 Physiol. Plant. 37: 218–222. 1976

Incorporation of 5-Aminolevulinic Acid in the Chlorophyll-Protein Complexes of the Moss *Ceratodon purpureus*

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(Received 14 January, 1976; revised 24 March, 1976)

Abstract

After one month of cultivation in the dark in inorganic medium the chloroplasts of protonemata of *Ceratodon purpureus* have larger grana than chloroplasts from light-grown cultures. Incubation of dark-grown material with ALA increases the chlorophyll content and chlorophyll *a/b* ratio. On polyacrylamide-gel electrophoresis, a preferential labelling of chlorophyll-protein complex I is obtained after treatment with (^{3}H) ALA in darkness. In contrast, in light, much higher activity is found in chlorophyll-protein complex II. The free pigment zone is highly labelled in both environments.

Introduction

Mosses synthetize chlorophyll in the dark as well as in the light (Kirk and Tilney-Bassett 1967, Chevallier 1975). In dark-grown plants, the chloroplasts have well-developed grana, but stroma lamellae are nearly absent (Valanne 1971, Chevallier 1974). An increase in the size of the grana has also been observed in algal cells placed in the dark (Dubacq and Puiseux-Dao 1974). Although higher plants are not capable of synthetizing chlorophyll in darkness, extreme shade plants contain very large grana stacks (*e.g.* Anderson *et al.* 1973).

The stacking capacity of lamellae has been connected with photosystem II components (*e.g.* Park and Sane 1971). However, in recent years the complete correspondence between grana and PS II has been seriously questioned (*e.g.* Bishop 1974), and recently Arntzen and Briantais (1975) came to the conclusion that appressed lamellae are not required for PS II activity, although some components of the PS II are a prerequisite of grana stacks.

The main aim of this paper was to isolate the chlorophyll-protein complexes of moss protonemata and to compare the distribution between these complexes of chlorophyll synthetized in the dark with the distribution of chlorophyll in material grown in the light.

Abbreviations: ALA, 5-aminolevulinic acid; Chl, chlorophyll; CP I, chlorophyll-protein complex I; CP II, chlorophyll-protein complex II; FP, free pigment zone; PS I, photosystem I; PS II, photosystem II; SDS, sodium dodecyl sulfate.

Material and Methods

The spore material [*Ceratodon purpureus* (Hedw.) Brid.] was collected from natural habitats in 1974 and 1975. The cultures were sterile liquid cultures in Erlenmeyer flasks, prepared as described earlier (Valanne 1966, 1971). The basic nutrient solution used in all cultures was the *Micrasterias* solution developed by Waris (1953). Since the germination of Ceratodon in darkness in inorganic medium does not progress beyond swelling of the spore, it was not feasible to obtain enough chloroplasts from cultures grown all the time in the dark. Therefore cultures were first grown for two weeks in rhythmic light (18 h light, 6 h darkness, 21°C, 320 $\mu W/cm^2$ Philips TLF 40 W/55) and then transferred to the dark for one month. It was assumed that most of the chlorophyll synthetized in the light would be decomposed during one month in darkness, since in higher plants the half-life of chlorophyll has been shown to be shorter than two weeks (Kirk and Tilney-Bassett 1967). One month was also sufficient to produce the typical ultrastructure of dark-treated chloroplasts.

The 5-aminolevulinic acid was supplied to the protonemata according to Sundqvist (1969). The material used for studying its effect on chlorophyll synthesis in the dark was handled in green safelight (Kindermann Inactin Schutzfilter, pangrün Nr 2320 D, incandescent lamp 15 W). The nutrient solution was changed to 0.005 *M* 5-aminolevulinic acid hydrochloride (Fluka) in 0.05 *M* phosphate buffer, pH 7.5. The incubation time was always 24 h. The chlorophylls were extracted immediately after the treatment by grinding the protonemata in a glass homogenizer in 80%

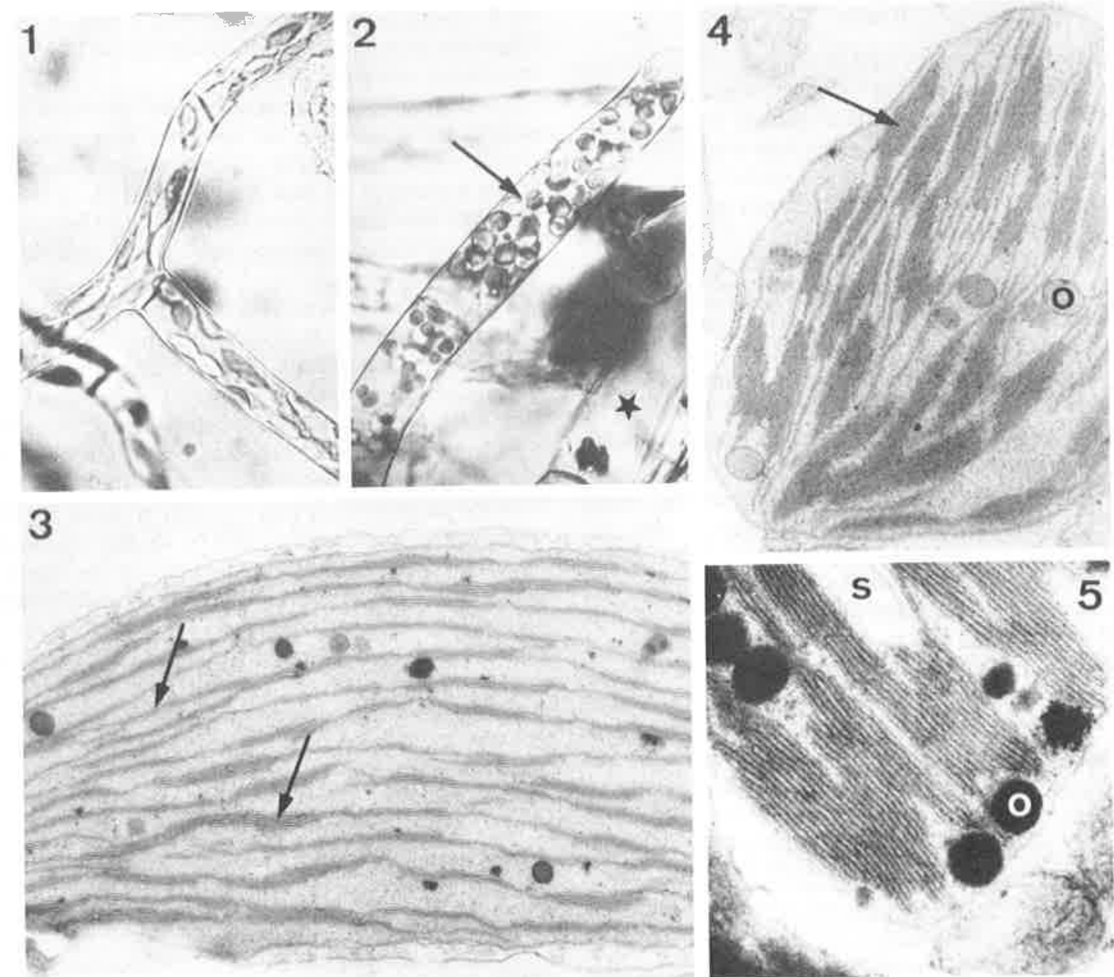
acetone under green safelight. The absorption spectra were recorded with a Perkin Elmer 402 spectrophotometer, and the chlorophylls were determined according to Arnon (1949).

The biosynthesis of the chlorophyll-protein complexes was studied by adding tritiated ALA, 5-amino (G-^{3}H) levulinic acid hydrochloride (the Radiochemical Centre, Amersham, England) to the incubation medium. In each experiment the material was harvested from five flasks (about 200 mg dry weight) and incubated for 24 h immersed in 5 ml of isotope solution containing 100 μCi/ml (specific activities of the batches varied from 2–2.7 Ci/mmol). The treatment was carried out in darkness for the dark-grown material and in the light for the light-grown material. The absorption of the radioactivity was not complete and the material was washed several times with the buffer at the end of the incubation.

For isolation of the chlorophyll-protein complexes, protonemata harvested from five Erlenmeyer flasks were ground in a glass homogenizer in an ice bath for 5 min. The medium was 0.05 *M* Tris-HCl, pH 8.0, containing 2 m*M* $MgCl_2$, and the isolation was performed by the method used by Alberte *et al.* (1972). The dark-grown material was handled in green safelight throughout. Finally the green pellet was homogenized for 5 min in an ice bath with sufficient 1% SDS to yield a 10:1 ratio of SDS:chlorophyll.

The SDS-extracted lamellar components were resolved by SDS polyacrylamide-gel electrophoresis, using the method described by Thornber (1970). The electrophoresis was carried out at 13 ± 1°C for 40 min. Densitometer tracings were obtained with a densitometer device attached to the Perkin Elmer 402 spectrophotometer. Unstained gels were scanned at 675 nm (absorption maximum of CP I in the gels) and the gels stained with amido schwarz at 620 nm.

The relative proportions of the various chlorophyll-containing bands were estimated by calculating the areas



Figures1–5. *Protonema of Ceratodon purpureus*. (1) Protonema grown for two weeks in rhythmic light, ×920. (2) Protonema grown for two weeks in light and then one month in darkness. Besides green cells (arrow) there are many empty cells (asterisk) in these cultures, ×920. (3) Chloroplast of prononema grown in light for two weeks, ×23,000. (4) Chloroplast of dark-grown cell, arrows indicate grana, O = plastoglobuli, ×23,000. (5) Portion of chloroplast of dark-grown cell with large plastoglobuli, s = starch, ×40,000.

under each recorder peak, by a height times width at half height method.

The gels were frozen rapidly at −25°C and immediately sliced by hand to discs of approximately 1 mm thickness. To measure the radioactivity of the gels, slices were placed in glass liquid scintillation vials, 0.1 ml of 30% H_2O_2 was added and the tightly capped vials were kept overnight at 50°C. After the vials had been cooled, 5 ml of Aquasol^R (NEN New England Nuclear) scintillation fluid was added to each. The radioactivity was counted on a Wallac Decem-NTL^{314} apparatus.

For electron microscopy, the material was fixed in glutaraldehyde-osmium tetroxide by methods described earlier (Valanne 1971). The prefixation of dark-grown material was always carried out in darkness. The grids were stained with Reynolds' lead citrate and examined with a Siemens Elmiskop I or AEI EM6B electron microscope.

Results

Compared with those of light-grown protonemata (Figure 1), the chloroplasts of dark-grown material are more rounded and have a more central location in the cells (Figure 2). A great part of the cells have lost their chloroplast in the darkness.

The ultrastructure of protonemata grown two weeks in the light shows chloroplasts with numerous bands of small grana consisting of 2–4 thylakoids (Figure 3). Stroma thylakoids are rare. After one month in darkness, the size of the grana has markedly increased (Figure 4). The grana can have deeply indented margins (Figure 5). The chloroplasts had mostly attained the same lamellar structure as that observed earlier in ungerminated swollen spores sown directly in the dark (Valanne 1971), but the number and size of the plastoglobuli were greater. There were also some starch grains in the chloroplasts in spite of the long incubation in the darkness.

The chlorophyll content of the protonema decreased in darkness in these liquid cultures (Table 1). On the other hand, a decrease in the chlorophyll content could also be seen in the material of the same age (6 weeks) grown throughout in the light. There was no significant difference between the Chl a:Chl b ratios at the beginning of the dark period (after two weeks in light) and after one month in darkness; but the treatment of dark-grown material with

Table 1. *The chlorophyll content of protonemata grown in light or in the dark* (two weeks in light + one month in dark).

Treatment	Chl $a+b$ mg/g dry weight	Chl a/b
Light, 2 weeks	5.16 ± 0.26	2.17 ± 0.17
Light, 6 weeks	1.95 ± 0.09	2.43 ± 0.11
Dark	1.09 ± 0.10	2.01 ± 0.14
Dark + ALA	1.50 ± 0.10	2.53 ± 0.07

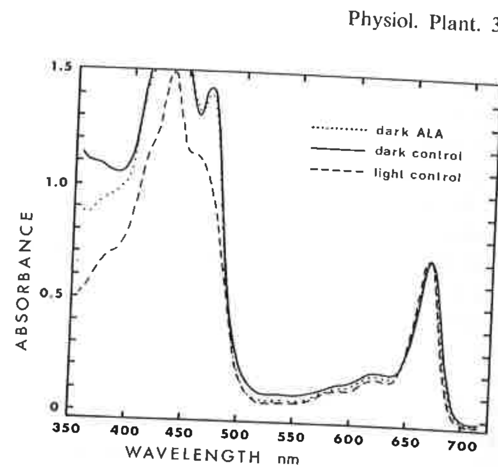


Figure 6. *Absorption spectra of 80% acetone extracts of Ceratodon protonemata grown two weeks in the light and after one month of incubation in darkness.*

ALA always increased the Chl a:Chl b ratio. It seems that Chl a was principally synthetized during the 24-h incubation in solution containing ALA.

The absorption spectra of chlorophyll extracted in acetone were rather similar for the light- and dark-grown protonemata except that higher absorptions were seen in dark-grown material in the blue region (Figure 6). Differences on the blue side can probably be due to differences in the carotenoid contents between light grown and dark-grown material.

The electrophoresis of SDS extracts of the photosynthetic membranes (Figure 7) showed the pattern known from higher plants (Brown *et al.* 1974). Although the densitometer tracings (Figure 8) of the gels revealed peaks better separated from each other in the light-grown material, there were no significant differences between the estimated areas of pigmented bands in the two materials (Table 2).

When the protonema was fed with (^{3}H) ALA, the labelling of the pigmented bands depended on the light conditions during the incubation period (Figure 9). In light the rate of incorporation was much faster and the highest radioactivities were

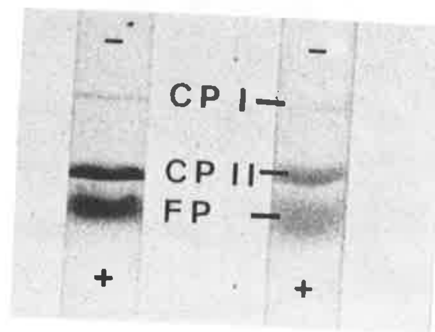


Figure 7. *Polyacrylamide-gel electrophoresis of SDS extracts of protonemata grown two weeks in light* (left) *and protonemata grown two weeks in light and then one month in darkness* (right). The gels are not stained.

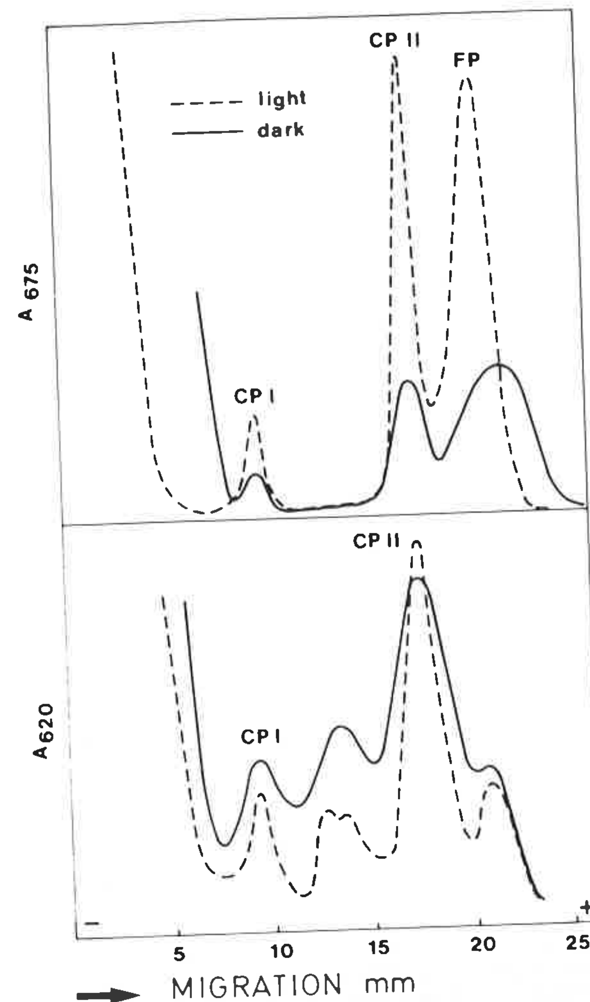


Figure 8. *Densitometer tracings of chlorophyll-protein complexes.* Above unstained gels scanned at 675 nm, below gels stained with amido schwarz and scanned at 620 nm.

found in CP II and FP, the distribution of activity matching the densitometer tracings of unstained gels. In darkness, CP I had rather high radioactivity compared with the small percentage of chlorophyll in this zone. Densitometer tracings of gels stained with amido schwarz revealed more protein in CP I in darkness than in the light. In the dark there was only low incorporation of (^{3}H) ALA in CP II, whereas higher activities were found in FP.

Table 2. *The percentages of chlorophyll associated with each chlorophyll-containing component.*

Treatment	CP I	CP II	FP
Light	6.2 ± 0.4	33.1 ± 0.9	60.7 ± 0.8
Dark	5.8 ± 0.4	32.2 ± 0.3	62.0 ± 0.5

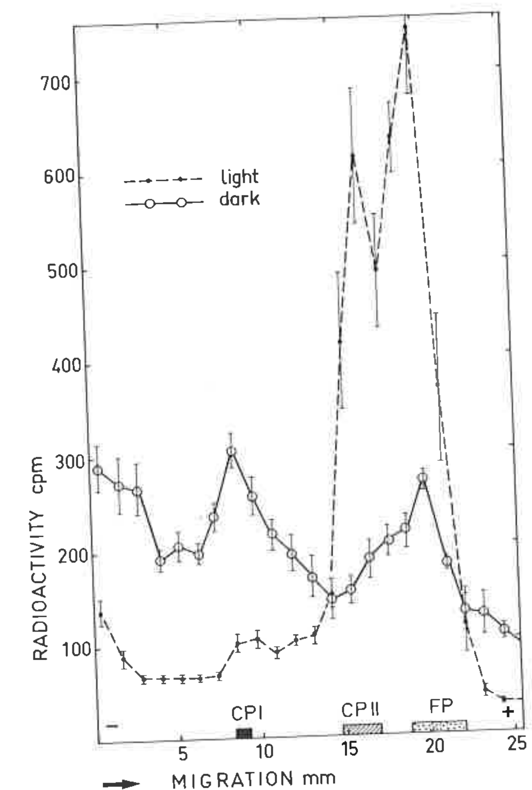


Figure 9. *The distribution of radioactivity in gels run with light grown material incubated in (^{3}H) ALA in the light and dark-grown material incubated in (^{3}H) ALA in the dark.*

Discussion

In the present investigation the percentages of chlorophyll associated with CP I and CP II are lower than those generally found in higher plants (Brown *et al.* 1974, Shiozawa *et al.* 1974, Thornber and Highkin 1974, Thornber 1975). The large amount of free pigment indicates, however, that the low percentages in the chlorophyll-protein complexes may be due to our methods. The temperature (13°C) used during electrophoresis was probably too high. On the other hand, in contrast to *Spinacia* and *Cucurbita*, the CP I of Ceratodon protonemata has been found to be very stable even at higher temperatures (unpublished results).

The highly stacked lamellae in the dark-grown protonemata led us to expect that only CP II would be synthetized in the dark. The results show that this was not the case; the dark-grown material incorporated most of the (^{3}H) ALA in CP I. There are reports that more protein complex I than complex II is synthetized in darkness in higher plants, too (Rémy *et al.* 1972, Argyroudi-Akoyunoglou and Akoyunoglou 1973). In higher plants during greening in flash light, chlorophyll is added to pre-existing protein in etioplasts (Hiller *et al.* 1973), whereas in dark-grown mosses the chlorophyll is synthetized and bound directly to CP I.

The rather high radioactivity found in the free pigment zone of dark-grown material, might partly be due to chlorophyll lost from CP II (*see* Genge *et al.* 1974); but the lack of radioactivity in CP II was not seen in the light-grown material. Nor does it seem likely that prolongation of dark incubation after treatment with ALA would change the results since the distribution of chlorophyll between CP I and CP II was so different in dark-grown and in light-grown material. Moreover, it has been observed that chlorophylls once bound to one polypeptide do not migrate to another (Guignery *et al.* 1974. Henriques and Park 1974).

Many papers in the literature point to the conclusion that lack of stroma lamellae, such as found here in the dark-grown protonemata, indicates non-functioning chloroplasts. Chloroplasts with only grana lamellae are found in overwintering organs (Vintéjoux 1973), roots (Wroblewski 1973), phloem (Jupin *et al.* 1975), and chloroplast mutants (Susalla and Mahlberg 1975). No direct correspondence has been found between a high capacity for stacking and PS II activity in higher plants (Björkman 1973, Smith and Sjolund 1975). In mosses, however, Chevallier (1975) reports that thicker grana of dark-incubated spores of *Funaria* indicate very high photosynthetic activity. We have also obtained photosynthetic activity in Ceratodon grown one month in darkness (in preparation).

This work was supported by the Finnish National Research Council for Sciences. We express our thanks to Mrs. Ulla-Maija Suoranta for technical assistance in electron microscopy.

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