Properties of Protochlorophyllide and Chlorophyll(ide) Holochromes from Etiolated and Greening Leaves

K. W. Henningsen, S. W. Thorne, and N. K. Boardman
Division of Plant Industry, Commonwealth Scientific and Industrial Research Organization, Canberra, 2601, Australia

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Abstract
Protochlorophyllide and chlorophyll(ide) holochromes (Pchl-H and Chl-H) were extracted from dark-grown and greening seedlings with saponin and partly purified by ammonium sulfate fractionation. Sephadex gel filtration in the presence of saponin showed that the photoactive saponin Pchl-H from dark-grown leaves of bean (Phaseolus vulgaris L. cv. Redlands Pioneer) or pea (Pisum sativum L. cv. Greenfeast) has an apparent molecular weight of about 170,000, compared with about 70,000 to 80,000 for the saponin Pchl-H from barley (Hordeum vulgare L. cv. Svalöfs Bonus). Photoconversion of saponin Pchl-H from dark-grown barley seedlings yields Chl-H with an absorption maximum at 678 nm, and with no change in apparent molecular weight. Above 0°C, a spectral shift from 678 to 672 nm follows, and a change in apparent molecular weight from about 65,000 to 29,000 is observed.

Saponin Chl-H extracted from barley leaves illuminated for 15 minutes has an absorption maximum at 670 nm and an apparent molecular weight greater than 100,000. This chlorophyll holochrome has photosystem I activity and is eluted together with the cytochromes. Saponin holochrome extracted from barley leaves returned to darkness after a light period, contains chlorophyll(ide) and protochlorophyllide complexes. Gel chromatography yields a complete separation of Chl-H (apparent molecular weight > 100,000) and photoactive Pchl-H (63,000).

It is proposed that Chl-H dissociates into a chlorophyll(ide) a carrier protein complex and a photoenzyme, before the incorporation of chlorophyll into the lamellar membrane.

Spectrofluorimetry on partially photoconverted preparations of saponin holochrome from barley, bean, and pea gave no indication for resonance energy transfer from protochlorophyllide to chlorophyllide. The saponin holochromes gave high polarization values, in contrast with bean holochrome extracted without the aid of detergents and bean leaves.

Dark-grown seedlings of angiosperms contain a small amount of Pchl which is photoconverted to Chl a on illumination of the plants. The photoactive Pchl is associated with the prolamellar body membranes of the etioplasts (15, 16). After the photoconversion of Pchl a series of spectral changes occur in vivo. Immediately following photoconversion, the absorption maximum of Chl a is at 678 nm. There is then rapid shift in the absorption maximum to 684 nm (5, 10) followed by a much slower shift from 684 to 672 nm (23). Seedlings returned to darkness reaccumulate Pchl to about the same level as that of dark-grown seedlings (23).

A protochlorophyllide-protein complex, termed protochlorophyllide holochrome (Pchl-H), with a mol wt of about 600,000 (1) can be isolated from dark-grown bean seedlings without the aid of detergents (18, 24). With the detergents, Triton X-100 and saponin, Pchl-H of lower mol wt (100,000–300,000) can be obtained from bean leaves (14, 21). Subunits of Pchl-H with an apparent mol wt of 63,000 have been isolated from dark-grown barley seedlings with the aid of saponin (14).

Fluorescence measurements at liquid nitrogen temperature of leaves, isolated prolamellar body membranes, and non-detergent Pchl-H, indicated that the pigment molecules are organized into energy-transferring units (6, 16, 27). From circular dichroism spectra on bean Pchl-H, it was concluded that the pigment molecules are present in aggregated form, probably as dimers (19, 22). On the other hand, spectrofluorimetry (14) and circular dichroism spectroscopy (K. W. Henningsen, A. Kahn, and C. Houssier, unpublished data) on barley Pchl subunits gave no indication of multiple chromophores.

A change in size or conformation of Pchl-H has been postulated after in vivo or in vitro photoconversion. Bean Chl-H was found to be lighter in sucrose density gradients than Pchl-H (4). In contrast, the Chl-protein complex, obtained from leaves illuminated for 30 min or longer was heavier than Pchl-H (2, 4).

Barley Pchl-H subunits were indistinguishable by Sephadex gel filtration from their immediate in vitro photoproduction absorbing at 678 nm (14). But a shift in the absorption maximum of the subunits from 678 nm to 672 nm correlated with changes in the circular dichroism spectrum at 580 to 590 nm (8). In the present work, gel filtration and analytical ultracentrifugation were used to study the relative molecular sizes of Pchl-H and Chl-H formed under various conditions, both in vivo and in vitro.

Materials and Methods

Plant Material. Seedlings of barley (Hordeum vulgare L. cv. Svalöfs Bonus), bean (Phaseolus vulgaris L. var. Redlands Pioneer), and pea (Pisum sativum L. var. Greenfeast) were grown in darkness at 25°C in vermiculite moistened with nutrient solution. The bean and pea seeds were soaked over-

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1 K.W.H. was supported by a travel grant from the Danish National Science Research Council.
2 Permanent address: Institute of Genetics, University of Copenhagen, Denmark.
3 Abbreviations: Pchl: protochlorophyllide; H: holochrome.
night in tap water (20 C) before planting, and the seedlings were watered with tap water. The terminal 5 to 6 cm of the primary leaves of barley seedlings were used on the 6th day after planting, and the primary leaves of bean and pea seedlings on the 9th day. Manipulations of plant material were carried out in a dim green safelight, except for the specified periods of illumination. The collected leaf material was immediately placed on ice and used for extraction as soon as possible.

Leaf material from barley and bean was placed on aluminum foil in contact with ice, and illuminated for 1 min with white light (1 x 106 erg cm-2 sec-1) from a xenon lamp, filtered through a 10-cm layer of water. The light intensity was measured with a YSI-Kettering Model 65 Radiometer (Yellow Springs Instrument Company, Ohio). Intact seedlings of barley were illuminated at 25 to 27 C with white light from a bank of 40-w fluorescent tubes (Philips, type daylight): at leaf level the intensity was 4 x 106 erg cm-2 sec-1 or 450 ft-c (measured by a Weston illumination meter).

**Extraction of Holochrome with Saponin.** All preparations were carried out at 0 C under a dim green safelight. The extraction procedure was as described previously (14), except for the following variations. In some experiments, 3.6% of saponin (Merck, white pure) was used instead of 1.2% for the extraction of the holochromes. Following ammonium sulfate fractionation, these preparations were resuspended with 1.5% saponin (instead of 0.5% saponin) in 0.1 M Tricine-NaOH buffer, pH 8.5, using 0.7 ml of saponin solution per g fresh weight of leaves. The resuspended material was frozen to -18 C. After thawing, the sample was clarified by centrifugation for either 1 hr at 27,000g or 20 min at 85,000g. When further purification was needed, the sample was diluted with an equal volume of 1.5% saponin in Tricine buffer, and refractonated with ammonium sulfate. The precipitate obtained at 75% of saturation was collected by centrifugation for 30 min at 27,000g. The precipitate was resuspended in 0.5 ml of 1.5% saponin in Tricine buffer, and dried overnight at 0 C against the same medium. The dried preparation was clarified by centrifugation for either 1 hr at 27,000g or 20 min at 85,000g. With some preparations of Chl-H, ammonium sulfate fractionation was omitted. Preparations could be stored for several months at -18 C without loss of activity.

**Extraction of Pchl-H from Bean Leaves Without Detergent.** Leaves were homogenized in 0.1 M Tricine-NaOH buffer, pH 8.5, containing 50% glycerol, (2 ml/g fresh weight) in a Sorvall omnimixer for four 30-sec periods at maximum speed with intermittent periods of cooling. Two ml of grinding medium were used per g fresh weight of leaves. The homogenate was filtered through four layers of Miracloth (Cal Biochem) and fractionated with ammonium sulfate. The fractions precipitating at 0 to 40% and 40 to 75% saturation were collected by centrifugation at 27,000g for 30 min, resuspended in Tricine buffer containing 20% (v/v) glycerol, and dried overnight against the resuspension medium. After dialysis, the solutions were clarified by centrifugation for 1 hr at 100,000g.

**Photoconversion of Pchl.** Maximum photoconversion was obtained by illumination for 10 sec at 0 C with white light from a tungsten lamp (Philips Photoflood, 150w) filtered through a 2-cm layer of water. The intensity was 1 x 106 erg cm-2 sec-1.

**Gel Filtration.** Sephadex G-200 was soaked in 0.1 M Tricine NaOH buffer, pH 8.5, at 0 C and packed in a column (2.5 x 45 cm) equipped with a cooling jacket maintained at 0 C. The columns were equilibrated and eluted with Tricine buffer containing either saponin (0.5 or 1.5% [w/v]) or 20% (v/v) glycerol at flow rates of 0.4 to 0.6 ml/min. Eluate was collected in 4-mI fractions. The void volumes (Vo) of the columns were determined with blue dextran 200 (Pharmacia). Relative Pchl concentration was determined at 644 nm and Chl at the absorption maximum in the region 670 to 678 nm. The relative concentration of photoactive Pchl was determined from light minus dark difference spectra. The elution profile of cytochromes was obtained by measuring the difference in extinction at 558 nm and 573 nm in dichloromethane reduced minus untreated difference spectra. The values determined for the chromatography constant (Kc) allowed the apparent molecular weights to be obtained from the manufacturer's specifications for the gels.

**Ultracentrifugation.** Sedimentation velocity analyses of saponin Pchl-H and Chl-H preparations were determined in a Spincro Model E analytical ultracentrifuge at 50,740 rpm and 3 C. The samples were loaded into the cell in dim room light and exposed to strong green light only during photographic recording of the schlieren pattern. Orange light was sometimes used to follow the sedimentation of the pigments by visual inspection.

**Fluorescence Measurements.** Fluorescence emission and excitation spectra were recorded on a fully corrected fluorescence spectrophotometer, constructed as described earlier (3). The band widths of the excitation and emission monochromators were ±1.5 nm and ±1.0 nm, respectively. For fluorescence measurements at 77 K the samples were dissolved in 63% (v/v) glycerol. The sample holder could be maintained at temperatures ranging from 77 K to 50 C.

Fluorescence polarization measurements were carried out with a polaroid type (Leitz) polarizer/analyser set. The E vector of the excitation light was vertically polarized. For each set of excitation and emission wavelengths, a grating correction factor (G = I1/I1') using horizontal polarized excitation was determined and used to correct the actual measurement. The polarization value was obtained from p = (I1 - GI1)/(I1 + GI1).

**RESULTS**

**PROPERTIES OF PCHL-H FROM UNIILLUMINATED LEAVES**

**Absorption Spectra.** Similar absorption spectra were obtained for barley, bean, and pea Pchl-H extracted with saponin and resembled an earlier spectrum of barley saponin Pchl-H (8, 14). The absorption maximum varied with the concentration of saponin; 642 to 644 nm in 0.5% saponin and 644 to 646 nm in 1.5% saponin. Nearly all of the Pchl-H extracted with saponin precipitated between 40 and 75% saturation with ammonium sulfate. However, bean Pchl-H extracted without detergent was found in the 0 to 40% fraction as well as the 40 to 70% fraction. The former fraction had an absorption maximum at 641 nm, compared with 637 nm for the latter fraction. When the 40 to 70% fraction was transferred to a saponin solution, the absorption maximum shifted from 637 to 644 nm.

Photoconversion of saponin Pchl-H gave Chl-H with an absorption maximum of 677 to 679 nm. More than 80% of the Pchhl was photoconvertible. At temperatures above 0 C, the absorption maximum shifted from 677 to 679 nm to 672 nm. For Chl-H in 0.5% saponin the spectral shift was complete in 45 to 60 min at 20 C and about 120 min at 10 C. In 1.5% saponin 120 to 240 min was required at 20 C to complete the shift. A decrease in absorbance of about 10 to 20% preceded or accompanied the spectral shift (Fig. 4). Photoconversion of the 637 nm-absorbing Pchl-H fraction, extracted from bean without detergent, gave Chl-H with an absorption maximum at 673 nm.

**Fluorescence Measurements.** The fluorescence spectra at 77 K of pea saponin Pchl-H and Chl-H (0.5% saponin with
of 63% [v/v] glycerol) are shown in Figure 1. Here comparison is made of the pea saponin holochrome before photoconversion, at partial (40%) photoconversion prior to freezing and at maximum photoconversion. The fluorescence emission bands at 648 and 684 nm are due to Pchl and Chl a, respectively. The spectrum of the partially converted sample indicates a lack of energy transfer from Pchl to Chl a. The intensity of the fluorescence emission at 648 nm was about 45% of that of the unilluminated sample and it correlates reasonably well with the degree of photoconversion. In contrast, leaves (27) or nondetergent bean holochrome (16) at a comparable degree of photoconversion gave very low fluorescence emission intensities from the remaining photoactive Pchlide and a large increase in the fluorescence emission from Chl a, due to energy transfer from Pchl to Chl a. Further evidence for a lack of energy transfer in the pea saponin holochrome was provided by comparing the fluorescence emission spectrum of a 1:1 mixture of unilluminated and maximally converted saponin holochrome (not shown) with the fluorescence emission spectrum of the partially converted sample. Both spectra were very similar. Barley and bean saponin holochromes gave similar spectra to those shown in Figure 1 for pea saponin holochrome.

Fluorescence emission maxima and optimal polarization measurements on barley and bean Pchl-H and Chl-H and bean leaves are compared in Table I. Pea saponin Pchl-H gave results very similar to those for barley Pchl-H. Where fluorescence excitation was made within the first singlet absorption band, the polarization for barley or bean saponin holochrome gave high values; before conversion, after photoconversion and also following the 678 to 672 nm spectral shift. Chl a extracted from barley leaves in ethanol gave, after dilution in glycerol, a high polarization value, \( p = 0.45 \) (excitation 650 nm, emission 674 nm) at 20°C, while Pchl under similar treatment gave \( p = 0.44 \) at 20°C and \( p = 0.39 \) at 77 K (excitation 615 nm, emission 630 nm).

These relatively high fluorescence polarizations of saponin Pchl-H and Chl-H are consistent with the lack of energy transfer between chromophores in these preparations. The nondetergent bean holochrome, which shows energy transfer gave a much lower polarization and, as expected, the fluorescence from leaves was almost completely depolarized, because of the massive energy transfer between chromophores in leaves (27).

**Apparent Molecular Weight.** In previous studies (14), an apparent mol wt of 63,000 was obtained for barley saponin Pchl-H. This barley Pchl-H in either 0.5% saponin or 1.5% saponin gave an apparent mol wt of more than 100,000. Similar results were obtained in the present investigation using both Sephadex G-100 and G-200. Pea Pchl-H in 0.5% saponin showed a similar behavior to that of bean Pchlide.

By increasing the saponin concentration from 1.2 to 3.6% more photoactive Pchlide was extracted. After ammonium sulfate fractionation, the Pchlide-H was resuspended in 1.5% saponin and chromatographed on Sephadex G-200 (Fig. 2). The apparent mol wt of the major fraction was estimated as 170,000 while the minor fraction appeared to have a mol wt of about 500,000. Thus, the smallest unit of Pchlide-H extracted from bean or pea with saponin is considerably larger than that from barley. Figure 2 indicates that cytochromes are not associated with the major fraction of bean Pchlide-H, but they are eluted with the minor holochrome band of higher mol wt and are also found in a separate band of low apparent mol wt (23,000).

Pchlide-H extracted from bean leaves without detergent was analyzed on Sephadex G-200 with Tricine buffer containing 20% glycerol as eluting medium. Both fractions obtained by ammonium sulfate precipitation gave an elution profile similar to that of blue dextran, indicating an apparent mol wt of 500,000 or more. The cytochromes eluted with the Pchlide-H.

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**Fig. 1.** Low temperature (77 K) fluorescence emission and excitation spectra of saponin Pchl-H and Chl-H from pea, — — : unilluminated; — — : illuminated to convert about 40% of the chlorophyllide; — : maximal photoconversion. Emission spectra was obtained with 440 nm excitation light. The excitation spectra for 648 and 684 nm fluorescence emission are shown.

**Table I. Fluorescence Polarization of Pchl-H and Chl-H from Barley and Bean, and of Bean Leaves in Vivo**

<table>
<thead>
<tr>
<th>Material</th>
<th>Emission Maximum Wavelength</th>
<th>Excitation Wavelength</th>
<th>Fluorescence Polarization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Barley saponin Pchl-H</td>
<td>646</td>
<td>620</td>
<td>0.30</td>
</tr>
<tr>
<td>Bean saponin Pchl-H</td>
<td>650</td>
<td>620</td>
<td>0.27</td>
</tr>
<tr>
<td>Bean nondetergent Pchl-H</td>
<td>652</td>
<td>620</td>
<td>0.13</td>
</tr>
<tr>
<td>Etiolated bean leaf</td>
<td>655</td>
<td>620</td>
<td>0.05</td>
</tr>
<tr>
<td>Barley or bean Chl-H in 0.5% saponin, at 5°C (maximum photoconversion)</td>
<td>F684</td>
<td>650</td>
<td>0.40-0.43</td>
</tr>
<tr>
<td></td>
<td>685</td>
<td>650</td>
<td>0.44</td>
</tr>
<tr>
<td>Barley or bean Chl-H in 1 63% glycerol, at 5°C</td>
<td>685</td>
<td>660</td>
<td>0.44</td>
</tr>
<tr>
<td>Bean leaf at 0°C (maximum photoconversion)</td>
<td>694</td>
<td>630</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td></td>
<td>660</td>
<td>0.03</td>
</tr>
<tr>
<td>Barley or bean Chl-H in 0.5% saponin at 20°C (spectral shift completed)</td>
<td>679</td>
<td>630</td>
<td>0.26-0.29</td>
</tr>
<tr>
<td></td>
<td></td>
<td>650</td>
<td>0.35-0.40</td>
</tr>
<tr>
<td>Bean leaf</td>
<td>680</td>
<td>650</td>
<td>0.00</td>
</tr>
</tbody>
</table>

1 min light at 3 C, 2 min light at 5 C, followed by 60 min at 20 C.
In a previous study (14), it was shown that the behavior of barley or bean Pchl-H on Sephadex gels was not altered by photoconversion of Pchl to Chl a. The photoconversion and gel filtration were performed at 0°C, and sucrose or glycerol was present before and after gel filtration. Thus, conditions were optimal for maintaining the Chl a in the 678-nm absorbing form (8, 19, 22). It appears that Pchl-H and its immediate photopproduct, in vitro, Chl-H absorbing at 678 nm, have an identical hydrodynamic behavior.

We have now examined the gel filtration behavior of barley saponin Chl-H following the spectral shift from 678 to 672 nm. A sample of barley saponin Pchl-H was photoconverted and then incubated at room temperature for 1 hr to enable the spectral shift from 678 to 672 nm to be completed. The Chl-H absorbing at 672 nm was mixed with barley Pchl-H and subjected to gel filtration on Sephadex G-200 in darkness. Each column fraction was divided into two; one-half remained unilluminated, while the other was illuminated to give maximum photoconversion. The elution profile of Chl-H (672 nm) was obtained by following absorbance at 672 nm of the unilluminated samples, while the profile of photoactive Pchl-H was constructed by plotting the absorbance difference at 678 nm between the illuminated and unilluminated samples (Fig. 3). From the measured chromatographic constants (K_m) of 0.44 and 0.62, apparent mol wt of 75,000 and 29,000 were obtained for saponin barley Pchl-H and saponin Chl-H (672 nm form).

**Fig. 2.** Elution profiles of bean saponin Pchl-H, in 1.5% saponin, from a Sephadex G-200 column equilibrated and eluted with Tricine buffer, pH 8.5, containing 1.5% saponin. •: photoactive Pchl-H; ■: cytochromes. The elution profile of blue dextran is also shown (---). The profiles are normalized to absorbance 1.0 for the most concentrated fractions.

Ultra centrifugal analyses of saponin Pchl-H showed three peaks with sedimentation coefficients of 23.3, 10.3, and 1.99 at 3°C. Pigments absorbing orange light appeared to be associated with the slowest sedimenting peak. A sedimentation coefficient of 2.00 was obtained for bovine serum albumin (mol wt 67,000) at 3°C in 0.5% saponin containing 1% NaCl. These results, together with the gel filtration data, suggest that saponin Pchl-H is associated with the 1.99S peak. Relative to the peak at 1.99S, the amplitudes of the 23.3 and 10.3S peaks were 0.1 and 0.4, respectively.

The sedimentation pattern of Chl-H (672 nm form) revealed two peaks with sedimentation coefficients of 9.7 and 1.64, but further work with absorption optics is needed to establish the relative pigment contents of the bands. These preliminary measurements appear to support the conclusion from gel filtration data that saponin Chl-H (672 nm form) has a much smaller mol wt than saponin Pchl-H or Chl-H (678 nm form).

**Fig. 3.** Elution profiles of barley saponin holochrome (in 0.5% saponin) from a Sephadex G-200 column equilibrated and eluted with Tricine buffer, pH 8.5, containing 0.5% saponin. ▲: PChl-H; ■: Chl-H (672) formed in vitro, absorption maximum at 672 nm; ---: elution profile of blue dextran.

**PROPERTIES OF CHL-H EXTRACT FROM ILLUMINATION LEAVES**

Chl-H was extracted with saponin from leaves at the early stages of greening. With the exception of leaves illuminated for 2 min at 25°C, the yield of soluble pigment complexes was comparable to that obtained with unilluminated leaves (30-40% of the leaf pigment). However, the pigment complexes from greening leaves tend to form insoluble aggregates during freezing and thawing or on precipitation with ammonium sulfate.

**Absorption Spectra.** Chl a absorbing at 682 to 684 nm in vivo was formed by illuminating barley or bean leaves at 0°C for 1 to 2 min. Lowering the temperature to 0°C inhibits the spectral shift to 672 nm. The leaves were then extracted with saponin at 0°C to give saponin Chl-H with absorption maxima at 680 nm (barley) and 682 nm (bean). Saponin Chl-H was stable overnight at 0°C and for several months at -18°C. At 20°C, the absorption maximum of the barley Chl-H shifted from 680 to 672 nm in about 55 min. Bean Chl-H showed a decrease in absorption at 682 nm, followed by the spectral shift to 672 nm (Fig. 4); the isosbestic point at 676 nm indicates a quantitative conversion of the 682 nm form to the form absorbing at 672 nm. Extraction of the pigment into acetone confirmed that the Chl content remained constant during the spectral shift.

If barley leaves illuminated for 2 min at 25°C were extracted with saponin at room temperature, a very low yield of a soluble pigment complex absorbing at 668 nm was obtained.
It is likely that the spectral shift from 682 to 672 nm had occurred during the extraction. Perhaps the Chl-H is labile during the spectral shift and the pigment easily detached from the holochrome protein in the presence of detergent.

A high yield of saponin Chl-H was obtained from barley leaves illuminated from 15 min to 4 hr with 450 ft-c of white light. Between 15 min and 1 hr illumination in vivo, the absorption maximum was at 671–672 nm. The red shift in vivo from 672 to 678 nm commenced after about 1.5 hr illumination and was complete after 5 hr. The saponin Chl-H from leaves illuminated for 15 min had an absorption maximum at 670 nm, but that from barley leaves greened for from 1 to 4 hr had a maximum at 675 to 677 nm. In barley leaves returned to darkness after a brief illumination, Pchl reaccumulated in 1 hr to about 70% of the original level of the dark-grown leaves.

**Gel Filtration and Photochemical Activity.** Gel filtration of the saponin Chl-H extraction from barley leaves illuminated for 15 or 30 min showed an elution peak close to the front (Fig. 5), indicating an apparent mol wt in excess of 100,000. Cytochromes also eluted near the front, suggesting that they might be associated with Chl-H. The observation that plastids isolated from barley leaves illuminated for 15 min have photosystem I activity (12) supports this view.

Saponin Chl-H from barley leaves illuminated for 15 and 30 min have excellent photosystem I activity when measured as a rate of oxygen uptake per unit Chl (Table II). The activity was comparable to that of plastids isolated from barley leaves greened for 24 hr (12). Chl-H extracted from leaves illuminated for 1 or 2 hr showed lower photosystem I activity per unit of Chl.

Chl-H and Pchl-H, extracted with saponin from barley leaves which had been returned to darkness after a period in the light, were also examined by gel filtration on Sephadex G-100 in darkness. The elution profile of the Chl-H was constructed from the absorbance at 672 nm of unilluminated aliquots of the eluate (Fig. 6). Other aliquots of the eluate were illuminated to give maximum photoconversion and the elution profile of the photoactive Pchl-H determined from the absorbance at 677 nm in illuminated minus unilluminated difference spectra. The behavior of the saponin Chl-H on the gel was similar to that of the Chl-H from the illuminated leaves not returned to darkness. On the other hand, the saponin Pchl-H behaved like the Pchl-H of unilluminated leaves. The elution profile of the cytochromes in these preparations (Fig. 6) was similar to the elution profile of the Chl-H. There was no indication of an association either between the Pchl-H and the cytochromes or between Pchl-H and Chl-H.

**DISCUSSION**

The apparent mol wt (51,000–75,000) for barley saponin Pchl-H obtained with Sephadex G-200 in the present study is in excellent agreement with the previous reported value of 63,000 which was determined from gel filtration on Sephadex G-100 (14). The saponin Pchl-H extracted from barley leaves
which were returned to darkness after a period in the light is very similar in its gel filtration behavior to the saponin Pchl-H of etiolated leaves. Previously, it was shown that Pchl-H, extracted without detergents from bean seedlings, returned to darkness after a light period had similar sedimentation properties to those of Pchl-H from dark-grown seedlings (2).

On transfer of nondetergent bean Pchl-H to saponin, a red shift is observed in the absorption maximum. Nondetergent bean Pchl-H holochrome is relatively unstable compared with saponin Pchl-H, which suggests that saponin interacts with the Pchl-H to increase its stability.

The smallest photoactive unit of Pchl-H (apparent mol wt, 170,000) obtained from bean even with a high concentration of saponin is considerably larger than barley Pchl-H (51,000–75,000). The larger photoactive unit could conceivably be a dimer or trimer, although the spectroscopic results do not provide evidence for multiple chromophores in the saponin holochromes. The lack of transfer of excitation energy, the high fluorescence polarization, and the lack of exciton interaction (K. W. Henningsen, A. Kahn and C. Houssier, unpublished data) with Pchl-H of 170,000 mol wt are consistent with the presence of a single Pchl per holochrome. But we cannot exclude the possibility that the 170,000 mol wt subunits contain two or three noninteracting chromophores.

Light converts the nondetergent bean Pchl-H fraction with an absorption maximum at 637 nm directly to Chl-H absorbing at 673 nm. The formation of the 672 nm form of Chl directly from Pchl has previously been observed in vivo with young leaves (17), in chloroplast mutants of barley (30), and in leaf material subjected to various physical or chemical treatments (7, 9, 11).

Light converts Pchl-H preparations with absorption maximum at 641 to 646 nm to Chl-H with absorption maximum at 677 to 679 nm. In agreement with earlier observations (8, 19, 22), we have not detected a red shift of the 678 nm absorption maximum prior to the shift to 672 nm in any of the preparations of holochrome. The rate of the shift from 678 nm to 672 nm depends on the temperature (8, 19, 21, 22) and on the saponin to holochrome ratio. In holochrome extracted from bean with a high concentration of saponin the spectral shift is extremely slow. With such preparations, it was possible to show that a decrease in the extinction coefficient of Chl preceded or accompanied the spectral shift. In the case of barley saponin Chl-H, changes in the circular dichroism correlated with the spectral shift from 678 to 672 nm. It has been proposed that the changes in circular dichroism are the result of a conformational relaxation of the holochrome protein (8). The changes in apparent mol wt of the Chl-H subunit after completion of the spectral shift suggest that a dissociation of the holochrome subunit follows such a conformational relaxation. We propose that the Chl-H subunit dissociates into a Chl-carrier protein complex and a photoenzyme. The observed change in apparent mol wt (supported by changes in the sedimentation behavior) of the holochrome is greater than expected for a conformational change alone, although a conformational change might affect the binding of saponin in the complex and thereby influence the hydrodynamic behavior of the holochrome subunit.

Evidence has been presented for a dissociation of Chl-H extracted from bean leaves without detergent (4). Transformation of an aggregated or dimeric form of Chl into a monomeric form has been inferred from circular dichroism and fluorescence spectroscopy of holochrome from bean (19, 22). These nondetergent preparations presumably contained holochrome of high mol wt, i.e., assemblies of holochrome subunits. In such preparations, a distinction between disaggregation of a subunit assembly and a dissociation of the subunit is not possible.

Some observations on leaf material may be explained in terms of the proposed dissociation of the Chl-H subunit into a pigment-protein complex and a photoenzyme. Fluorescence spectroscopy has shown that resonance energy transfer from Pchl to Chl $a$ absorbing at 672 nm is possible before, but not after the increase in fluorescence quantum efficiency of the newly formed Chl $a$ (27, 28). This suggests that the newly formed Chl $a$ molecule is translocated to a new position in the lamellar membrane. The increase in fluorescence efficiency of newly formed Chl $a$ (K. W. Henningsen and S. W. Thorne, unpublished data) corresponds with the appearance of photochemical activity in greening leaves of barley (12). These observations suggest that newly formed Chl $a$ is translocated from the site of photoconversion to the reaction centers of the photosystems.

The existence of a photoenzyme (site for photoconversion of Pchl) is expected since the number of sites at which photoactive Pchl can accumulate remains constant during the early stages of greening (20, 26, 28). Thus, the photoenzyme appears to be used repeatedly, and this requires a translocation of the newly formed Chl away from the original site on the photoenzyme. Assuming that the number of molecules of the photoenzyme remains constant, the observation that photoactive Pchl is reaccumulated on holochrome separate from Chl-H is strong evidence for the translocation of the newly formed Chl $a$.

The spectroscopic and structural changes observed with preparations of holochrome subunits can be related to the structural reorganization of the prolamellar body membranes. Photoconversion of Pchl initiates tube transformation (the loss of the regular membrane arrangement in the prolamellar body [15, 29]). The spectral shift of newly formed Chl $a$ from 684 to 672 nm in vivo, precedes or accompanies the dispersal of
the prolamellar body membranes into the primary lamellar layers (7, 11, 13). If the 684 to 672 nm shift in vivo is related to the 678 to 672 nm shift of the subunit, it appears that tube transformation and the early stages of membrane dispersal reflect a conformational relaxation of the Chl-H in the prolamellar body membranes. At a later stage of the dispersal process, when the tubular membranes are changed into perforated sheets of membranes, the Chl a-carrier-protein is translocated from the photoenzyme to the reaction center sites in the lamellar membranes.

Although pigment-protein complexes can be extracted from green leaves by saponin, they behave very differently on the Sephadex gels from the saponin PchI holochromes and their photoproducts in vitro. It seems likely that when Chl is incorporated into the lamellar membranes and becomes associated with the electron transport system, it is not extracted in the same way as the PchI-H of the etiolated leaf. The apparent association of the cytochromes with the Chl complex may be due to a lack of efficiency of saponin in dissociating the Chl complex of the greening system.

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