Distribution and Nonphotochemical Transformation of Phytochrome in Subcellular Fractions from Pisum Epicotyls

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ABSTRACT
In etiolated pea (Pisum satiun L. cv. Alaska) shoots about 3% of the total extractable phytochrome was found in the mitochondrial fraction and about 4.5% in the microsomal fraction, while over 70% was soluble in the 105,000g supernatant. The value of ΔΔA per milligram of protein was significantly higher in the 105,000g supernatant than in these particulate fractions. The percentage conversion of Pr to Pfr was approximately proportional to the total dose of red light in every subcellular fraction tested, unless the dose approached a saturation level. After a brief irradiation of intact shoots with red light at 26°C, each subcellular fraction showed different patterns of dark transformation in vivo at 26°C: that is, the amount of the particulate-bound phytochrome increased immediately after the irradiation, and a reversion of Pfr to Pr was indicated for the first 2 hr in the 12,000g supernatant, but not at all in the mitochondrial and microsomal fractions. The amounts of Pr in the mitochondrial and microsomal fractions did not change during the dark incubation, while those in the 12,000g supernatant increased with time. Similar results were obtained with apical shoot segments after exposure to red light at 0°C and a subsequent dark incubation on moist filter paper at 26°C.

Although the intracellular distribution of phytochrome had long been an open question, recent studies revealed that spectrophotometrically detectable phytochrome was located not only in the cytosol (3, 5) but was also found in various particulate fractions such as the 1,500 to 40,000g pellet from an etiolated oat seedling homogenate (20), the 1,000 to 20,000g pellet from maize coleoptiles and pumpkin hooks (19), the 500 to 17,000g pellet (14) and the 17,000 to 100,000g pellet (15) from squash hypocotyls, the 1,000 to 7,000g particle fraction from bean hypocotyls (11) and purified mitochondria from etiolated pea epicotyls (12). Thus, evidence has accumulated that phytochrome is also located in particulate fractions.

Many earlier attempts have failed to correlate the spectrophotometrically detectable status of in vivo phytochrome with the physiological responses to red and far red light (6, 8). Briggs and Chon (2) explained this paradox by postulating that a small, physiologically active fraction of phytochrome is much more easily converted to Pfr than is bulk phytochrome. Amounts of particle-bound phytochrome in etiolated tissues of oat (20), corn (19), and squash (14) were indicated as 4%, 6%, and 7.5%, respectively, indicating that only a small fraction of the total extractable phytochrome from dark-grown tissues is bound to particle fractions, most being in the cytosol. The amounts of bound phytochrome increased significantly after exposure of the tissues to red light, depending upon the pH (19) and Mg concentration (14,15) in the extraction medium, the temperature at irradiation time (13), and the rate of Pfr decay (13). Thus, there might be a possibility that particle-bound phytochrome is the above-mentioned "small, active fraction" of in vivo phytochrome controlling physiological responses.

The aims of the present work were to demonstrate the intracellular distribution of phytochrome in totally etiolated tissues of pea shoots before and after light treatments, to see the relationship between the amount of incident irradiation energy and the degree of photoconversion of phytochrome in each subcellular fraction, and to determine the fates of Pfr in each fraction during the dark incubation.

MATERIAL AND METHODS
Plant Material, Light Sources, and Irradiation. Seeds of Pisum satiun L. cv. Alaska, purchased from Watanabe Seed Co., Kogota, Miyagi, Japan, were germinated in a dark room at 26°C on vermiculite saturated with water. Apical 2-cm long portions of the shoots were cut with scissors from 5-day-old etiolated seedlings under dim green safe light and were kept in the dark at about 0°C on crushed ice until used in the experiments.

Red light was provided by three 20-w fluorescent tubes (Toshiba FL 20 SW) behind a 3.2-mm thick sheet of red Plexiglas (Rohm and Haas, No. 2444). Far red light was supplied by one 500-w incandescent lamp (Matsushita, reflector lamp) behind about 10 cm of water and a 3.2-mm thick black Plexiglas (Rohm and Haas, V-58015).

The prechilled segments were irradiated with 0.75 w m⁻² red light for 3 min, or with 15 w m⁻² far red light for 2 min or both, at 0°C.

Extraction and Fractionation Procedures. For preparation of particulate fractions, 10 g of shoot segments were placed on ice for 15 min or longer, and were homogenized by grinding with a pestle in a chilled mortar with 20 ml of 10 mM tris-HCl buffer, pH 7.2, containing 0.4 M sucrose, and 1 mM dithiothreitol. After filtration through two layers of cheesecloth, the extract was adjusted to pH 7.2 with 0.5 M tris solution, centrifuged for 10 min at 10,000g, and the supernatant further centrifuged for 10 min at

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12,000g. The pellet from the 12,000g centrifugation was washed with 20 ml of the extraction buffer described above, and centrifuged at 400g for 5 min. The supernatant from the 400g centrifugation was further centrifuged for 10 min at 7,000g, and the pellet was resuspended in 1.75 ml of the same extraction buffer. This suspension was used as the “mitochondrial” fraction. This entire procedure was carried out in a cold room at 3°C under a dim green safe light.

After preparation of the mitochondrial fraction, the supernatant from the first 12,000g centrifugation was further centrifuged at 105,000g for 30 min, and the 12,000 to 105,000g sedimentary particle fraction was suspended in 1.5 ml of the extraction buffer. This suspension is called the “microsomal” fraction. The extraction protocol and the terminology of the fractions are shown in Figure 1.

The amounts of protein in the samples were determined by the method of Lowry et al. (10).

Phytochrome Determination. Phytochrome was determined with a dual-wavelength difference spectrophotometer (Hitachi Ltd., Japan, Model 261), as previously described (16). For the assay, suspensions were pipetted into a hollow cylinder with glass windows (10 mm in diameter, 16 mm in path length), and shoot segments were packed in a cuvette (6 mm in diameter, about 5 mm in path length). The cell was maintained at about 0°C with crushed ice before and during measurement.

Each value in Tables and Figures of this paper represents an average of 2 to 6 samples from at least two separate experiments.

RESULTS

Intracellular Distribution of Phytochrome in Etiolated Pea Shoots. An attempt was made to see how phytochrome is distributed in the subcellular fractions obtained by the routine extraction procedure from 10 g of apical 2-cm shoots of 5-day-old totally etiolated pea seedlings. Table 1 clearly demonstrates that only 2.7% of total extractable phytochrome was bound to the mitochondrial fraction and 4.5% to the microsomal fraction, while about 70% was in the cytosol, and that the Δ(ΔA) per milligram of protein value with the 105,000g supernatants was significantly higher than that of the particulate fractions. Percentages of protein distribution with the subcellular fractions were not always consistent with those of phytochrome distribution.

Effect of Red Light Irradiation on Photoconversion in Subcellular Fractions. Apical 2-cm segments were kept on ice in the dark for at least 15 min before red light treatment, and were irradiated with 0.75 w m⁻² red light for various period of time at about 0°C. The percentage of Pfr transformed was determined with intact segments and with subcellular fractions from such segments (Fig. 2). The percentage of conversion of Pr to Pfr is simply dependent upon total incident energy of red light in intact tissue, the mitochondrial fraction, and both the 10,000 g and the 105,000 g supernatants, irrespective of their phytochrome concentrations. In the microsomal fraction, the percentage of Pfr was somewhat higher than in other fractions at a dosage higher than 16 joules m⁻².

Table 1. Intracellular Distribution of Phytochrome in Totally Etiolated Tissues of Pea Epicuticle

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Phytochrome Conc.</th>
<th>Protein Conc.</th>
<th>Specific Activity</th>
<th>Distribution of Protein</th>
<th>Distribution of Phytochrome</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Δ(ΔA) X 10⁶/mg protein</td>
<td>mg/mg protein/10⁵/g fresh wt</td>
<td>%</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>1000g Supernatant</td>
<td>769 ± 20</td>
<td>4.48 ± 0.02</td>
<td>170 ± 10</td>
<td>86.1 ± 3.5</td>
<td>100 ± 2.3</td>
</tr>
<tr>
<td>Mitochondrial</td>
<td>196 ± 1</td>
<td>1.80 ± 0.01</td>
<td>109 ± 10</td>
<td>3.55 ± 0.3</td>
<td>4.1 ± 0.3</td>
</tr>
<tr>
<td>12000g Supernatant</td>
<td>720 ± 20</td>
<td>4.34 ± 0.02</td>
<td>167 ± 10</td>
<td>80.0 ± 3.5</td>
<td>92.9 ± 3.5</td>
</tr>
<tr>
<td>Microsomal</td>
<td>174 ± 1</td>
<td>2.36 ± 0.01</td>
<td>74 ± 10</td>
<td>8.77 ± 0.3</td>
<td>10.2 ± 0.3</td>
</tr>
<tr>
<td>105000g Supernatant</td>
<td>608 ± 20</td>
<td>4.08 ± 0.02</td>
<td>151 ± 10</td>
<td>70.2 ± 3.5</td>
<td>81.5 ± 3.5</td>
</tr>
</tbody>
</table>

Values are means with standard deviations.

Fig. 1. Extraction procedure for subcellular fractions and the terminology of the fractions.

Fig. 2. Effect of red irradiation energy on conversion of Pr into Pfr with intact tissues of apical pea shoots (O), the mitochondrial fraction (C, M), 12,000 g supernatant (B, S), microsomal fraction (O, M), and cytosol (O, C). Arrows identify dots if they are piled. The segments were maintained at 0°C before and during the treatment.
Photoreversible Effect on Intracellular Distribution of Phytochrome. To observe the change, if any, of the intracellular distribution of phytochrome after the photoconversion of Pr to Pfr, shoot segments obtained from 5-day-old etiolated pea seedlings were kept on moist filter paper at 0 C, and were irradiated with 0.75 w m⁻² red light for 3 min on ice. The segments were returned to darkness at 26 C immediately after the treatment. The distribution of in vivo phytochrome in subcellular fractions of the segments was determined 2 hr after the irradiation by the extraction procedure (Table II). With the red light-treated segments, the phytochrome content of all tested fractions decreased during the 2-hr incubation, although the protein concentrations were not significantly altered, and percentage of Pfr in particulate fractions (especially in mitochondrial fraction) was maintained at a higher level than in the 12,000g supernatant (Fig. 4).

The red light-induced changes of the phytochrome content were far-red reversible when the segments were irradiated with 15 w m⁻² far red light for 2 min immediately after the initial exposure to red light (Table II).

Dark Transformations of Pfr in Subcellular Fractions of Intact Shoots. Intact etiolated seedlings were irradiated with 0.75 w m⁻² red light for 3 min at 26 C, and were transferred to a dark room at 26 C for up to 3 hr. Apical 2 cm portion of the shoots were cut with scissors after the dark incubation, quickly transferred onto ice, and were then used as materials for fractionation. The amounts of total phytochrome and Pfr in the subcellular fraction were spectrophotometrically determined at 0, 0.5, 1, 2, and 3 hr after the irradiation (Fig. 3). The total amount of phytochrome increased immediately after the red light treatment in the particulate fractions but not in the 12,000g supernatant. However, the dark reversion of Pfr to Pr was observed during the first 2 hr in the supernatant fraction but not in the mitochondrial and microsomal fractions. Amounts of Pr appear not to change in the particulate fractions during the dark incubation, but increase in the 12,000g supernatant fractions.

![Figure 3](image_url)  
**Fig. 3.** Dark changes of phytochrome at 26 C in mitochondrial and microsomal fractions, and 12,000g supernatant of intact pea shoots after a brief irradiation with red light. Measured values were multiplied by a factor of 1.25 (9) for the corrected values of total phytochrome (○), Pfr measured (○), Pr calculated (○), total phytochrome of dark control (●).

![Figure 4](image_url)  
**Fig. 4.** Dark changes of phytochrome at 26 C in mitochondrial and microsomal fractions and 12,000g supernatant of pea shoot segments. Symbols used and the correction of total phytochrome are the same as in Figure 3.

The intracellular distribution of phytochrome in etiolated pea shoots was significantly changed immediately after a brief irradiation.

Table II. Effect of Red and Far Red Light on the Intracellular Distribution of Phytochrome in Etiolated Tissues of Pea Epicotyl Segments

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Fraction</th>
<th>Phytochrome Concn.</th>
<th>Protein Concn.</th>
<th>Specific Activity</th>
<th>Distribution of Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Δ(A) X 10⁻³ mg/mg extract</td>
<td>mg/ml extract</td>
<td>Δ(A) X 10⁻³ mg protein</td>
<td>mg protein/10 g fresh tissue</td>
</tr>
<tr>
<td>Red</td>
<td>1,000 g Supernatant</td>
<td>±37</td>
<td>441</td>
<td>4.70</td>
<td>94</td>
</tr>
<tr>
<td></td>
<td>Mitochondrial</td>
<td>126</td>
<td>±23</td>
<td>2.11</td>
<td>60</td>
</tr>
<tr>
<td></td>
<td>12,000 g Supernatant</td>
<td>417</td>
<td>±41</td>
<td>4.39</td>
<td>95</td>
</tr>
<tr>
<td></td>
<td>Microsomal</td>
<td>115</td>
<td>±38</td>
<td>0.27</td>
<td>±10</td>
</tr>
<tr>
<td></td>
<td>105,000 g Supernatant</td>
<td>366</td>
<td>±38</td>
<td>4.11</td>
<td>89</td>
</tr>
<tr>
<td>Red/FR</td>
<td>1,000 g Supernatant</td>
<td>±40</td>
<td>652</td>
<td>4.02</td>
<td>162</td>
</tr>
<tr>
<td></td>
<td>Mitochondrial</td>
<td>256</td>
<td>±23</td>
<td>2.07</td>
<td>124</td>
</tr>
<tr>
<td></td>
<td>12,000 g Supernatant</td>
<td>615</td>
<td>±64</td>
<td>3.63</td>
<td>164</td>
</tr>
<tr>
<td></td>
<td>Microsomal</td>
<td>270</td>
<td>±35</td>
<td>3.33</td>
<td>82</td>
</tr>
<tr>
<td></td>
<td>105,000 g Supernatant</td>
<td>578</td>
<td>±39</td>
<td>3.52</td>
<td>165</td>
</tr>
</tbody>
</table>

1 Values are means with standard deviations.
ation with red light. The bulk phytochrome in cytosol and the membrane-bound phytochrome in mitochondrial and microsomal fractions were equally photoconverted with the amount of photoconversion dependent on total incident energy (Fig. 2). These results do not support the hypothesis of Briggs and Chon (2) that the Zea etopic sensitivity paradox might result from a small, physiologically active fraction of phytochrome which is much more easily converted to Pfr than the bulk phytochrome. When the shoot tissues were irradiated with 10 joules m⁻² or more red light, causing 80% or more photoconversion of Pr to Pfr, the irradiation always caused a somewhat higher percentage of Pfr in the microsomal fraction than in any other fraction (Manabe, unpublished data). This result still does not support the above cited hypothesis by Briggs and Chon (2).

Manabe and Furuya (13) recently found that a brief irradiation with red light of pea shoot segments kept at 0°C resulted in very rapid binding of both Pr and Pfr to mitochondrial and microsomal fractions, and the effect was not far-red reversible. The rapid binding of phytochrome to the particulate fractions by red light irradiation at low temperature was clearly confirmed with both intact tissues (Fig. 3) and excised segments (Fig. 4) of etiolated pea shoots. Although this rapid, red light-induced increase of bound phytochrome at low temperature was not reversed by subsequently applied far red light, this effect appeared to be dependent on phytochrome because the dose response curve for the red light-induced bound phytochrome was almost identical to the dose response curve in the phototransformation of phytochrome (13).

Since the early work of Furuya and Hillman (7), dark transformations of phytochrome in pea tissues have been studied extensively by several workers (6), nothing is known about the fate of phytochrome in each subcellular fraction after an initial red light irradiation. The present work partly answers the question, showing that the pattern of phytochrome change in the 12,000g supernatant obtained from red light-treated shoots (Fig. 3) reflects that in intact pea tissues (7), whereas the dark reaction curves of the particulate-bound phytochrome (Figs. 3 and 4) are quite different from those of the 12,000g supernatant and the intact tissue. The amount of Pr in mitochondrial and microsomal fractions remains constant during the dark period, maintaining the level of Pr which had been induced by an initial red irradiation. In contrast, dark reversion of Pfr to Pr took place in the 12,000g fraction (Figs. 3 and 4) and intact epicytophy (7) during the first 2 hr of dark incubation. Boisard et al. (1) recently reported that in squash seedlings phytochrome destruction is related exclusively to the fraction that becomes membrane bound, while the soluble fraction of phytochrome remain constant. Pratt et al. (18) immunochromically demonstrated that in oat shoots the in vivo phytochrome destruction involves the loss of extractable protein moiety of phytochrome. The pattern of dark phytochrome changes in those particulate fractions is the same as that in intact coleoptiles of Zea (49, 17), Hordeum (9), and Oryza (16), while the pattern of the 12,000g supernatant appears to be comparable with that in root tips of Pisum (7), and hypocotyls of Phaseolus, Raphanus, and Glycine (9).

Quail et al. (19) found that brief exposure of maize coleoptiles and pumpkin hypocotyls to red or far-red light, or both, at 25°C increased the pelletability to 16 to 26% instantaneously in the presence of Mg²⁺, and that the pelletability of phytochrome decreased with time in the dark following first order kinetics with a half-time of about 50 min. A similar phytochrome decrease was seen in the particulate fractions obtained from the samples of red light-treated tissues of pea shoots in extraction buffer without Mg²⁺ or Ca²⁺ (Figs. 3 and 4). When the pea shoot segments were briefly irradiated with red light at 0°C and subsequently incubated at 30°C in tris-HCl buffer containing dithiothreitol or EDTA, which inhibits Pfr decay, the contents of phytochrome in the mitochondrial and microsomal fractions were significantly enhanced with time, and the red light effect was reversed by far red light (13). Therefore, the process of Pfr decay in the dark apparently has an important role in the in vivo binding of phytochrome to the particulate fractions in pea tissues.

LITERATURE CITED