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Chlorophyll Formation in Greening Bean Leaves during the Early Stages

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ABSTRACT

In the evolution of the absorption spectrum of etiolated bean leaves (Phaseolus vulgaris L.) following illumination, a rapid photoconversion of 50% or more of the active protochlorophyllide at room temperature is followed by a shift of the chlorophyll(ide) absorption maximum: \( C_{678}^{+} \rightarrow C_{684}^{+} \rightarrow C_{672} \text{ nm} \). Kinetic studies at 2°C and the absence of an isosbestic point provide evidence for an intermediate between \( C_{678} \) and \( C_{684} \). A dramatically different evolution is observed following the photoconversion of only 5 to 30% of the active protochlorophyllide at room temperature. \( C_{672} \) appears within 30 sec, and no subsequent dark shift occurs during the following 90 min. At 0°C, conversion of 5% of the active protochlorophyllide produces a new species, \( C_{676} \), which converts progressively to \( C_{672} \) within 10 min. We interpret the results in terms of two photochemical steps operating in series for the complete conversion of active protochlorophyllide. Furthermore, there appears to be competition between an irreversible, terminal dark shift and the second light reaction. We propose a scheme based on dimers of protochlorophyllide reduced stepwise to dimers of chlorophyllide in two successive light reactions. The intermediate mixed protochlorophyllide-chlorophyllide dimer absorbs at 676 nm and displays a much faster dissociation to monomers than does the chlorophyllide-chlorophyllide dimer.
In etiolated leaves of higher plants, the terminal reactions of chlorophyll synthesis include a photochemical reduction of protochlorophyllide. Spectroscopic studies of this reaction exhibit a complex photochemical behavior and involve several wavelength shifts of the newly formed chlorophyllide (18). We will summarize some of the important aspects of this problem. (See ref. 11 for a more complete review of the subject.)

The reaction transforming protochlorophyllide into chlorophyllide can be very fast, completed in $10^{-3}$ or even $10^{-5}$ sec (13,15). However, analyses of the photochemical kinetics under light-limited conditions revealed a biphasic behavior, attributable to two first-order reactions (2,17). Godnev et al. provided good evidence for the first-order character of the initial stages of the reaction by demonstrating its one-quantum nature (8).

After complete photoconversion, chlorophyllide absorbing at 678 nm ($C_{678}$) transforms rapidly to chlorophyllide absorbing at 684 nm ($C_{684}$) (5,7). Butler and Briggs proposed that $C_{684}$ is an aggregated form of chlorophyllide and that the next shift [Shibata shift, yielding chlorophyll(ide) absorbing between 670 and 673 nm ($C_{672}$)] reflects a disaggregation (6). This idea finds strong support in recent results showing that, in protochlorophyllide holochrome and in homogenized material from bean leaves, protochlorophyllide before illumination and chlorophyllide after illumination are arranged as dimers, which then dissociate in parallel with the blue-shift in the wavelength of maximum absorption (16,14).

Studies based on fluorescence spectroscopy at intermediate photoconversion suggest a more complex picture. Thorne (19) showed that the spectral properties of the products of partial photoconversion depend on the rate of the dark reactions. He interpreted his results as indicating
the presence of an additional photochemical intermediate absorbing at 668 nm. Litvin and Belyaeva (12) were also led to a scheme involving two successive photochemical reactions.

We have reinvestigated the problem of chlorophyllide absorption shifts following partial photoconversion, using absorption spectroscopy near 0 C instead of fluorescence spectroscopy at -180 C as in the previous studies. This was done in order to avoid possible structural artifacts occurring during the assay at very low temperatures and uncertainties in the real concentrations of different species owing to possible variations in their relative quantum yields of fluorescence. Our studies were done on etiolated bean leaves. We have already reported a parallel investigation on homogenized material from similar leaves (14).

We present a simple scheme to account for our observations. It is predicated on the presence of dimeric protochlorophyllide as the basic active component in the etioplasts and emphasizes the role of pigment-pigment interactions in interpreting the spectroscopic properties.

MATERIALS AND METHODS

Red kidney beans (Phaseolus vulgaris L.) were grown for 11 + 1 days in vermiculite in complete darkness at 21 C (room temperature). After harvesting, the leaves were handled under dim green light which had no measurable effect.

Absorption spectra were measured using a Cary 14 spectrophotometer equipped with a scattered-transmission attachment (Model 1462) and a red sensitive photomultiplier (Dumont 6911). A folded single leaf was attached with translucent tape over a small aperture in a 1 mm thick brass plate.
The plate was then fitted into a thermostated brass cell holder; this had three openings; the one above, for inserting the leaf, was then closed with tape, and the two lateral openings, for the spectrophotometer beam, were covered with ground glass diffusing plates. The aperture in the brass plate was sufficiently small that all the transmitted light had passed through the leaf. The photocathode of the photomultiplier tube was at about 3 cm from the leaf. During cooling the windows of the cell holder were flushed with dry nitrogen gas. The temperature of the leaf was determined with a copper-constantan thermocouple, whose EMF was measured with a Keithley 610 B microvoltmeter.

Absorption spectra were recorded with either a suitable number of layers of tissue paper and of translucent tape or, more commonly, a folded single leaf placed in the reference beam. This reference leaf remained at room temperature. Also we generally used the expanded (0-0.1 absorbance) scale of the spectrophotometer. In a typical experiment, run from 790 to 650 nm, at 0.5 nm-sec⁻¹, the percentage of photoconversion induced by the measuring beam of the spectrophotometer was around 0-1%.

The photoconversion was effected either in monochromatic light or in white light. In the first case we used the spectrophotometer beam itself, carefully blocking the reference leaf, with the slit at maximum opening and using a variable lamp voltage to control the intensity. Illumination in white light was done in situ using a tungsten lamp (Sylvania, Truflector DFA; 115 v, 150 w; operated at variable voltage) and mirrors. For the experiments involving initial partial conversions, a subsequent complete conversion allowed us to calculate the extent of the partial conversion. After an initial recording of the usually smooth
base line, the leaves, their temperatures, and their geometric arrangements were not altered.

RESULTS

Absorbance Changes after Complete Photoconversion. The absorption spectra of two superimposed folded bean leaves are shown in Figure 1, before illumination, just after and at two later times. These spectra are in good agreement with those reported by Bonner (5). Just after illumination we found a peak at 678 nm which then shifted to 684 nm, slowly at -8 C but within 30 sec at room temperature. Next, we observed the regular Shibata shift (to 672 nm), which takes 20 to 30 min at room temperature (18). We observed a similar pattern after either a short (5 sec) illumination in white light or after a long (2 min) illumination in monochromatic light, provided in the latter case that the temperature is low enough. There was always a noticeable hyperchromicity accompanying the 678 → 684 nm shift.

Another way of studying the 678 → 684 nm shift is by measuring the evolution of the absorbance at a single wavelength after a relatively short (6 sec) illumination (Fig. 2). In these experiments a time of ca. 10 sec elapsed between the end of the illumination and the beginning of recording of the absorbance. The curves in Figure 2 clearly show that the kinetics is not identical at the different wavelengths, attesting that the process is not a simple one. Gassman et al. (7) previously observed the lack of an isosbestic point during this shift. We find that, at all wavelengths between 665 and 700 nm, the absorbance increases during the
first seconds following the end of the illumination, which is consistent with the absence of a isosbestic point in this region.

**Progressive Illumination at Room Temperature.** A 10-sec illumination at an intensity sufficient to give 100% photoconversion at room temperature, permits the detection of C$_{684}$ and then the slow Shibata shift to 672 nm. However, if the 10-sec illumination is carried out at low intensity so as to effect only 5 to 30% of the photoconversion, the absorption peak of the product is initially observed at 672.5 nm in the difference spectrum (corresponding to ca. 672 nm for the true position of the absorption maximum), in less than 30 sec. Figure 3 shows such a difference spectrum and shows, in addition, that the absorption at 672 nm, increases linearly with illumination.

Litvin and Belyaeva (12) found a similar spectroscopic species and confirmed that it contained chlorophyll, which we will call C$_{672}$. Once formed, C$_{672}$ is stable for at least 90 min in the dark at room temperature. The peak position does not change upon cooling to -5 C. We found a behavior similar to that shown in Figure 3 for various illumination wavelengths between 640 and 670 nm, as well as for white light.

Increasing the light intensity during a 10-sec illumination produces a progressive long wavelength shift for the first detected absorption maximum: between 672 nm at <5% and 684 nm at >50% of photoconversion. The subsequent dark shift, if any, is always toward short wavelengths and is relatively slow, as is the Shibata shift (18).

After accumulating 25-30% of the chlorophyll as the C$_{672}$ form, a complete photoconversion gives a product absorbing at 682.5 nm, with a pronounced shoulder near 670 nm, as shown in Figure 4 (a,b). It seems
that we are observing a mixture of \( C_{672} \) and \( C_{684} \), in various proportions; and that \( C_{672} \), once formed, is not capable of transformation to \( C_{684} \). A fast complete phototransformation gives the chlorophyllide entirely as the \( C_{684} \) form (Fig. 4c).

**Progressive Illumination at 0°C.** A short illumination at 0°C, so as to transform less than 5% of the active protochlorophyllide, gives a peak located at 676±1 nm in the difference spectrum. Subsequently there is a progressive shift to 672.5 nm during the next 10 min in the dark. The rate of this shift is strongly temperature-dependent. It occurs in less than 30 sec at 21°C.

If the light intensity is higher the peak is found between 676 and 684 nm a few minutes after the illumination. The difference spectrum is relatively stable; but the maximum shifts slowly to shorter wavelengths, especially at small percentage photoconversion. At more than 50% conversion the shift is very slow; it takes more than 1 hr at 0°C.

Figure 5 represents the difference spectra obtained at several levels of illumination. It is difficult to describe these observations quantitatively. The half bandwidth of the difference spectrum varies in a regular way:

<table>
<thead>
<tr>
<th>% conversion</th>
<th>4</th>
<th>7</th>
<th>16</th>
<th>31</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \lambda_{\text{max}} ), nm</td>
<td>675.5</td>
<td>676.5</td>
<td>679.5</td>
<td>680.5</td>
<td>683.5</td>
</tr>
<tr>
<td>( \Delta \lambda_{1/2} ), nm</td>
<td>23</td>
<td>25</td>
<td>27</td>
<td>26</td>
<td>24.5</td>
</tr>
</tbody>
</table>

We believe that at low and high conversion we have single components, but that there are mixtures of the two components at intermediate conversions.

In the range 0-30% of photoconversion, the rate of chlorophyll(ide) formation is practically constant at a given illumination intensity.
Moreover, upon illumination at 0°C, the wavelength of absorption of the product depends primarily on the extent of photoconversion, not on the light intensity: 20% of photoconversion obtained in 5 sec or in 15 min each gives a $\lambda_{\text{max}}$ at 680 nm. (At room temperature the result is different: $\lambda_{\text{max}}$ is 672.5 nm at low light intensity and 680 nm at high intensity).

**DISCUSSION**

The results of this study indicate a clearly non-linear behavior in the spectroscopic properties of etiolated leaves upon illumination either at room temperature or at 0°C. At high fractional conversion (50%) we observe the usual progression: 678 → 684 → 672 nm, but at low conversion we see only 676 → 672 nm. Intermediate conversions exhibit intermediate behavior.

In order to interpret these results, as well as to account for similar results published previously (12,19), we propose the mechanism outlined in Figure 6. The scheme is based on the existence of dimers as the basic structural elements of protochlorophyllide in the etioplast photoenzymes. The results that we have obtained on etiolated leaf homogenates (12) are best explained by this hypothesis. Although there are significant differences between the absorption band positions of intact etiolated leaves and their homogenates, we suppose that the basic structural properties are similar. Support for this supposition comes from the studies of Sironval et al. (17) on intact leaves, where the kinetics of photoconversion are biphasic just as they are for the leaf homogenates (14) or for purified protochlorophyllide holochrome (2).
The scheme in Figure 6 accounts for the results of the present study in the following way: Partial conversion (<30%) of P-P\(_{650}\) produces predominantly the heterodimeric species P-C\(_{676}\), which dissociates to C\(_{672}\) and P monomeric species slowly at 0°C and rapidly at room temperature. Because of the speed of this dissociation at room temperature, we did not observe the P-C\(_{676}\) species as an intermediate at room temperature; however, Litvin and Belyaeva (12) found evidence for its presence following a 2-4 msec flash if they then cooled the leaf rapidly to -196°C.

If sufficient light is given initially to convert all of the active protochlorophyllide, then both of the light steps indicated in Figure 6 occur and C-C\(_{678}\) is the first product observed. This rapidly rearranges in the dark to form C-C\(_{684}\), which then dissociates slowly to form the monomeric C\(_{672}\). The identity of the absorption maxima suggests that this is the same species as the final chlorophyllide product following partial conversion; however, this assignment must await confirmation on the basis of additional measurements.

The scheme of Figure 6, which is the simplest one that accounts for the dimeric nature of protochlorophyllide (650) in the etiolated leaf, is also consistent with a wide range of kinetic results obtained by other authors [See, for example, the most recent scheme of Litvin and Belyaeva (12), with which the formulation in Figure 6 is kinetically identical.]

In addition to using partial illumination, it is possible to obtain directly a short wavelength form of chlorophyll by carrying out the photoconversion in very young (2-3 day old) seedlings (1). In this case C\(_{673}\) appears to form directly from a 635 form of protochlorophyllide. As a straightforward
extension of our model we propose that $P_{635}$ is a monomeric form of active protochlorophyllide which converts to $C_{673}$ without going through the $C_{676}$ intermediate. By treating etioplast preparations with saponin, Henningsen and Kahn (9) have recently isolated a photoconvertible subholochrome particle that exhibits simple first-order photochemical kinetics and appears to contain only one protochlorophyllide per particle. This result demonstrates that dimers are not a necessary prerequisite for photoconversion. Leaves which are grown for 5 days or longer accumulate $P-P_{650}$ in addition to $P_{635}$. The presence of these two photoconvertible components may account for the observation of Sironval et al. (17) using monochromatic actinic light that simple first-order photochemical kinetics is observed using 647 nm actinic light, but that biphasic kinetics is seen when 630 nm light is used. In this connection one may assume that partial conversion of $P-P_{650}$ produces $C_{672}$ together with a monomeric form of protochlorophyllide that exhibits a shorter wavelength (635 nm?) absorption.

It has been reported that the shift from $C-C_{678}$ to $C-C_{684}$ occurs without an isosbestic point (5), and the studies shown in Figure 2 confirm the report of Gassman et al. (7) that a transient spectroscopic intermediate participates in this transformation. This may be a species between $C-C_{678}$ and $C-C_{684}$, as suggested by the two arrows in Figure 6, or it may arise via a hitherto undetected intermediate in the conversion of $P_{635}$ to $C_{672}$, which is presumably occurring in a parallel process.

In homogenates and holochrome preparations the picture is both simpler and more ambiguous (14,16). In the presence of high concentrations (2 M) of sucrose, the dissociation of the pigment dimers is prevented and one sees evidence of the simple two-step process $P-P \xrightarrow{hv} P-C \xrightarrow{hv} C-C$. 
In the absence of high sucrose concentrations dissociation of the C-C species does occur, but in neither medium does the absorption spectrum provide evidence for a transformation analogous to $C_{678} \rightarrow C_{684}$. There is, however, a rather pronounced alteration in the shape of the circular dichroism spectrum in going from high to low sucrose medium, which suggests the occurrence of alternative conformations of the dimeric chlorophyllide species. Furthermore, there is no direct evidence in homogenates for the step P-C $\rightarrow$ P + C in the absence of sucrose; however, it may occur with little effect upon either the absorption or the circular dichroism spectrum. These are questions which clearly deserve further study.

In leaves, the formation of monomeric $C_{672}$ is not the end of the story. These species presumably rapidly become involved in building pigment complexes which soon exhibit photosynthetic competence (4). The structure and evolution of these complexes define one of the most appealing and challenging problems in the field of chloroplast development.

CONCLUSION

A scheme is presented to account for the complex kinetics of the photoconversion of protochlorophyllide to chlorophyllide in etiolated leaves. It is based in part on the occurrence of a dimeric form of protochlorophyllide which is converted to dimeric chlorophyllide via two photochemical steps in series. Both the intermediate heterodimer, obtained by partial photoconversion, and the final chlorophyllide dimer, perhaps following an internal rearrangement, then dissociate in the dark to monomeric forms. Furthermore, it is proposed that a smaller amount of
monomeric protochlorophyllide is present in the leaf and is convertible
directly to monomeric chlorophyllide. While this scheme is held to be
tentative at the present time, it is consistent with a large body of
published data on this process and it has the virtue of assigning specific
molecular structures to species that have hitherto been presented largely
as undefined spectroscopic forms. We consider its chief value to be that
it permits rather definite questions to be asked in future studies of the
process.
ACKNOWLEDGMENTS

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LITERATURE CITED


FIGURE CAPTIONS

Fig. 1. Absorption spectra of two superimposed, folded bean leaves in the etiolated state (1), and after complete photoconversion followed by 5 min (2), a further 25 min (3) and a further 18 min (4) of darkness. Temperature, -8 C; reference beam, air. Illumination by Cary monochromator, λ = 650 nm, full slit width, for 2 min.

Fig. 2. Absorption of single, unfolded bean leaves as a function of time following a 6 sec illumination using white light (complete photoconversion) at +2 C. Different leaves were used at each of several wavelengths, as indicated on the curves. The absorption spectra 10 min after illumination exhibited maxima at 684 nm.

Fig. 3. The difference in the absorption spectra of illuminated and unilluminated bean leaves. The relatively low illumination intensity (Cary monochromator, λ = 650 nm, full slit width) was incident for (a) 10 sec, (b) 30 sec, (c) 50 sec (27% conversion). Experiment carried out at room temperature. Inset: dependence of the absorbance change at 672 nm on the duration of illumination. A single, folded bean leaf was used in each spectrophotometer beam.

Fig. 4. The difference in the absorption spectra of bean leaves illuminated first with low intensity light, as in Fig. 3, sufficient to convert (a) 30.6% (b) 15.5% of the active protochlorophyllide and then a short, bright illumination to complete the photoconversion. (c) Leaf which received only the short bright illumination. The lower curve is the difference between (a) and (c). All experiments at room temperature and normalized to the same ΔA at 683 nm.
Fig. 5. The difference in the absorption spectra of illuminated and unilluminated bean leaves at 0°C for different illumination intensities. Time of illumination, 10 sec. Curves 1-4 represent increasing illumination intensities, sufficient to give 4, 16, 31 and 100% conversion, respectively. The curves have been normalized to give the same peak heights. A single, folded bean leaf was used in each spectrophotometer beam for each experiment.

Fig. 6. A proposed scheme to account for the spectroscopic changes observed in etiolated bean leaves following illumination.
Fig. 1
Fig. 2

Absorbance

Time (Min.)

Light on

Light off

684

675

692

668

730

XBL725-4643
Fig. 4
Fig. 5
\[ P - P_{650} \xrightarrow{h\nu/k_1} P - C_{676} \xrightarrow{h\nu/k_2} C - C_{678} \xrightarrow{\text{dark}} C - C_{684} \]

\[ \text{dark} \]

\[ P + C_{672} \]

\[ 2C_{672} \]

\[ P_{635} \xrightarrow{h\nu} C_{672} \]

Fig. 6
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