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The Conversion of Photoinactive Protochlorophyllide$_{633}$ to Phototransformable Protochlorophyllide$_{650}$ in Etiolated Bean Leaves Treated with δ-Aminolevulinic Acid[1]

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ABSTRACT

The relationship of phototransformable protochlorophyllide to photoinactive protochlorophyllide has been studied in primary leaves of 7- to 9-day-old dark-grown bean (*Phaseolus vulgaris* L. var. Red Kidney) seedlings. Various levels of photoinactive protochlorophyllide, absorbing at 633 nm *in vivo*, were induced by administering δ-aminolevulinic acid to the leaves in darkness. Phototransformable protochlorophyllide, absorbing at 650 nm *in vivo*, was subsequently transformed to chlorophyllide by a light flash, and the regeneration of the photoactive pigment was followed by monitoring the absorbance increase at 650 nm *in vivo*. A small increase in the level of protochlorophyllide$_{633}$ causes a marked increase in the extent of regeneration of protochlorphyllide$_{650}$ following a flash. High levels of the inactive pigment species, however, retard the capacity to reform photoactive protochlorophyllide. A nonstoichiometric and kinetically complex decrease in absorbance at 633 nm *in vivo* accompanied the absorbance increase at 650 nm. The half-time for protochlorophyllide$_{650}$ regeneration in control leaves was found to be three times longer than the half-time for conversion of chlorophyllide$_{678}$ to chlorophyllide$_{683}$ at 22 C. The results are consistent with the hypothesis that protochlorophyllide$_{633}$ is a direct precursor of protochlorophyllide$_{650}$ and that the protein moiety of the protochlorophyllide holochrome acts as a "photoenzyme" in the conversion of protochlorophylide to chlorophyllide.

Etiolated leaves of bean seedlings contain at least three species of PChld.[2] These pigment species can be identified by their absorption maxima *in vivo* at −196 C in the red region of the spectrum (1, 5, 15). The first, P_{650}, is associated with the phototransformable Pchld holochrome (20). This species is transformed by a brief pulse of light to Chld absorbing at 678 nm *in vivo* (3, 9, 21). The second Pchld species, P_{637}, occurs to a lesser extent in etiolated bean leaves but to a larger extent in etiolated leaf extracts containing the Pchld holochrome (1, 4, 5, 15). It is also transformed to Chld by a pulse of light. The third species, P_{628}, is present in low levels in etiolated bean leaves (1, 12, 15, 20), but its concentration may be increased by administering ALA to the leaves in darkness (2, 8, 11, 12, 22, 24). P_{650} and P_{637} can be converted to P_{628} by heat, acid, freezing and thawing, and by treatment with a variety of compounds (4–6, 13). P_{628} is not directly phototransformable to Chld but, under some circumstances, seems to act as a precursor of P_{650} (10, 11, 18, 23, 24).

The experiments reported here were undertaken to study the conversion of P_{628} to P_{650} in both ALA-treated and control leaves. The results provide further evidence that P_{628}, termed PChld$_{633}$ in this report, can act as a precursor of P_{650}, presumably by combining with the protein moiety of the holochrome ("apophotoenzyme") following phototransformation of endogenous P_{650}.

MATERIALS AND METHODS

Growth and Manipulation of Plant Material. Seeds of *Phaseolus vulgaris* L. var. Red Kidney were germinated in moistened vermiculite in total darkness for 7 to 9 days at about 22 C. All manipulations of plant material were performed under a dim green safelight (6). Primary leaves were excised and a single leaf was mounted against the interior wall of a 1-cm glass cuvette, as described previously (6).

Excised leaves were placed in Petri dishes on filter paper moistened with 10 mM ALA, pH 6.5 (Sigma Chemical Co.). The tissue was vacuum infiltrated several times to accelerate uptake of the solution and then incubated in darkness at 22 C.

***In Vivo* Spectrophotometry.** Spectral measurements were carried out at 22 C with a Hitachi-Perkin Elmer Model 356 spectrophotometer, as described previously (6). The slit was open to 0.125 mm (0.5 nm).

Leaves mounted in the cuvette were irradiated in the spectrophotometer with a reflectorized xenon-filled flashtube held approximately 3 cm from the sample (6). The arc was discharged with a Zeiss Ukatron UN60 microflash unit at 60 W-sec (1 msec duration), and the intensity was saturating with respect to the Pchld holochrome transformation (6).

RESULTS

Effect of Various Levels of PChld$_{633}$ on PChld$_{650}$ regeneration. The effect of various levels of PChld$_{633}$ on the extent of PChld$_{650}$ regeneration after a light flash is shown in Figure 1. Etiolated 8-day-old bean leaves were incubated in 10 mM ALA in darkness for 0, 1, 2, 3, or 4 hr (Fig. 1A, curves a–e). The level of PChld$_{633}$ increases with time of incubation. The level of PChld$_{650}$ is, however, not significantly affected until the

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[2] Abbreviations: PChld: protochlorophyllide; Chld: chlorophyllide; ALA: δ-aminolevulinic acid.

3rd hr. After a saturating light flash, the absorbance at 650 nm diminishes but then recovers 19, 41, 53, 53 and 54%, respectively, during the first 60 sec after the flash (Fig. 1C, a–e; Table I). Thus, a small increment in the level of $PChld_{633}$ (within 1–2 hr after infiltration) increases the extent of $PChld_{650}$ regeneration within 1 min after a flash.

The absorption spectrum of each leaf recorded 3 min after the light flash is shown in Figure 1B. Shoulders at 650 nm are apparent. In addition, the presence of large amounts of $PChld_{633}$ seems to shift the position of the Chld peak from 683 nm to 672 nm. It has been suggested that the displacement of the Chld absorption peak to shorter wavelengths in leaves containing ALA-PChld is due to an aggregation of Chld with the excess PChld (9).

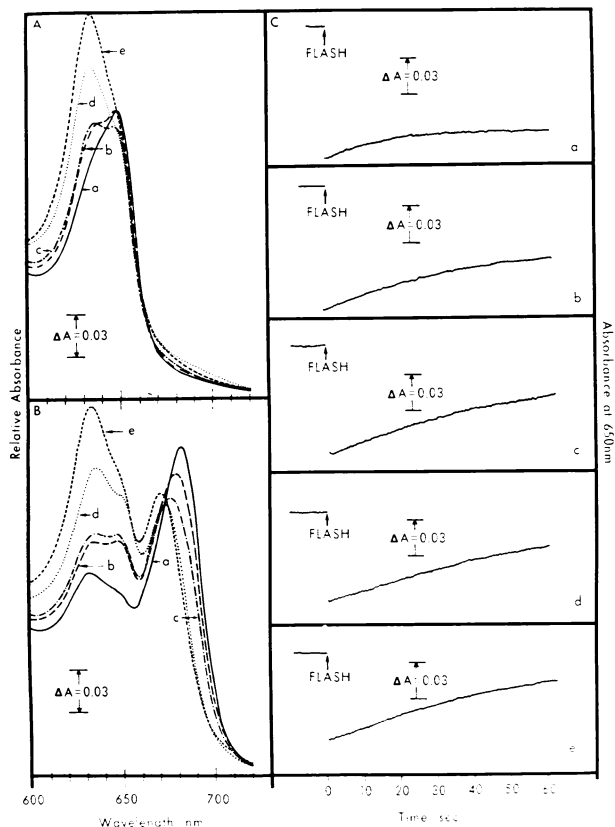

FIG. 1. A: Absorption spectra *in vivo* of etiolated 8-day-old bean leaves infiltrated with 10 mM ALA for the following periods in darkness: (a) 0 hr (b) 1 hr (c) 2 hr (d) 3 hr (e) 4 hr. B: Absorption spectra of the leaves in A recorded 3 min after a flash of light. C: Absorption changes at 650 nm *in vivo* before and immediately following a saturating light flash, using the leaves in A.

Prolonged incubation of the leaves with ALA in darkness has a deleterious effect upon the amount of Chld formed after the actinic light flash and upon the regeneration of $PChld_{650}$. By the 3rd hr, a significant loss in $PChld_{650}$ is apparent as judged by a smaller drop in the absorbance at 650 nm after the flash (Fig. 1C, d,e; Table I). This loss in $PChld_{650}$ becomes especially noticeable after 12 and 24 hr of incubation with ALA (Fig. 2C). The amount of Chld formed as a result of a flash is similarly diminished (Fig. 2B). In addition, the rate of $PChld_{650}$ regeneration falls to near 0 by 24 hr (Fig. 2C). The extent of $PChld_{650}$ regeneration within 1 min after a flash also diminishes with time of infiltration with ALA, especially when expressed as a capacity relative to a fixed level of $PChld_{650}$ (Table I). Levels of $PChld_{650}$ in control leaves which are incubated on H_2O for up to 24 hr do not appreciably change. The ability to regenerate $PChld_{650}$ after a flash is also not affected in control leaves.

Conversion of $PChld_{633}$ to $PChld_{650}$. An 8-day-old etiolated bean leaf was infiltrated with 10 mM ALA for 8 hr and then subjected to a series of light flashes (Fig. 3). The absorbance at 650 nm and 633 nm was monitored simultaneously after each of three flashes, using the 356 spectrophotometer in the dual wavelength mode (Fig. 3, A and B). The absorbance at 650 nm increases while that at 633 nm decreases after flash No. 1. However, there are two unusual features associated with these changes which are worth noting: (*a*) the absorbance change at 633 nm is much smaller than that at 650 nm, and (*b*) the absorbance at 633 nm increases during the first 10 to 20 sec and then decreases. These changes are qualitatively and quantitatively similar to those which occur when $PChld_{633}$ in H_2S-treated etiolated bean leaves reverts to $PChld_{650}$ as H_2S is released from the tissue (6).

The absorbance changes after flashes No. 2 and No. 3 are similar to those following flash No. 1, except that their magnitude diminishes with each flash (*cf.* Fig. 6, ref. 24). However, the initial rapid rise at 633 nm is not affected (Fig. 4, ref. 6). Fig. 3C shows the absorption spectra of the leaf after each flash. It is clear that the elevated pool of $PChld_{633}$ is being transformed to Chld via $PChld_{650}$ (Fig. 4, ref. 24).

The log of the absorbance change at 650 nm was plotted as a function of time for each of three ALA treatment periods (Fig. 4). In each case the change is roughly linear, following

Table I. *Regeneration of Protochlorophyllide$_{650}$ in Etiolated Bean Leaves Treated with δ-Aminolevulinic Acid*

The number of experiments is given in parentheses. The 95% confidence limits calculated from a *t*-test follow each determination.

Treatment	A_{633}/A_{650}	$-\Delta A_{650}$ after Flash	% Regeneration of A_{650} within 1 Min after Flash	
			Based on actual $-\Delta A_{650}$	Normalized for a $-\Delta A_{650}$ = 0.100
Control (6)	0.775 ± 0.048	0.094 ± 0.0073	19 ± 5	18 ± 5
ALA, 1 hr (7)	0.839 ± 0.041	0.088 ± 0.016	41 ± 7	36 ± 6
ALA, 2 hr (7)	1.05 ± 0.113	0.078 ± 0.023	53 ± 6	41 ± 5
ALA, 3 hr (8)	1.17 ± 0.160	0.074 ± 0.015	53 ± 11	39 ± 8
ALA, 4 hr (12)	1.32 ± 0.116	0.073 ± 0.016	54 ± 8	39 ± 6
ALA, 8 hr (5)	1.48 ± 0.142	0.063 ± 0.023	45 ± 13	28 ± 3
17′ dark → flash #2	1.32 ± 0.360	0.047 ± 0.010	54 ± 13	25 ± 6
17′ dark → flash #3	1.31 ± 0.315	0.040 ± 0.012	48 ± 17	19 ± 7
ALA, 12 hr (4)	1.44 ± 0.156	0.057 ± 0.020	41 ± 14	24 ± 8
ALA, 24 hr (4)	1.51 ± 0.072	0.043 ± 0.0093	8.5 ± 9	3.6 ± 3.9

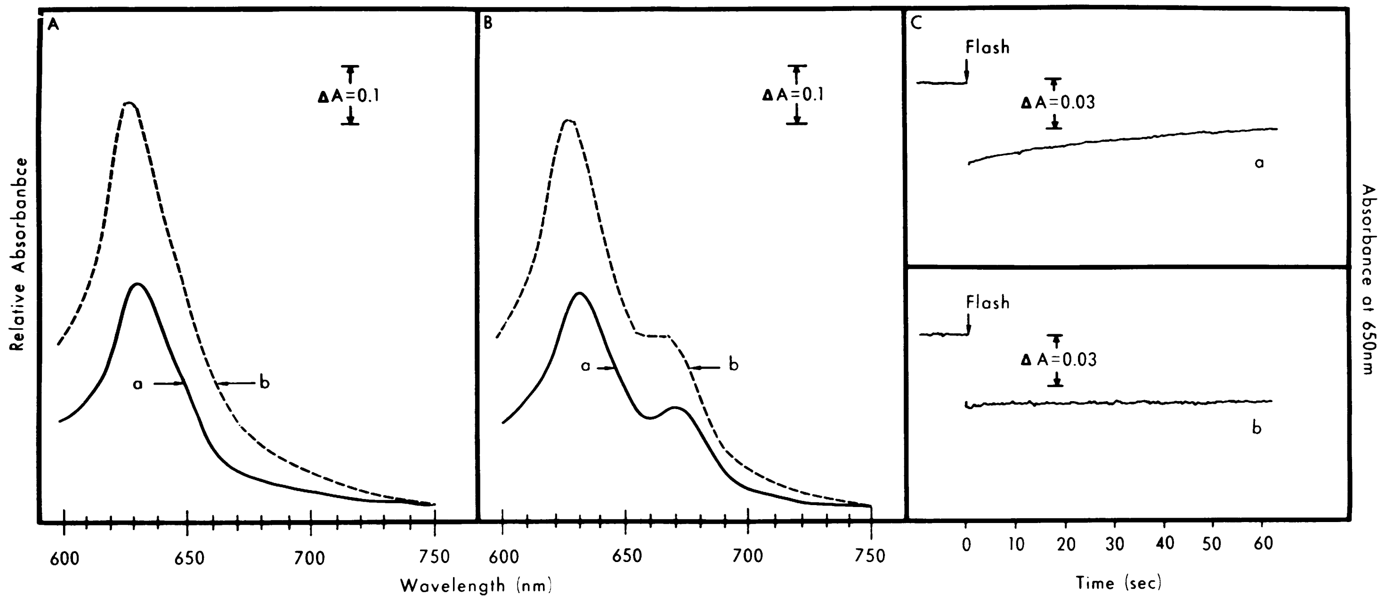

FIG. 2. A: Absorption spectra *in vivo* of etiolated 8-day-old bean leaves infiltrated with 10 mM ALA for (a) 12 hr (b) 24 hr in darkness. B: Absorption spectra of the leaves in A recorded 3 min after a flash of light. C: Absorption changes at 650 nm *in vivo* before and immediately following a saturating light flash, using the leaves in A.

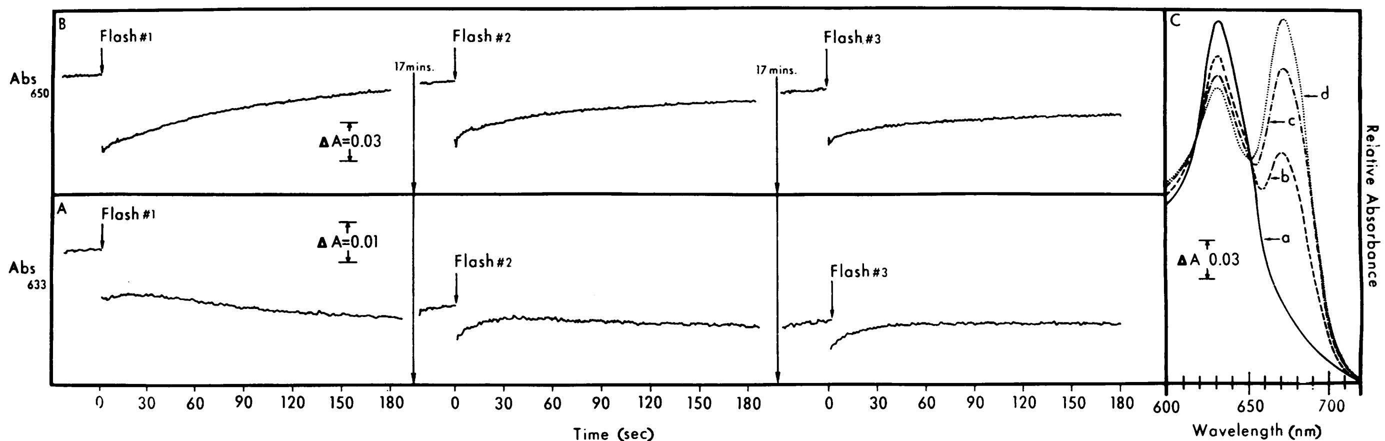

FIG. 3. Absorption changes at (A) 633 nm and at (B) 650 nm following three sequential flashes of light in 8-day-old etiolated bean leaves infiltrated with ALA for 8 hr in darkness. C: Absorption spectra *in vivo* of the leaves from A and B recorded (a) before any irradiation, (b) after flash #1, (c) after flash #2, (d) after flash #3.

first order kinetics, although there seems to be a systematic deviation from linearity in two of the treatments. The absorbance changes at 633 nm do not transform to linear plots. There is no single isosbestic point between 633 nm and 650 nm during the course of these absorbance changes.

Relation of $PChld_{650}$ Regeneration to the $Chld_{678 \rightarrow 683}$ Change. The regeneration of $PChld_{650}$ presumably represents recombination of the holochrome protein with PChld molecules at the expense of $PChld_{633}$ (free PChld?) (11, 18, 24). Gassman *et al.* (9) have suggested that the rapid shift in absorption of $Chld_{678}$ to $Chld_{683}$ after a light flash represents the diffusion of Chld off the holochrome protein moiety (but not necessarily away from other pigment molecules). Thus, if both of these hypotheses are true, one would expect that the freeing of sites on the apoprotein (*i.e.* the $Chld_{678} \rightarrow Chld_{683}$ change) would have to be as fast or faster than the reconstitution of the holochrome complex from free PChld and the apoprotein (the absorbance increase at 650 nm). To test this supposition, the absorbance change at 692 nm was measured simultaneously with the absorbance change at 650 nm, using the 356 spectrophotometer in the dual wavelength mode (Fig. 5). The change at 692 nm is a measure of the $Chld_{678 \rightarrow 683}$ change (3, 9). It can be seen that the change at 692 nm in a control leaf is completed prior to the change at 650 nm (Fig. 6).

It was not possible to compare accurately the time course of the absorbance change at 692 nm with the change at 650 nm in ALA-treated leaves because of the rapid shift of $Chld_{683}$ to shorter wavelengths in such tissue; *i.e.* the absorbance at 692 nm after a flash in leaves treated with ALA for 4 hr increases in the first 30 sec but then decreases for several minutes. These changes reflect the instability of the $Chld_{683}$ species in the presence of excess PChld. In leaves containing large amounts of ALA-PChld (9) or in weakly irradiated control leaves (17), the $Chld_{683}$ species does not form from $Chld_{678}$; instead, a species with absorption maximum at 675 to 676 nm appears. This $Chld_{675}$ species is presumably a mixed PChld-Chld dimer (17) or polymer (7) which rapidly converts to a $Chld_{672}$ (monomer?) species.

One explanation for the loss in $PChld_{650}$ and the loss in the capacity to regenerate $PChld_{650}$ after a flash in leaves treated with ALA for long periods is that some energy source which may be needed for P_{650} synthesis and/or maintenance may be depleted in the excised leaves during the biosynthesis of $PChld_{633}$ from ALA. Murray and Klein (18) have shown that anaerobiosis prevents the conversion of ALA to $PChld_{633}$ in etiolated bean leaves. Horton and Leech (14) report the participation of ATP in the synthesis of $PChld_{650}$ from $PChld_{633}$ in isolated maize etioplasts. $PChld_{650}$ was also stabilized in the presence of ATP (14). The following compounds were found to inhibit $PChld_{633}$ formation from ALA to the extent indicated over a 6-hr incubation period: 10 mM sodium arsenate, 46%; 10 mM sodium levulinate, 38%; 1 mM dinitrophenol, 32%. Only dinitrophenol inhibited the absorbance increase at 650 nm after a flash once appreciable $PChld_{633}$ had formed.

If 50 mM sucrose containing 100 mM ascorbic acid was administered to etiolated bean leaves concomitant with 10 mM ALA over a 21-hr period, partial restoration of the capacity to regenerate $PChld_{650}$ after a flash was accomplished. However, sucrose plus ascorbate did not preserve the concentration of $PChld_{650}$ at control levels during this period.

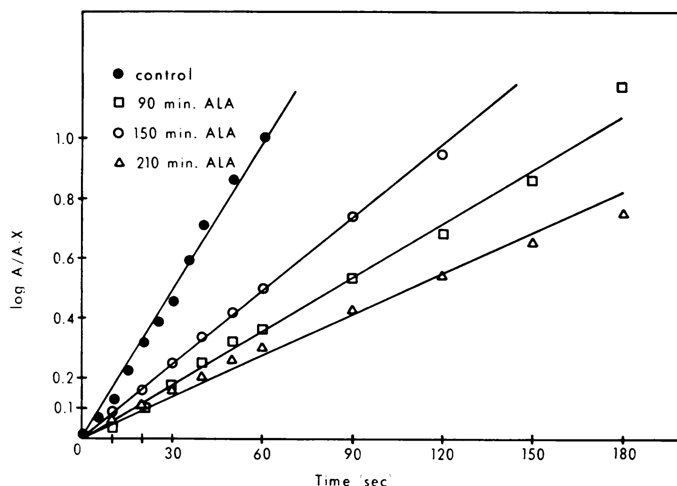

FIG. 4. Absorbance changes at 650 nm following a light flash in etiolated 8-day-old leaves infiltrated with ALA for various periods in darkness. The changes are expressed as log A/A-X where A is the total absorbance regeneration at 650 nm after the flash and X is the absorbance regeneration at any time, t. The straight lines were fitted to the data by the method of least squares.

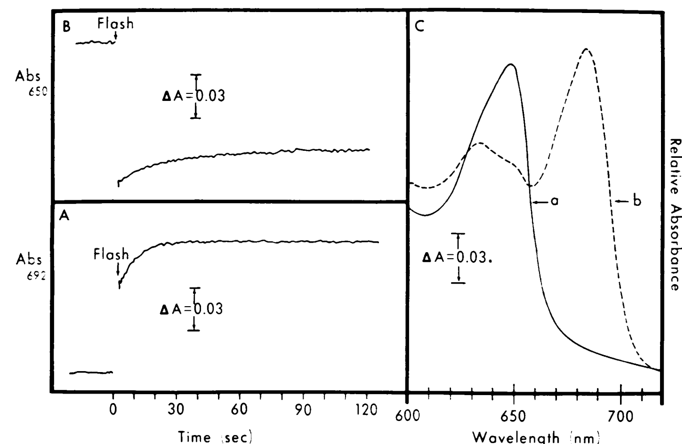

FIG. 5. Absorbance changes at (A) 692 nm and at (B) 650 nm in an 8-day-old etiolated bean leaf following a flash of light. C: Absorption spectra *in vivo* of this leaf (a) before and (b) 5 min following the light flash indicated in A and B.

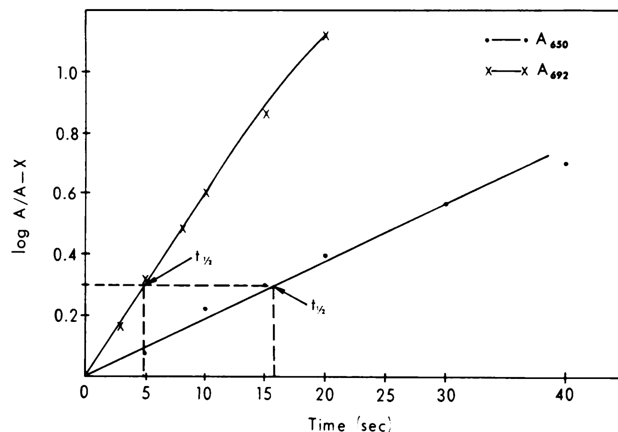

FIG. 6. Absorbance changes in Fig. 5 transformed to a log plot as explained for Fig. 4.

DISCUSSION

Treatment of etiolated bean leaves with ALA for several hours has at least two effects upon the regeneration of $PChld_{650}$ after a flash: (*a*) the extent of regeneration is increased (Fig. 1, Table I), and (*b*) the time course of the regeneration is prolonged (Fig. 4). Sundqvist (23) has reported that ALA treatment of etiolated wheat leaves increases the amount of Chld formed during low level irradiation after a light flash. He also indicates that ALA treatment prolongs the time course of Chld synthesis.

Prolonged treatment with ALA, however, causes a loss in $PChld_{650}$ and a diminution in the capacity to regenerate $PChld_{650}$ as compared to untreated leaves. High levels of ALA-PChld have been reported (23) to reduce the rate of Chld formation from $PChld_{633}$ in wheat leaves subjected to continuous low level irradiation after photoconversion of $PChld_{650}$. Steer (22) has reported that ALA-derived PChld diminished flash-induced chlorophyll synthesis in barley leaves. The reason for this inhibition is not immediately clear. Several explanations are possible, one of which being the exhaustion of some factor necessary for $PChld_{650}$ synthesis and depleted by $PChld_{633}$ synthesis from ALA. This factor could be ATP, as mentioned above. However, sucrose is only slightly effective in restoring the capacity to regenerate $PChld_{650}$ after a flash in ALA-treated leaves. It is ineffective in maintaining the level of $PChld_{650}$ to that of control leaves. By partially denaturing $PChld_{650}$ with NaN_3, Sundqvist (23) has shown that the level of $PChld_{650}$ controls the capacity of etiolated wheat leaves to synthesize Chld under continuous irradiation.

Another explanation for the inhibition of $PChld_{650}$ regeneration in leaves containing large amounts of ALA-PChld is that the excess PChld may be a different pigment species from the endogenous phototransformable PChld or from the PChld generated by brief (*i.e.* 2–3 hr) treatment with ALA. However, Sundqvist (23) finds that ^{14}C-ALA treatment of etiolated wheat leaves gives rise to ^{14}C-Chl upon subsequent illumination. He interpreted his results as an indication that ALA-derived PChld could freely exchange with the PChld on the PChld holochrome (*i.e.* $PChld_{650}$). The PChld derived from ALA treatment is apparently chromatographically and spectroscopically identical to the PChld of untreated bean leaves (7). Thus, ALA-PChld is probably chemically identical to the endogenous

PChld. On the other hand, ALA-PChld has a slightly different fluorescence emission peak *in vivo* from the endogenous nontransformable PChld (7). It would appear, then, that ALA-$PChld_{633}$ may be in a different physical state or environment from endogenous $PChld_{633}$, such as in a large aggregate. This aggregate may restrict the ability of the individual PChld monomers, dimers, or oligomers to bind to the holochrome protein after phototransformation of $PChld_{650}$.

Another anomaly in the regeneration of $PChld_{650}$ in ALA-treated leaves is the loss in the rate and extent of regeneration of the photoactive complex after each in a series of flashes (Fig. 3; Fig. 3, ref. 12; Fig. 6, ref. 24). Sundqvist (23) suggests that the inability to transform most of the ALA-PChld to Chld in continuously irradiated wheat leaves may be due to the exhaustion of the hydrogen donor for the holochrome or to product inhibition by the large amounts of Chld which build up.

The slow absorbance decrease at 633 nm after a light flash in ALA-treated leaves is about 10-fold smaller than the simultaneous absorbance increase at 650 nm. However, the half bandwidth of $PChld_{633}$ is about 50% greater than that of $PChld_{650}$ (6). Thus, $PChld_{633}$ might be expected to have a smaller extinction coefficient at its absorption maximum than that of $PChld_{650}$ at 650 nm. Heating etiolated wheat leaves at 45 C for 15 min causes a loss in absorption *in vivo* at 650 nm which is three times greater than a concomitant increase in absorption at 633 nm (23). This would tend to support the contention that the extinction coefficient of $PChld_{633}$ is smaller than that for $PChld_{650}$.

The mechanism by which $PChld_{633}$ is converted to $PChld_{650}$ remains to be satisfactorily elucidated. The complexity of the absorption change at 633 nm may be compounded by the overlap in the absorption bands of the two PChld species. However, the lack of a single isosbestic point between 633 and 650 nm during the time course of these absorbance changes indicates that more than one reaction is taking place. Perhaps, the $PChld_{633}$ rapidly disaggregates (or aggregates) and the smaller (larger?) pigment units then bind to the holochrome protein moiety. At the same time, the nascent Chld ($Chld_{678}$) diffuses away from the apoprotein, while the oxidized reducing group is being reduced by a source of reducing power.

The studies reported here as well as previous studies (2, 8, 10, 11, 18, 19, 23–25) indicate that nontransformable $PChld_{633}$ may serve as a precursor of $PChld_{650}$ and that the holochrome protein may act as a "photoenzyme" (9, 12, 25) in the conversion of PChld to Chld, at least in the early stages of greening. Further studies are needed to determine whether the holochrome protein functions enzymatically throughout the entire greening process or whether additional holochrome protein synthesis is required, as seems to be the case in some other organisms (16).

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