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Molecular Properties of Phytochrome [and Discussion]

M. Furuya, W. Haupt and M. Furuya

Phil. Trans. R. Soc. Lond. B 1983 **303**, 361-375
doi: 10.1098/rstb.1983.0099

Phil. Trans. R. Soc. Lond. B **303**, 361–375 (1983)
Printed in Great Britain

Molecular properties of phytochrome

By M. Furuya

Biology Department, Faculty of Science, University of Tokyo, Hongo, Tokyo 113, *Japan*
and Division of Biological Regulation, National Institute for Basic Biology, Okazaki 444, *Japan*

Chromopeptides with molecular masses of *ca.* 114, 62, 56, 40, 39 and 33 kDa were prepared from pea phytochrome by limited proteolysis. Absorption and circular dichroism spectra were determined and proton uptake and release investigated. The data indicate how long the chromopeptide chain must be for photoreversible changes between P_r and P_{fr} or between P_{659} and P_{bl}.

Double flash-photolytic and low-temperature spectroscopic studies on the phototransformation pathways from P_r to P_{fr} and from P_{fr} to P_r of native and degraded chromopeptides were carried out under different conditions, demonstrating that the pool size of kinetically detectable intermediates in a sample changed reversibly depending upon monomer size, and microenvironmental factors such as pH and temperature.

Six monoclonal antibodies against rye phytochrome and six against pea phytochrome were raised and investigated in terms of the sites of phytochrome determinants, species specificity, and influence on spectral and other molecular properties.

1. Introduction

Developmental and physiological processes in plants are controlled by not only genetic information but also by changes in the physical and chemical factors of the environment. Among the latter, light is well known to be the most evident and crucial factor in plants (Smith 1976, 1982). Phytochrome and blue–near-u.v. light-absorbing pigments are widely distributed in the plant kingdom as phototransducers and act for a variety of photomorphogenetic responses (Furuya 1968; Senger 1980; Shropshire & Mohr 1983), and each developmental process such as dormancy induction, dormancy breakage, cell division, growth and differentiation is properly progressed under a collaboration of these two photoreceptor systems (Furuya 1978, 1980).

Phytochrome is a chromoprotein with two distinct and photointerconvertible forms, a form absorbing red light, P_r, and a form absorbing far-red light, P_{fr} (Butler *et al.* 1959). Phytochrome can be isolated from plant tissues and purified by conventional and affinity procedures, so spectrophotometrical and molecular properties of phytochrome *in vitro* have been intensively studied in the past two decades, and the results accumulated in the literature have been repeatedly reviewed by Pratt (1978, 1979, 1982*a*, *b*). I therefore do not intent to present a general review of this subject, but should like to introduce here some recent results obtained in my laboratories.

2. Subunit size and spectral properties

Since the discovery of phytochrome (Butler *et al.* 1959), photoreversible absorbance changes between P_r and P_{fr} have been the most prominent property of phytochrome. Although absorption spectra *in vivo* result from all the pigments existing in the sample tissues (figure 1*a*),

difference spectra after actinic red and far-red light irradiations clearly separate phytochrome from other pigments (figure 1*b*). Thus the detection of phytochrome both *in vivo* and *in vitro* has been most widely based upon this spectral property.

Phytochrome has been isolated from various plant tissues and purified by either conventional or immunoaffinity procedures (see Pratt 1982*a*). In the early days of phytochrome study, the

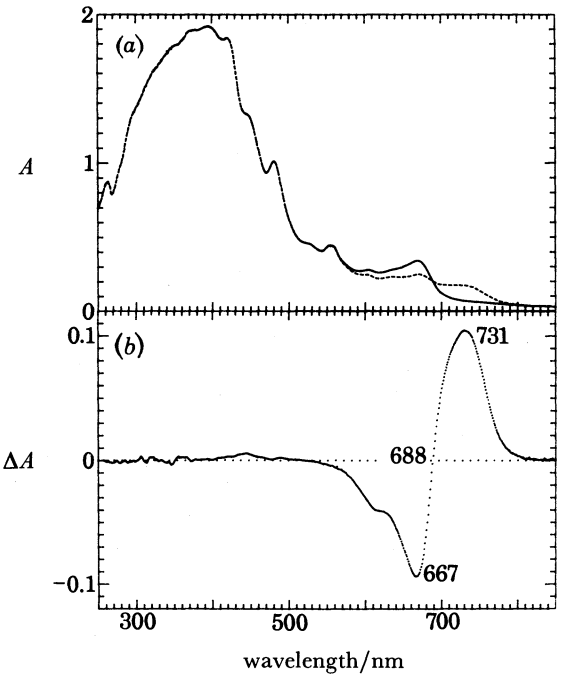

FIGURE 1. Absorption (*a*) and difference (*b*) spectra of etiolated pea hook tissue (Y. Inoue, unpublished data). ——, Totally dark-grown tissue; ---, after irradiation with red light.

monomer size of phytochrome was reportedly believed to be *ca.* 60 kDa (Mumford & Jenner 1966). However, phytochrome was later found to consist of two identical chromopeptides, each of molecular mass *ca.* 120 kDa (Briggs & Rice 1972). By now it is well established that the former, 'small' phytochrome, is produced from the latter, 'large' phytochrome, by proteolytic degradation (Pratt 1982*a*). It has, however, long been questioned why the absorption maximum of purified 'large' phytochrome in the literature (*ca.* 724–725 nm; figure 3*a*) was somewhat, but significantly, shorter than that observed *in vivo* (730–732 nm; figure 1). Recently it became evident that phytochrome isolated from oats as P_{fr} absorbs at longer wavelengths than that extracted as P_r and that the long-wavelength absorbing phytochrome apparently shows a larger molecular mass on gel electrophoresis than that reported as 'large' phytochrome (Epel 1981; Baron & Epel 1982). Further, Vierstra & Quail (1982*a*, *b*) demonstrated that 'native' oat phytochrome is homogeneous, with a monomeric molecular mass of 124 kDa, whereas 'large' phytochrome purified by conventional procedure is heterogeneous with molecular masses of 118, 114 and 112 kDa. It appears that the 'native' phytochrome can be prepared whenever endogenous proteolysis is properly prevented during extraction and purification. An example of rye phytochrome is presented in figure 2, which confirms the above conclusion. An immunoaffinity purification procedure provides a major band of

124 kDa monomer at the final purification step irrespective of the spectral form in which phytochrome is extracted (K. T. Yamamoto, unpublished).

The spectral properties of 'small' phytochrome are generally very similar to those of 'large' phytochrome (Pratt 1978, 1979). The next step is to characterize the autonomous chromophore-containing subregion of phytochrome (chromophore domain) that can exhibit the

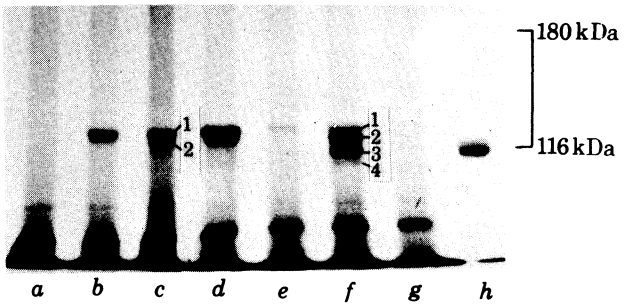

FIGURE 2. Sodium dodecyl sulphate polyacrylamide gel electrophoretograms of immunoprecipitated rye phytochrome (K. T. Yamamoto, unpublished). Phytochromes immunoprecipitated from crude extracts as P_{fr} (*a*, *b*) or P_r (*c*) were compared with those immunoprecipitated from purified phytochrome preparations obtained by conventional procedures as P_{fr} (*d*, *e*) or P_r (*f*, *g*). Conventionally purified pea phytochrome was also included in the electrophoresis for comparison (*h*). Immunoprecipitations were performed with monoclonal anti-rye phytochrome antibody (AR3)-coated *Staphylococcus aureus* cells (*b*, *c*, *d*, *f*) according to the method of Vierstra & Quail (1982*a*). A monoclonal antibody to rat liver cell membrane glycoproteins (Fukumoto *et al.* in preparation) was also used as a control for non-specific binding of antibodies (*a*, *e*, *g*). The immunoprecipitates were subjected to sodium dodecyl sulphate polyacrylamide electrophoresis (Laemmli 1970) using 30 g l^{-1} acrylamide stacking gel and 50 g l^{-1} separating gel.

photoreversible absorbance change by itself. We have prepared five chromophore-containing fragments from pea phytochrome of 114 kDa in the P_r form by limited proteolysis with trypsin, thermolysin and chymotrypsin, and determined the absorption and circular dichroism (c.d.) spectra, and the fluences required for photoconversion (Yamamoto & Furuya 1983). The fragments of 62 and 56 kDa that were 'small' phytochrome (figure 3*b*) showed a photoreversible transformation between P_{667} and P_{722} like 'large' phytochrome (figure 3*a*). Smaller fragments of 40, 39 and 33 kDa showed an absorption maximum at 657–660 nm (P_{658}), which was transformed to a bleached form (P_{bl}) after a brief exposure to red light (figure 3*c*). P_{bl} was transformed back to P_{658} by far-red light at a fluence that was *ca.* 10 times that needed for the conversion of P_{722} to P_{667}. The transformation between P_{658} and P_{bl} was repeatedly photoreversible. Both P_{658} and P_{bl} showed negative c.d. bands in the red region like P_{667}, whereas P_{722} has a positive band in the far-red region. As far as the size of the chromophore domain of phytochrome is concerned, the smallest fragment that showed photoreversible transformation between P_r and P_{fr} was a 56 kDa chromopeptide obtained by thermolysin digestion, and the largest one that no longer exhibited the P_r–P_{fr} photoconversion was a 40 kDa fragment prepared by trypsin digestion. This fact indicates that the molecular mass of the chromophore domain of phytochrome is at most 56 kDa. This conclusion is consistent with that in a previous report (Stoker *et al.* 1978).

The fragments of 40 kDa or smaller obtained by limited trypsin digestion did not show the typical photoreversible conversion between P_r and P_{fr}, but exhibited an atypical photoreversible conversion between new spectral forms, P_{658} and P_{bl}, by actinic red and far-red light. The fluence response of the conversion showed that incomplete conversion was due to

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photoequilibrium between P_{658} and P_{bl}. These facts show that digestion of the 56 kDa fragment to the 40 kDa fragment distorts the confrontation around its chromophore, but that the 40 kDa fragment still maintains a certain structure that is essential for the photoreversible spectral change of phytochrome.

It is interesting to note that the absorption spectrum of P_{bl} with 33–40 kDa fragments

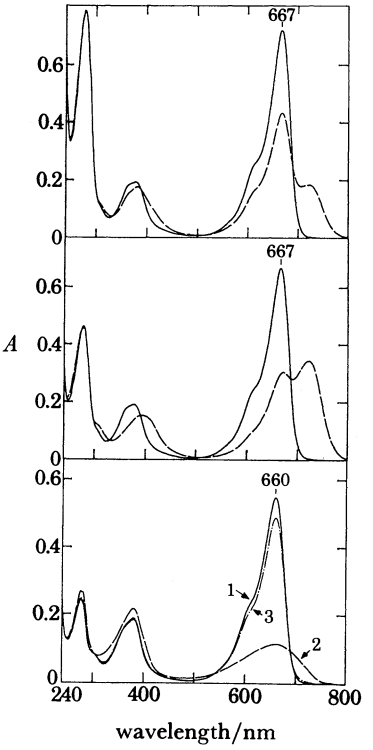

FIGURE 3. Absorption spectra of (*a*) 114 kDa, (*b*) 62 kDa and (*c*) 40 kDa chromophore-containing fragments of pea phytochrome in 0.1 M sodium phosphate (pH 7.8), 1 mM Na_2EDTA and 0.25 mM dithiothreitol (Yamamoto & Furuya 1983). The absorption spectra were determined at 3 °C with a dual-wavelength difference spectrophotometer (Hitachi model 557) with 1 cm light-path quartz cuvettes and a slit width of 1 nm. (*a*), (*b*) Solid line, P_r; broken line, red-light-induced photostationary state. (*c*) Curve 1, an initial spectrum before actinic irradiation; curve 2, a spectrum after saturating red light irradiation; curve 3, a spectrum after saturating far-red light irradiation after the red light.

(Yamamoto & Furuya 1983) was essentially the same as that of phytochrome in the presence of urea (Butler *et al.* 1964), divalent metallic ions (Pratt & Cundiff 1975), anilinonaphthalene sulphonate (ANS) (Hahn & Song 1981), liposomes (Furuya *et al.* 1981), and a triterpenoid saponin (Konomi *et al.* 1982), although phytochrome did not show the repeated photoconversion between P_{659} and P_{bl} in the presence of these substances, with the exception of the saponin. In fact, P_{bl} was formed as a result of the interaction of the saponin with one or more intermediates produced during the phototransformation of P_r to P_{fr} (Konomi *et al.* 1982), because the addition of the saponin to phytochrome after red light irradiation did not produce P_{bl} as effectively as did red light irradiation of P_{658}, which was formed immediately after the saponin was added to P_{667}. For ANS it was postulated that phototransformation intermediates of phytochrome were selectively complexed with ANS, resulting in P_{bl} (Hahn & Song 1981). In addition, phytochrome in the presence of ANS showed a stronger bleaching of P_r, a

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significant loss of photoreversibility and much smaller negative c.d. band of the bleached form, indicating that the spectral forms observed in the presence of ANS were distinct from those observed in the 33–40 kDa fragments.

In conclusion, only three patterns of photoreversible spectral changes of phytochrome (table 1) have been found under experimental conditions so far tested: namely (1) phototransformations between P_{660} and P_{730} in the 'native' form; (2) that between P_{666} and P_{725} in 'large' and 'small' phytochrome; and (3) that between P_{658} and P_{bl} in 33–50 kDa chromopeptide fragments and in 'small' or 'large' phytochromes in the presence of the above chemicals. Therefore, the phytochrome chromophore appears to take only a few quasi-stable spectral states under various microenvironment rather than a great number of different states, which may result in diverse spectra.

TABLE 1. MONOMER SIZE AND SPECTRAL PROPERTIES OF PHYTOCHROME IN VARIOUS PLANTS

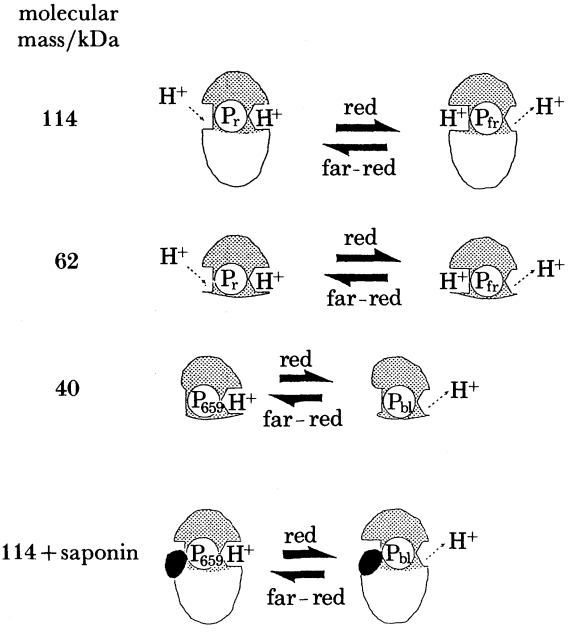
sample	monomer molecular mass/kDa	P_r max.	difference spectra/nm isosbestic point	P_{fr} max.	reference
in vivo					
oat (coleoptile)	.	666	687	729	Inoue (unpublished)
(mesocotyl)	.	668	689	730	
corn (mesocotyl)	.	669	690	732	
rye (coleoptile)	.	665	688	733	
pea (hook)	.	667	688	730	
in vitro					
'native'					
oat	124	660	.	730	Vierstra & Quail (1982)
rye	124	662	684	731	Inoue & Yamamoto (unpublished)
'large'					
oat	118, 114	665	.	722	Vierstra & Quail (1982)
rye	124–118	665	687	729	Inoue & Yamamoto (unpublished)
pea	114	667	.	722	Yamamoto & Furuya (1983)
'small'					
oat	62	666	.	725	Rice *et al.* (1973)
rye		665	687	726	Inoue (unpublished)
pea	62	667	—	722	Yamamoto & Furuya (1983)
degraded					
pea	33–40	658		bleached	Yamamoto & Furuya (1983)

3. PHOTOREVERSIBLE PROTON UPTAKE AND RELEASE

Proton uptake and release have been proposed in hypothetical models for phytochrome phototransformation (Rüdiger 1980; Lagarias & Rapoport 1980). In this connection, we recently reported the first crucial evidence of photoreversible proton transfer in unbuffered solutions of both 'large' and 'small' pea phytochromes (Tokutomi *et al.* 1982). When pH changes of dialysed phytochrome solutions were measured with a semimicro-combination pH electrode, red light irradiation caused an alkalinization of the solutions in the pH range 5.2–7.6 and an acidification in the pH range 7.6–9.2 (Tokutomi *et al.* 1983*a*). The pH changes were

fully reversed by a subsequent irradiation with far-red light, and the effects of red and far-red light were repeatedly photoreversible.

These photoreversible pH changes of unbuffered pea phytochrome solutions occurred concomitantly with not only optically measured absorbance changes between P_r and P_{fr} that were induced by actinic light, but also in dark reversion of P_{fr} to P_r. The net numbers of



matic illustration of proton uptake and release in unbuffered solution of pea phytochrome. For details, see the text.

protons released from, or taken up to, phytochrome were dependent upon the degree of absorbance change at 730 nm. The alkalinization in the dark took place slowly in parallel with P_{fr} dark reversion, whereas the acidification in the dark showed an initial rapid phase correlated to the rapid absorbance increase at 667 nm (Tokutomi *et al.* 1983*a*). This finding is consistent with the observation of Sarkar & Song (1981) of the effect of D_2O on phototransformation and dark transformation of oat phytochrome.

Unbuffered solution of pea 'large' phytochrome in the pH range 7.4–7.8 was transiently acidified at an early step of the process after irradiation with red light. The pH titration curves of the solutions with both P_r and a photostationary state under red light irradiation were determined, and the resultant curve of the latter showed an upward shift at pH 5.4–7.6 and a downward shift at pH 7.6–9.2. The results on the transient acidification and the light-induced shift of pH titration curves suggest that each phytochrome molecule has one or more proton release sites and one or more uptake sites, as shown in figure 4, and that the former possibly has one or more groups with a pK of *ca.* 6.0 and undergoes an upward pK shift, while the latter may have one or more groups with a pK of 9.2 or higher and undergoes a downward shift (Tokutomi *et al.* 1983*a*).

Finally, proton uptake or release, if any, during phototransformation between P_{659} and P_{bl}, which were described in the previous section (see figure 3*c*), were measured. When an unbuffered solution of proteolytically prepared 40 kDa chromopeptide of pea phytochrome

was irradiated with red light, an acidification of the solution was induced at pH 7.0–8.7 as 'large' pea phytochrome, but no alkalinization was observed at any pH range tested. Similarly, an acidification (but no alkalinization) was observed in 'large' pea phytochrome solution in the presence of 0.8 mM soyasaponin I (Tokutomi *et al.* 1983*b*), indicating that the phototransformation between P_{659} and P_{bl} results in the acidification but not in the alkalinization.

4. Phototransformation pathways and intermediates

Photoreversible absorbance changes of phytochrome result from a series of physical and chemical changes in the phytochrome molecule that are induced by photon capture by the chromophore and its intramolecular effects on protein moiety. Although P_r and P_{fr} are spectrophotometrically detectable as stable forms at physiological temperature, numerous short-lived intermediates of phytochrome have been characterized in both directions of phototransformation *in vivo* and *in vitro* by flash kinetic spectroscopy and low-temperature spectroscopy.

(*a*) *Flash photolysis*

Four intermediates of 'small' oat phytochrome were originally demonstrated to occur through two kinetically identifiable stages by a flash photolysis technique on the pathway from P_r to P_{fr} (Linschitz *et al.* 1966), and two more were added to the later stage (Pratt & Butler 1970). Recently the phototransformation intermediates from P_r to P_{fr} were re-examined with 'large' phytochrome of pea (Shimazaki *et al.* 1980; Cordonnier *et al.* 1981) and oat (Cordonnier *et al.* 1981; Pratt *et al.* 1982), demonstrating that phototransformation pathways from P_r to P_{fr} of 'large' phytochrome took place, as with 'small' phytochrome, through at least three reaction stages. These include a photoinduction of I_{692}, a dark decay process of I_{692} to I_{bl} on a microsecond timescale and the appearance of P_{fr} from I_{bl} on a millisecond or longer timescale. The last two dark processes were separable kinetically into three distinct reactions (figure 5). Flash activation of intermediates present during the first decay stages resulted in their photoconversion back to P_r within 8 μs, and during the second stage converted them to another transient intermediate stage, which decayed thermally to P_r within 2 ms (Pratt *et al.* 1983).

In contrast, significantly less attention has so far been paid to the phototransformation of P_{fr} to P_r (e.g. 'small' oat phytochrome (Linschitz *et al.* 1966; Pratt & Butler 1970) and 'large' pea phytochrome (Inoue *et al.* 1982)). One of the difficulties for such a study is due to the dark

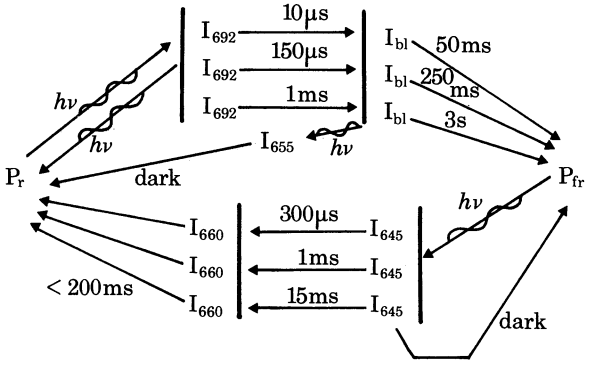


Figure 5. A general scheme showing probable phototransformation pathways and intermediates of phytochrome. The data are taken from Shimazaki *et al.* (1980), Inoue *et al.* (1982) and Pratt *et al.* (1983).

reversion of P_{fr} to P_r, which occurs in parallel with the phototransformation. When P_{fr} of 'large' pea phytochrome was excited by a 715 nm laser flash light (Inoue *et al.* 1982), the maximum amount of phototransformation intermediates was produced by a pulse of 50 mJ, which resulted in *ca.* 65% P_r at the photostationary state. A difference spectrum between an intermediate measured 10 μs after flash excitation and P_{fr} showed an absorbance increase at 651 nm and a decrease at 724 nm, indicating a formation of the first detectable intermediate (I_{645} in figure 5). The difference spectrum of this earliest intermediate showed a peak at 651 nm, which was similar to the previously reported data on 'small' oat phytochrome (Linschitz *et al.* 1966; Pratt & Butler 1970). Although Kendrick & Spruit (1977) found an intermediate named *lumi*-F *in vivo* and *in vitro*, the difference spectrum of 'large' pea phytochrome did not show any positive peak at 730 nm, so that the existence of *lumi*-F seems dubious in this sample. The observation that the absorbance of 'large' pea phytochrome increased in both red and far-red spectral regions in the dark after an excitation with a far-red laser flash light (Inoue *et al.* 1982) indicates that some of the induced intermediates were transformed back to P_{fr} besides the phototransformation from P_{fr} to P_r (figure 5). Considering this dark process, the decay curve of I_{645} was determined at 554 nm, at which the absorbance of the intermediate rapidly decreased. The resultant curve was kinetically resolved into three reactions with rate constants of 2500, 590 and 48 s^{-1}.

Spectral changes in the visible and near-u.v. region during phototransformations from P_r to P_{fr} (Shimazaki *et al.* 1980) and from P_{fr} to P_r (Inoue *et al.* 1982) became available with the use of a custom-built multichannel transient spectral analyser that can determine the absorbance change in the spectral range between 350 and 800 nm at once with a sampling time of 300 μs beginning 10 μs after a flash excitation (Furuya *et al.* 1983). Difference spectra between phototransformation intermediates and P_r showed that a small but significant increase at 400–410 nm and decrease at 360 nm took place 10–260 μs after a red light laser flash, and those between intermediates and P_{fr} indicated that an increase in absorbance at 370–380 nm and a decrease around 415 nm occurred 10–310 μs after a far-red flash. Using these data on intermediate spectra, Sugimoto *et al.* (1983) analysed theoretically the change of chromophore structure of phytochrome during the phototransformations in terms of wavelength and oscillator strength of absorption by using the zero-differential approximation of molecular orbital theory for π-electrons. The effects of a point charge and a point dipole on the optical absorption of phytochromobilin intermediates were examined by the stationary perturbation theory for degenerate states. The results indicate that the *cis–trans* photoisomerization of pyrrole ring D, if any, occurred within 10 μs after a laser-flash excitation of phytochrome and that the conformation of phytochromobilin and the protein moiety did not significantly change during the examined period of phototransformations in both directions.

The results on phototransformation pathways of 'large' pea and oat phytochrome obtained by laser flash photolysis in my laboratory are summarized in figure 5. The three elementary reaction stages in each direction were kinetically separatable in both directions of the phototransformation pathway: namely, I_{692}, I_{bl} and P_{fr} were found to be formed on the way from P_r to P_{fr} (Pratt *et al.* 1982), and similarly I_{645}, I_{660} and P_r were involved on the way from P_{fr} to P_r (Inoue *et al.* 1982). Each stage in both directions consisted of three kinetically distinct reactions (figure 5).

(*b*) *Heterogeneity of monomer size*

A question thus arises as to whether the triple pathways in each stage result from subunit heterogeneity of the samples, such as the 124, 118 and 114 kDa monomers (table 1), or whether

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it happens even with a sample of homogeneous monomer size. To answer this question, we have carefully extracted homogeneous samples of 124 and 62 kDa rye phytochrome (figure 2), and re-examined the phototransformation pathways. The results clearly showed that three kinetically distinguishable reactions were separated with both homogeneous samples, and that when the mixture of 124–118 kDa samples was measured, we separated more than four stages of P_{fr} appearance from I_{bl} (Inoue, Yamamoto & Furuya, unpublished).

(*c*) *Low-temperature spectroscopy*

The phototransformation of P_r to P_{fr} in 'small' oat phytochrome was mainly studied by low-temperature spectroscopy (Spruit 1975), and that in 'large' oat phytochrome was recently reported by Song *et al.* (1981). We have therefore intensively investigated the spectral changes accompanying phototransformation in both directions by low-temperature spectroscopy with 'large' pea phytochrome (Sasaki *et al.* 1983*a*, *b*), using the same samples as in the above studies on laser-flash photolysis (Shimazaki *et al.* 1980; Inoue *et al.* 1982; Pratt *et al.* 1983). It is fruitful to compare the data obtained by these two different techniques. It became evident that an irradiation of P_r with red light below −80 °C yielded an intermediate, I_{693}. Part of this intermediate reverted to P_r if kept above −130 °C, and the residue was converted to a 'bleaching form (I_{bl})' with two components. On warming, it was finally converted to P_{fr} at −40 °C or higher. In the phototransformation of P_{fr} to P_r an intermediate (I_{670}) was formed below −80 °C, which was converted by warming above −80 °C to I_{665}, with two components. The intermediates were then transformed to P_r via I^*_{665}. The elementary pathways separated in this work are shown schematically in figure 6.

It cannot yet be decided whether the courses of the conversion from I_{693} to P_{fr} and from I_{670} to P_r are parallel or sequential. However, we found many similarities between experimental results obtained by flash photolysis near room temperature and those by low-temperature spectrophotometry. One of the typical examples is that the spectral changes in the conversion of I_{693} were similar to those observed by Shimazaki *et al.* (1980). As already described, the formation of I_{693} was dependent on temperature, owing to the dark reversion of I_{693}-I to P_r above −130 °C. Moreover, because I_{693}-II was converted to I_{bl}, composed of two molecular species, it was concluded that I_{693} should consist of three components.

In the phototransformation of P_{fr} to P_r, the rate of formation of I_{670} decreased as the temperature was lowered, and at least three intermediates (I_{665} (low), I_{665} (high) and I^*_{665}) were observed in the course of the thermal conversion of I_{670} to P_r. The large spectral shift due to photoreaction of P_{fr} to I_{670} suggests that I_{670} may have a π-electron system of the chromophore

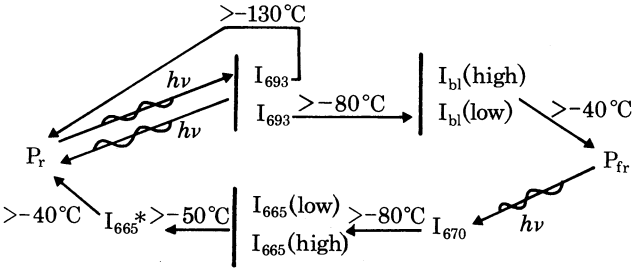

FIGURE 6. A scheme for the photoreaction cycle of phytochrome proposed on the basis of low-temperature spectrophotometry (Sasaki *et al.* 1983*b*). For details see the text.

close to that of P_r. The conversion of I_{670} to P_r in the dark might be induced by conformational changes of the protein moiety surrounding the chromophore.

5. Monoclonal antibodies to phytochrome

The molecular properties and function of phytochrome have been immunochemically studied by using multiclonal antibodies (Hopkins & Butler 1970; Rice & Briggs 1973; Pratt 1973; Cordonnier & Pratt 1982). As the conventional antibodies produced by multiple immunochemical determinants showed diverse antigenic specificity, their application in the analysis of phytochrome's molecular structure has been limited, and in particular they cannot be used to identify the entity governing the action of phytochrome. The recently developed method of producing monoclonal antibodies (Köhler & Milstein 1975), however, provides an antibody of undoubted specificity and unlimited supply, and it is possible to produce monoclonal antibodies that are specific to different determinants of the surface of the phytochrome molecule.

We have recently succeeded in producing six monoclonal antibodies (AR1 to AR6) to rye phytochrome (Nagatani *et al.* 1983*a*) and six (AP1 to AP6) to pea phytochrome (Nagatani *et al.* 1983*b*) by fusing spleen cells from immunized BALB/c mice with NS-1 myeloma cells. All the clones have been stably producing each monoclonal antibody in ascitic fluids of pristane-treated BALB/c mice into which the hybridomas were intraperitoneally injected. The binding ability of the monoclonal antibodies (mAbs) to phytochrome, which were coupled to either sheep red blood cells (s.r.b.c.) or Sepharose 4B, were determined by radioimmunoassay. The six mAbs to rye phytochrome had titres (defined as the dilution giving 50% binding in radioimmunoassay) of between $1:8\times10^4$ and $1:8\times10^5$.

The results of immunoelectrophoresis of these mAbs with the use of rabbit anti-mouse IgG_1 serum indicated that two of the six ARs were of the IgG_2 type and the other four were of the IgG_1 type. It is very important to know the determinant sites of the phytochrome molecule that resulted in each mAb. Thus the reactivities of each mAb to 'small' phytochrome and variously digested chromopeptides were measured by the inhibition assay (Mason & Williams 1980). All six mAbs of rye phytochrome were able to bind to the 40 kDa fragments, although the ratios of the molar concentrations giving 50% inhibition for 'small' phytochrome to that for 'large' phytochrome were different. Namely, the ratios for AR1, AR2, AR3 and AR4 were significantly higher than those for AR5 and AR6, whereas the ratios for AR5 and 6 were lowest, in the neighbourhood of 1.5.

Absorption spectra of P_r, and that at red-light-induced photoequilibrium, were measured after incubation of 1 μM P_r with *ca.* 10 μM crude IgG in phosphate-buffered saline at 4 °C in the dark overnight. None of the six IgGs affected the spectra. This fact that the binding of mAbs to phytochrome does not influence the photoreversible spectral change suggests a possibility that determinants of all the tested mAbs are located in the peptide regions that do not affect the chromophoric domain of phytochrome. Reactivities of the mAbs against 'large' rye phytochrome were determined with P_r in the dark or with phytochrome under continuous red light at 4 °C overnight to check the difference between affinities of the mAbs to P_r and P_{fr}. Their affinities to P_r and P_{fr} were indistinguishable in radioimmunoassay.

The cross-reactivity of the twelve available mAbs toward phytochromes provided from four dicotyledonous and three monocotyledonous species was examined by inhibition assay.

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AR1–AR6 did not cross-react with pea phytochrome at the maximum concentration tested (0.4 mg ml^{-1}), whereas AR5 and AR6 did cross-react with 'large' oat phytochrome (table 2). It is interesting to note that AR6 showed a stronger affinity to 'large' oat phytochrome than to 'large' rye phytochrome. The results so far obtained are summarized in table 2. At present, none of the mAbs react with both monocotyledonous and dicotyledonous phytochromes.

TABLE 2. CROSS-REACTIVITY OF THE MONOCLONAL ANTIBODIES OF RYE AND PEA PHYTOCHROMES TOWARD PHYTOCHROMES PREPARED FROM VARIOUS PLANT SPECIES

phytochrome prepared from	anti-rye phytochrome antibodies						anti-pea phytochrome antibodies					
	AR1	AR2	AR3	AR4	AR5	AR6	AP1	AP2	AP3	AP4	AP5	AP6
rye (*Secale cereale*)	+	+	+	+	+	+	−	−	−	−	−	−
oat (*Avena sativa*)	−	−	−	−	+	+	−	−	−	−	−	−
rice (*Oryza sativa*)	−	−	−	−	−	−	−	−	−	−	−	−
pea (*Pisum sativum*)	−	−	−	−	−	−	+	+	+	+	+	+
mung bean (*Vigna radiata*)	−	−	−	−	−	−	−	−	−	(+)	+	+
radish (*Raphanus sativus*)	−	−	−	−	−	−	−	−	−	−	(+)	+
morning glory (*Pharbitis nil*)	−	−	−	−	−	−	−	−	−	−	−	−

Symbols: +, positive reaction; (+), weak positive reaction; −, negative reaction.

Cundiff & Pratt (1975) reported the presence of antibodies that bound to 'large' phytochrome but not to 'small' phytochrome by double diffusion assay. All the six mAbs to rye phytochrome in this work, however, reacted with both 'large' and 'small' phytochromes. In this connection it must be mentioned that the assay system used for the screening of these six mAbs to rye phytochrome employed phytochrome coupled with s.r.b.c. (Nagatani *et al.* 1983 *a*). Considering that phytochrome is a multifunctional protein with a chromophoric and a hydrophobic domain and that the hydrophobic domain responsible for the photoinduced increase of hydrophobicity is separated by trypsin digestion for the chromophoric domain, accounts for the phototransformation between P_r and P_{fr} (Tokutomi *et al.* 1981). Although the mechanism of the binding of proteins with s.r.b.cs is poorly understood (Goding 1976), it is possible that phytochrome used in the assay binds to s.r.b.cs through hydrophobic interaction so that the hydrophobic region of phytochrome is hidden by the surface of the s.r.b.cs. In fact, when we used Sepharose 4B instead of s.r.b.cs, we obtained mAbs that showed a greater variety of binding site (Nagatani *et al.* 1983 *b*).

6. CONCLUDING REMARKS

As phytochrome-mediated photoreversible responses in plants must result from the difference of molecular properties between P_r and P_{fr}, extensive attempts have been made to find such differences. Many studies, however, failed to find such differences on physical and chemical nature of phytochrome (table 3). Recently, some molecular properties of either the chromophore or the apoprotein have been demonstrated to show differences between P_r and P_{fr} (table 4). This information should be a great help in understanding the primary action of phytochrome, but it is also true that no one yet knows how phytochrome induces photomorphogenetic reactions.

Molecular properties of phytochrome have been studied mainly with 'large' and 'small'

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Table 3. Molecular properties of phytochrome that showed no differences between P_r and P_{fr}

property tested	reference
c.d. spectra in u.v. region	Tobin & Briggs (1973)
n.m.r. spectra of aliphatic protons	Song *et al.* (1982)
exposed tyrosine and carboxyl groups	Hunt & Pratt (1981)
electrophoretic mobility	Briggs *et al.* (1968)
surface charges (isoelectric focusing)	Hunt & Pratt (1981)
sedimentation velocity	Briggs *et al.* (1968)
elution profiles of gel exclusion and brushite chromatography	Briggs *et al.* (1968)
affinity for anti-'small' phytochrome sera	Pratt (1973) Rice & Briggs (1973)
affinity for anti-'large' phytochrome sera	Cundiff & Pratt (1975)
affinity for monoclonal anti-'large' phytochrome antibodies	Nagatani *et al.* (1983*a*)

Table 4. Molecular properties of phytochrome that showed differences between P_r and P_{fr}

property tested	P_r	P_{fr}	reference
spectral lability in the presence of urea	<		Butler *et al.* (1964)
spectral lability in the presence of Me^{2+}	<		Pratt & Cundiff (1975)
spectral lability in the presence of $KMnO_4$	<		Hahn *et al.* (1980)
exposed cysteine and histidine	<		Hunt & Pratt (1981)
exposed tryptophan	<		Sarkar & Song (1982)
n.m.r. spectra of aromatic and –NH-proton resonance region	<		Song *et al.* (1982)
exchangeable proton	<		Hahn & Song (1982)
proton uptake and release	<		Tokutomi *et al.* (1982)
affinity for alkyl groups	<		Yamamoto & Smith (1981) Tokutomi *et al.* (1981)
affinity for Cibacron blue F3GA	<		Smith (1981)
affinity for ANS	<		Hahn & Song (1981)
affinity for liposomes	<		Kim & Song (1981)
energy transfer from FMN to phytochrome	+	–	Song *et al.* (1981)

phytochrome in the past. But, although it seems likely that 124 kDa monomers are 'native' phytochrome, many questions remain unsolved as discussed by Quail (this symposium). As we now can routinely provide 'native' phytochrome, it would be important to re-examine the molecular properties of phytochrome. Furthermore, we really need to know the differences between monomers, dimers and other polymers of phytochrome, if any, with their physical and chemical properties, and it is especially important to demonstrate whether or not phytochrome in cells exists as a dimer, as it does in solution.

Comparing the knowledge that we had a decade ago, we can say that the progress has been enormous, but it is very important to continue investigating the molecular properties of phytochrome. However, it would be more crucial to discover the partner substance(s) that transduce the signal from a phytochrome molecule to cell membranes. In this connection the evidence for the different dichroic orientation of P_r and P_{fr} at cell membranes in *Mougeotia* (Haupt *et al.* 1969) and *Adiantum* (Kadota *et al.* 1982) encourages us to work in such direction.

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The research on the molecular properties of phytochrome in my laboratories was supported in part by Grants-in-Aid for special research on photophysiology in 1978–81 and for group research on phytochrome in 1981–3. This work has been made possible by collaboration with many experts in various fields, to whom I should like to make grateful acknowledgement of their help. I am specially indebted to Dr Y. Inoue, Dr K. T. Yamamoto and Dr S. Tokutomi for their thorough and painstaking efforts during this work.

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Discussion

W. Haupt (*Institut für Botanik und Pharmazeutische Biologie, Erlangen, F.R.G.*). In Professor Furuya's scheme with the phytochrome intermediates, I realized that only light-dependent reversions of I_{692} and I_{bl} to P_r were shown. This is in contrast to the classical Kendrick–Spruit scheme, according to which dark reversions (or dark relaxations) to P_r also occur. Is this a definite revision of the classical scheme?

M. Furuya. No. I cannot say anything on the dark reversion from our present work. What I talked about is that I_{692} and I_{bl} went back to P_r, but not to P_{fr}, by a second laser flash excitation. This is the first evidence found at room temperature, and tends to confirm the results obtained by low-temperature spectroscopy.

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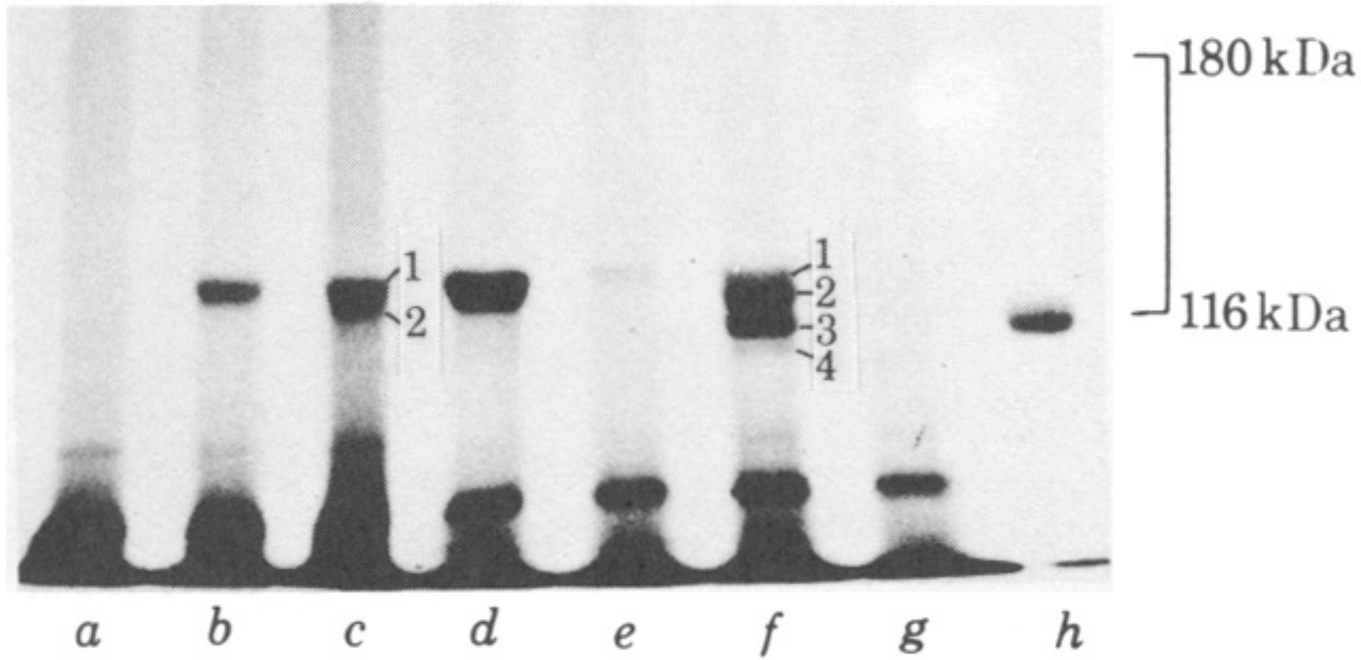

FIGURE 2. Sodium dodecyl sulphate polyacrylamide gel electrophoretograms of immunoprecipitated rye phytochrome (K. T. Yamamoto, unpublished). Phytochromes immunoprecipitated from crude extracts as P_{fr} (*a*, *b*) or P_r (*c*) were compared with those immunoprecipitated from purified phytochrome preparations obtained by conventional procedures as P_{fr} (*d*, *e*) or P_r (*f*, *g*). Conventionally purified pea phytochrome was also included in the electrophoresis for comparison (*h*). Immunoprecipitations were performed with monoclonal anti-rye phytochrome antibody (AR3)-coated *Staphylococcus aureus* cells (*b*, *c*, *d*, *f*) according to the method of Vierstra & Quail (1982*a*). A monoclonal antibody to rat liver cell membrane glycoproteins (Fukumoto *et al.* in preparation) was also used as a control for non-specific binding of antibodies (*a*, *e*, *g*). The immunoprecipitates were subjected to sodium dodecyl sulphate polyacrylamide electrophoresis (Laemmli 1970) using 30 g l^{-1} acrylamide stacking gel and 50 g l^{-1} separating gel.