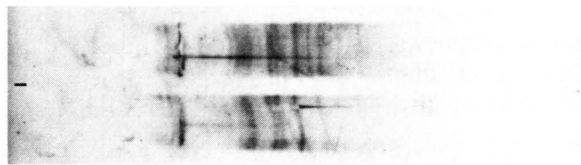

Fig. 7. Immunoblot after isoelectric focusing with urea of crude extracts from etiolated oat seedlings. Upper part = A. sativa cv. Pirol; lower part = A. sativa cv. Garry. Servalyte, pH 5−7 (60%), pH 6−7 (18%), pH 5−8 (6%), and pH 3−10 (16%); final conc. 3.1%. After capillary blot onto nitrocellulose, phytochrome bands were visualized with the monoclonal antibody Z3B1 [18].

One reason for different migration in electrophoresis would be a difference in the apparent size of the molecules. In this case, Pfr should have an apparently smaller size than Pr. We consider this possibility unlikely for the following reason: Lagarias and Mercurio [4] had found a higher retention time for Pr than for Pfr in size exclusion chromatography; they discussed an apparently larger molecular volume for Pfr than for Pr. This was confirmed by Jones and Quail [14] for phytochrome in Tris-buffer in the pH range 7.2 to 9.2; no difference between Pr and Pfr was found at pH 6.2. It is highly unlikely that photoconversion leads to conformational changes resulting in a different shape for Pfr and Pr in such a way that an apparent larger size for Pfr is found by size exclusion chromatography and an apparent smaller size for Pfr is found by electrophoresis in the same buffer. So far detected conformational differences between Pr and Pfr seem to involve only certain parts of the peptide chain [3−6, 15]. It is anyhow

questionable whether this could cause a significantly different overall shape of the phytochrome molecule.

An alternative explanation for the observed difference between Pr and Pfr could be a difference in the surface charge as outlined in the Introduction. A higher negative net charge of Pfr, compared with Pr would explain all observations discussed above, namely faster migration of Pfr than of Pr in electrophoresis and in size exclusion chromatography at high pH as well as lack of any difference at low pH.

The electrofocusing experiments confirmed the higher negative charge of Pfr comparing with Pr. This had not been reported before. Hunt and Pratt [16] used partially degraded phytochrome (114/118 kDa fragments) which had been eluted from an immuno-affinity column with 3 M Mg Cl$_2$; this treatment leads to spectral denaturation [17]. It is therefore not surprising that these authors did not find any difference between Pr and Pfr during electrofocusing. Vierstra and Quail [17] investigated only SDS-denatured phytochrome by electrofocusing; therefore they could not detect any difference between Pr and Pfr.

A higher negative net charge of Pfr could either mean that positive charges which are at the surface of Pr disappear during photoconversion or, alternatively, that negative charges which are in the interior part of the peptide chain in Pr become exposed upon photoconversion to Pfr. Differentially exposed regions of Pr and Pfr have recently been localized [6]. The N-terminal region (amino acids 1−70) which is preferentially exposed in the Pr form contains 8 basic and 10 acidic residues; it is therefore no candidate for induction of a differential surface charge. One region which is exposed only in the Pfr form has been localized around K-753; it is not known, however, how far this region is extended to either one or both sides from K-753. The region from residue 743 to residue 763 contains 3 acidic and 4 basic residues, the region from residue 733 to residue 773 contains 5 acidic and 5 basic residues. This region is therefore also an unlikely candidate for differences in the surface charge. However, the region from the tetrapyrrole chromophore (at C-322) to E-354 contains only one basic residue, namely H-323 close to the tetrapyrrole chromophore and 9 acidic residues. Since this region is exposed only in the Pfr form [6] one expects more negative surface charges in the Pfr form than in the Pr form. The present results of elec-

trophoresis and isoelectric focusing confirm this hypothesis.

Differences between Pr and Pfr are often considered under the aspect of possible biochemical and physiological function of Pfr. One of the signal functions of Pfr from etiolated plants is the start of phytochrome destruction. Rogers et al. [7] considered phytochrome as a protein with rapid turnover and described the region 322 to 360 as PEST sequence characteristic for proteins which are rapidly turned over. These authors did not consider the stability of Pr and the start of destruction only after Pfr formation. Our present findings raise the general question whether PEST sequences have to be exposed at the protein surface in order to exhibit their signal function for protein degradation; in this case the different stability of Pr and Pfr could be understood. We have described another PEST sequence for phytochrome from etiolated oat seedlings at residues 537−547 [18]. This region, contrary to the region near to the chromophore is not conserved, as a PEST sequence, however, in phytochrome from etiolated cucumber seedlings [19]. It shall be interesting to know whether either one of the indicated PEST sequences will be found in the so-called "green" or type II phytochrome which is found in green plants and does not show any Pfr destruction [20].

Another aspect of exposure of many negatively charged groups upon Pfr formation is the possible interaction with divalent metal ions. It is well known that the Pfr form tends to form large, insoluble aggregates in the presence of Mg^{2+} or Ca^{2+} ions [21]. The Pr form does not show this property. Although it is not clear whether this in vitro pelletability is somehow related to pelletability in vivo [22] the exposure of negative charges could possibly contribute also to in vivo processes of sequestering and finally to redistribution of phytochrome in the plant cell [23].

The reason for the observed microheterogeneity of phytochrome is still unknown. Microheterogeneity may be the reason for relatively broad bands in "native" electrophoresis. Since sharp bands at 124 kDa are obtained in SDS gel electrophoresis, all isophytochromes must have the same size. Since heterogeneity by isoelectric focusing is not only found in the native protein but also in completely unfolded phytochrome we can exclude heterogeneity due to only different conformers or aggregates. Whereas previously detected heterogeneity of 60 kDa phytochrome fragments [24] could have been caused by

heterogenous proteolysis, we could show that our phytochrome sample is heterogenous without any proteolysis.

Artifactual *in vitro* modification during purification is unlikely because we find heterogeneity already for phytochrome in the crude extract. The most likely possibilities are (1) differential post-translational modification reactions which might occur *in vivo* or (2) genetic polymorphism. The latter possibility would coincide with the finding of at least 4 different phytochrome genes in oat [25] and the corresponding isophytochrome proteins [26]. It re-

mains to be shown whether there is lack of phytochrome microheterogeneity on the protein level *e.g.* in cucurbita where no genetic polymorphism has been found [19].

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