ABSORBANCE AND FLUORESCENCE PROPERTIES OF PROTOCHLOROPHYLLIDE IN ETIOLATED BEAN LEAVES

Merrill L. Gassman

Dept. of Biological Sciences
University of Illinois at Chicago Circle
Chicago, Illinois 60680

Received June 7, 1973

SUMMARY

Primary leaves of 7-to-9 day-old etiolated bean seedlings contain a species of protochlorophyllide which is not transformed to chlorophyllide by light; this pigment species exhibits an absorption peak at 631nm *in vivo* at -196° and a fluorescence emission peak at 639nm *in vivo* at room temperature. Heat-treatment of etiolated leaves converts the phototransformable protochlorophyllide holochrome to a pigment species with *in vivo* absorption and fluorescence peaks identical to those of endogenous nontransformable protochlorophyllide. Administration of δ-aminolevulinic acid to etiolated leaves causes the synthesis of nontransformable protochlorophyllide with an absorption peak also at 631nm *in vivo* at -196° but with a fluorescence emission peak at 643nm *in vivo* at room temperature. Heat-treatment of such leaves does not affect the position of these bands. The results indicate that protochlorophyllide which is derived from exogenous δ-aminolevulinic acid is in a physically different state from other forms of protochlorophyllide in the leaf.

Etiolated leaves of angiosperm seedlings contain at least three species of the chlorophyll precursor PChld[1]. These pigment species can be identified by their absorption maxima *in vivo* at -196° in the red region of the spectrum (1, 2, 3). The first, P_{650}, is characteristic of the PChld holochrome *in vivo*. This species is transformed by light to Chld (4). The second, P_{637}, occurs to a lesser extent in etiolated leaves but to a greater extent in etiolated-leaf extracts containing the phototransformable PChld holochrome (1). It is also phototransformable to Chld (1). The third species, P_{628}, occurs only in low concentrations in etiolated leaves but its levels increase when ALA is adminis-

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

tered to the leaves (5). P_{628} is not directly phototransformed to Chld but, under some circumstances, seems to act as a precursor of P_{650} (6, 7, 8). 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 (2, 9, 10, 11). The fluorescence properties of these three PChld species at -196° have been reported (1, 3).

The studies reported here were undertaken to characterize the nontransformable PChld species. The results indicate that this species has the same absorption maximum *in vivo* at -196° whether it is derived by heat denaturation of P_{650} and P_{637}, by ALA administration, or by a combination of these two treatments. However, the fluorescence emission peak at 22° of the heat-generated PChld species differs to a small but significant extent from that of the ALA-derived species. Thus, the possibility must be considered that the photoinactive PChld species derived from exogenous ALA treatment is in a physically different state or environment than that of heat-denatured PChld holochrome or the endogenous nontransformable PChld and, therefore, that there may be at least two types of nontransformable PChld in etiolated bean leaves.

MATERIALS AND METHODS

Bean (*Phaseolus vulgaris* var. Red Kidney) seeds were germinated in total darkness in moist vermiculite for 7-9 days at about 22°. All manipulations of plant material were carried out under a dim green safe-light (10). The *in vivo* absorption spectrum through two layers of primary leaf tissue was measured at -196° in a Hitachi Perkin-Elmer Model 356 spectrophotometer equipped with a cryogenic accessory. A single thickness of

filter paper served as a reference blank (10). Spectra were scanned at a speed of 60mm/min and recorded at a chart speed of 60mm/min. The slit on the 356 was set at 0.125mm (0.5nm).

Fluorescence emission spectra were recorded at 22° on a single layer of leaf tissue in the solid sample accessory of a Hitachi Perkin-Elmer Model MPF-2A Fluorescence spectrophotometer equipped with a R446S photomultiplier and operated in the "ratio" mode. The excitation monochromator was set at 445 nm with the exit slit open to 40nm. A 500nm long-wave cut-off filter was placed between the excitation monochromator and the leaf sample. The intensity of the exciting light was 14.5mW/cm^2 at the leaf surface. Fluorescence from the leaf surface was taken at right angles to the exciting beam and passed through a 600nm short-wave cut-off filter before entering the emission monochromator. The emission monochromator exit slit was set at 2nm. The spectra were scanned at the rate of 50nm/min and recorded at a chart speed of 50mm/min. The spectra were not corrected for the response of either the emission monochromator or the photomultiplier.

Phototransformation of PChld holochrome was achieved by discharging a xenon arc (60 W-sec)3 cm from the surface of the leaf at 22° (10). Fluorescence spectra were recorded from 3 to 5 minutes after the actinic flash.

RESULTS

The absorption spectrum at -196° of a 7-day-old etiolated bean leaf is shown in Figure 1A (curve a). An absorption peak is apparent at 650nm, characteristic of P_{650}. A smaller absorption maximum is observed at 637nm. If an etiolated leaf is irradiated with a light flash at 22° and then brought to -196° within a few seconds, curve e of Figure 1A is obtained. The peaks at 650nm

and 637nm have disappeared and a peak at 678nm, characteristic of the first Chld photoproduct (12, 13, 14) appears. In addition a peak at 628nm becomes apparent. There are also small peaks at 548, 552, and 556nm which are probably due to cytochromes. These data agree with those of Kahn et al (1, 3).

When 7-day-old bean leaves are infiltrated with 10mM ALA for two and four hours in darkness, curves b and c, respectively, of Figure 1A are obtained. The absorbance at 631-632nm increases with time of infiltration, indicating the accumulation of P_{628}. Curves a and b of Figure 1B indicate the absorption spectra of similar leaves infiltrated with ALA for three and six hours, respectively. By the sixth hour, the level of P_{650} has diminished. The reason for this is not clear. A more complete description of this phenomenon will be treated elsewhere. However, this loss in P_{650} is probably the reason why Chld synthesis in wheat leaves under continuous low-level irradiation is inhibited when the pool of ALA-generated P_{628} exceeds a certain level (15).

Curve d in Figure 1A is the absorption spectrum of an etiolated bean leaf which has been boiled in water for 30 seconds. Curve c in Figure 1B is the absorption spectrum of a similar leaf which was infiltrated with 10mM ALA for six hours in darkness followed by boiling in water for 30 seconds. Both curves exhibit a single, symmetrical absorption maximum at 631nm. In addition, an estimation of the band widths at half-maximum height of the 631nm absorption bands in these two curves gave a value of 28nm ± 2nm.

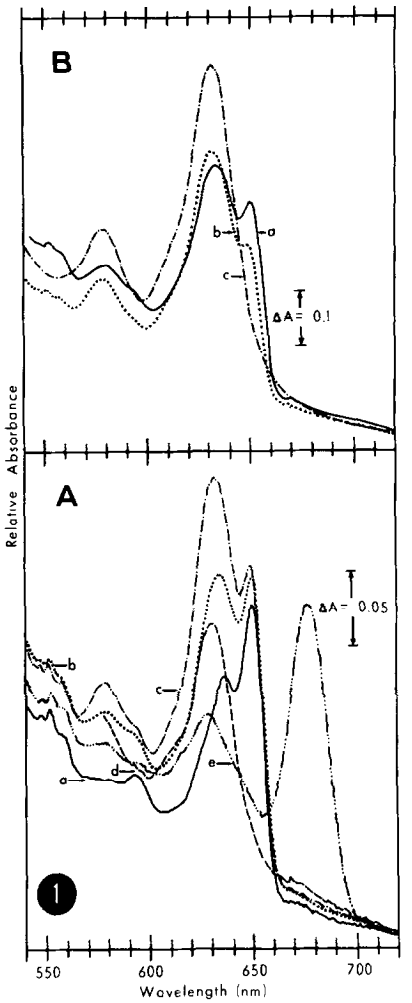
Figure 2 presents the fluoresence emission spectra of four etiolated leaves infiltrated with 10mM ALA for 0, 2, 4, or 6 hours in darkness. It can be seen that the fluorescence emission peak of PChld shifts from 639nm to 643nm as nontransformable PChld

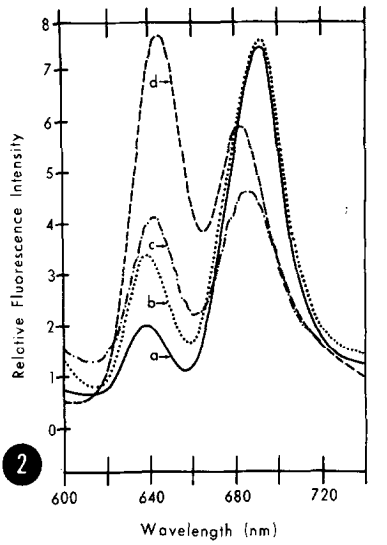
builds up in the leaf; i.e. curve a-639nm, curve b-639nm, curve c-640nm, curve d-643nm. These emission peaks were quite reproducible in other experiments. Heat-killed barley leaves have been reported to fluoresce at 638nm at room temperature (16). These results suggest that the endogenous nontransformable PChld emits at a slightly shorter wavelength than the PChld derived by ALA administration.

To test whether the fluorescence emission of heat denatured PChld holochrome was similar to either that of ALA-derived PChld or of endogenous nontransformable PChld, the experiment shown in Figure 3 was carried out. Curves a and c are of untreated leaves; curves b and d are of leaves which were infiltrated with 10mM ALA for 6 hours in darkness. Curves c and d depict leaves which were boiled in water for 30 seconds prior to recording the spectra. Curves a and c show emission bands at 639nm while curves b and d show emission bands at 643nm. Because of the large level of PChld in ALA-treated leaves as compared to controls, the emission band at 643nm in curve d was not appreciably shifted to shorter wavelengths by the 639nm emission band associated with heat-denatured PChld holochrome (curve c). The results indicate that the fluorescence emission peak of PChld in untreated leaves, either with or without heating, is at 639nm (curves a and c). However, the emission peaks of PChld in ALA-treated leaves, with or without heating, is at 642-643nm (curves b and d).

DISCUSSION

Evidence has been presented that the fluorescence, but not the absorption, properties of nontransformable PChld, whether endogenous or derived from heat-denaturation of PChld holochrome, are different from those of the nontransformable PChld derived





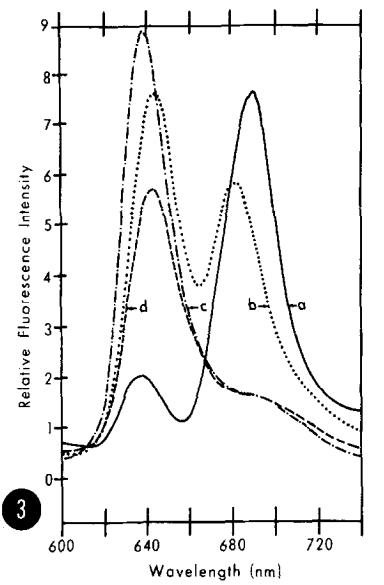


Fig. 1. The in vivo absorption spectra of etiolated bean leaves at -196°.
A. a. (——) control; b. (•••) treated with 10mM ALA for 2 hours; c. (•—•) treated with 10mM ALA for 4 hours; d. (– –) control, boiled in H_2O for 30 sec.; e. (••—) control, after flash of light
B. a. (——) treated with 10mM ALA for 3 hours; b. (•••) treated with 10mM ALA for 6 hours; c. (•—•) treated with 10mM ALA for 6 hours followed by boiling in H_2O for 30 sec.

Fig. 2. The fluorescence emission spectra of etiolated bean leaves at 22° following a flash of light.
a. (——) control; b. (•••) treated with 10mM ALA for 2 hours;
c. (•—•) treated with 10mM ALA for 4 hours;
d. (– –) treated with 10mM ALA for 6 hours.
The electrical gain for curve d was 1/9 that used in the other 3 curves.

from ALA administration. The reason for this difference is not clear but may reflect a difference in the environment of the pigments; e.g. a hydrophobic interior of a protein and/or the polymeric state of the pigments.

One explanation for the shift in the ALA-PChld emission peak to longer wavelengths is that other porphyrins may be synthesized from ALA and may emit fluorescence near 640nm, thereby shifting the apparent emission peak of PChld. This explanation is unlikely, however, for the following reasons: (1) only traces of magnesium protoporphyrin and protoporphyrin are found in ether extracts of ALA-treated leaves; (2) the PChld emission band at 643nm in ALA-treated leaves is quite symmetrical; and (3) the wavelengths used to excite fluorescence (425-465nm) are considerably displaced from the Soret bands of other porphyrins.

Another explanation for this shift in the ALA-PChld emission is that ALA-PChld may be a different chemical species from endogenous PChld. This is also unlikely based on the following: (1) If the pigments of ALA-treated etiolated bean leaves are extracted into acetone, washed with petroleum ether, transferred to ether, and purified by thin-layer chromatography on silica gel in benzene: ethyl acetate: ethanol (4:1:1), only 1 green spot with red fluorescence is obtained, at Rf = 0.54. Upon elution, this pigment has the same absorption and fluorescence properties in ether as PChld which is extracted from untreated leaves. (2) Sundqvist (15) reports that treating etiolated wheat leaves

Fig. 3. The fluorescence emission spectra of etiolated bean leaves at 22° following a flash of light.
a. (——) control; b. (····) treated with 10mM ALA for 6 hours;
c. (· — ·) control, boiled in H_2O for 30 sec.;
d. (— —) treated with 10mM ALA for 6 hours followed by boiling in H_2O for 30 sec.
The electrical gain for curve a was 9x that of curves b and c and 27x that of curve d.

with [^{14}C] ALA results in the formation of phototransformable [^{14}C] PChld; i.e. PChld associated with P_{650}. Thus, the PChld which forms from exogenous ALA treatment is probably chemically identical to the PChld which binds to the holochrome protein.

As the level of nontransformable ALA-PChld builds up in the etiolated leaf, the position of the Chld emission peak is shifted to lower wavelengths (curves c and d, Fig. 2; curve b, Fig. 3). This displacement in the Chld emission from 691 to 682nm is correlated with a displacement in its absorption maximum from 683 to 675nm at 22° in the presence of ALA-PChld (12). The rapid shift in the absorption band of the first Chld photoproduct, C_{678}, to shorter wavelengths (675nm), rather than to longer wavelengths (683nm), in the presence of large amounts of ALA-PChld was ascribed to an aggregation of Chld with PChld rather than with Chld (12). The displacement of the Chld emission peak to shorter wavelengths at increasing concentrations of PChld reinforces this explanation. Mathis and Sauer (17) have recently shown that a natural PChld-Chld mixed dimer can form in weakly irradiated etiolated bean leaves.

The shift in the PChld emission peak to longer wavelengths in ALA-treated leaves may also be ascribed to the formation of a PChld-Chld mixed dimer. However, the longwave shift in ALA-PChld emission occurs even in heat-treated leaves, when Chld cannot be produced (curve d, Fig. 3). Thus, this anomalous wavelength shift cannot be due exclusively to the formation of a PChld-Chld dimer.

Kahn *et al* (1, 3) attribute the 628nm absorption peak *in vivo* following phototransformation to both nontransformable PChld and to a secondary peak of Chld. Therefore, the true absorption maximum of the endogenous nontransformable PChld may lie at wave-

lengths longer than 628nm. This is supported by the position of the absorption peaks of these species at 631nm at -196° (curve d of Figure 1A, curve c of Figure 1B) and at 633-636nm at 22° (7, 10). Nevertheless, the apparently identical absorption spectra of the three types of nontransformable PChld described here and the discrepancy in their fluorescence spectra point out the necessity for using more than one method of analysis to identify the state of pigments in the intact leaf.

The effectiveness of these different forms of P_{628} as precursors of P_{650} is currently being investigated.

ACKNOWLEDGMENT

This investigation was supported in part by National Science Foundation grant GB 35627 and by a grant from the University of Illinois, Chicago Circle Research Board.

REFERENCES

1. Kahn, A., Boardman, N. K. and Thorne, S. W. (1970). J. Mol. Biol. 48, 85-101.
2. Dujardin, E., and Sironval, C. (1970). Photosynthetica 4, 129-138.
3. Boardman, N. K., Anderson, J. M., Kahn, A., Thorne, S. W., and Treffry, T. E. (1970). In Autonomy and Biogenesis of Mitochondria and Chloroplasts N. K. Boardman, A. W. Linnane, and R. M. Smillie, eds. pp. 70-84, North Holland, Amsterdam.
4. Shibata, K. (1957). J. Biochem. 44, 147-173.
5. Granick, S. (1963). Proc. V Int. Cong. Biochem. 6, N. M. Sissakian, ed. pp. 176-185, Pergamon Press, Oxford.
6. Granick, S., and Gassman, M. (1970). Plant Physiol. 45, 201-205.
7. Sundqvist, C. (1970). Physiol. Plant. 23, 412-424.
8. Murray, A. E., and Klein, A. O. (1971). Plant Physiol. 48, 383-388.
9. Butler, W. L., and Briggs, W. R. (1966). Biochim. Biophys. Acta 112, 45-53.
10. Gassman, M. L. (1973). Plant Physiol. 51, 139-145.
11. Henningsen, K. W. (1970). J. Cell. Sci. 7, 587-621.
12. Gassman, M., Granick, S., and Mauzerall, D. (1968). Biochem. Biophys. Res. Commun. 32, 295-300.
13. Sironval, C., Brouers, M., Michel, J.-M., and Kuiper, Y. (1968). Photosynthetica 2, 268-287.

14. Bonner, B. (1969). Plant Physiol. 44, 739-747.
15. Sundqvist, C. (1972). Ph.D. Thesis, Univ. of Goteborg.
16. Virgin, H., quoted by J. H. C. Smith and A. Benitez. (1955) In Modern Methods of Plant Analysis 4, K. Paech and M. V. Tracey, eds. pp. 142-196. Springer-Verlag, Berlin.
17. Mathis, P., and K. Sauer. (1973). Plant Physiol. 51, 115-119.