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RESTORATION OF A $P_{657-647}$ FORM FROM $P_{645-638}$ IN EXTRACTS OF ETIOLATED PRIMARY BEAN LEAVES

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SUMMARY

Measurements of absorption, fluorescence and fluorescence excitation spectra at liquid nitrogen temperature show that it is possible to restore in vitro a $P_{657-647}$ protochlorophyllide lipoprotein complex (PLC) starting from preparations which essentially contain the $P_{645-638}$ complex. The restoration is obtained by drying according to an experimental procedure which is described. Evidence for the attribution to the restored $P_{657-647}$ of a Soret absorption band at 460 nm is given.

INTRODUCTION

Etiolated primary bean leaves normally contain three forms of the PLC. We call them $P_{657-647}$, $P_{645-638}$ and $P_{633-628}$, the first subscript referring to the emission and the second to the red absorption wavelength. $P_{657-647}$ and $P_{645-638}$ are the photoactive forms of the leaf.

When an etiolated leaf is extracted in a buffer, the $P_{657-647}$ form which is predominant in the leaf disappears almost completely; it is transformed either into $P_{645-638}$ or into $P_{633-628}$ depending on the viscosity of the buffer [1]. Purified active preparations contain the $P_{645-638}$ form [2, 3].

In this paper, we show that a $P_{657-647}$ form may be restored from $P_{645-638}$ after extraction in vitro. We also show that a Soret band at 460 nm belongs to this $P_{657-647}$.

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Abbreviation: PLC, protochlorophyllide lipoprotein complex.

MATERIAL AND METHODS

Preparation of a fresh PLC precipitate

The method used is derived from Schopfer and Siegelman [2] and Henningsen and Kahn [3].

(*1*) 25 g (fresh weight) of 15-day-old etiolated primary bean leaves were homogenized in an ultra-turrax (Janke and Kunkel type TP 18/2) at full speed for 3 min with 100 ml of extraction buffer (0.05 *M* Tricine, 0.05 *M* KOH, 0.002 *M* $MgSO_4$ 7 H_2O, 0.001 *M* EDTA, 0.06% (v/v) Triton X 100.45% (v/v) glycerol; pH 8.6) in which 6 g of polyvinyl-pyrrolidone (PVP40 Sigma Chemicals) had been previously dissolved. The homogenate was filtered through three layers of 28 nm mesh nylon to remove the coarse debris and centrifuged at 78 500 *g* in a superspeed 65 MSE ultracentrifuge at −2° for 1 h.

(*2*) A 50% polyethylene glycol 6000 solution was added slowly to the supernatant of the first centrifugation while stirring to give a final concentration of 18%. The mixture was allowed to stand for 30 min and then centrifuged at 78 500 *g* in a superspeed MSE ultracentrifuge at −2° for 1 h. The precipitate obtained is named fresh PLC precipitate.

All these manipulations were made at +2° in the dark.

Drying the precipitate

The fresh PLC precipitate was divided into several small fractions (a few cubic millimeters).

The fractions were placed on a watch glass inside a dark box on anhydrous

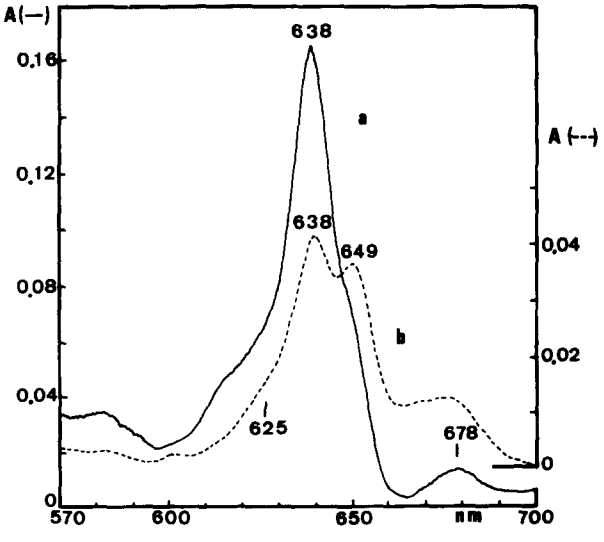


Fig. 1. Absorption spectrum at liquid nitrogen temperature of: (a) a fresh precipitate of the protochlorophyllide lipoprotein complex (fresh PLC); (b) a dried film of the precipitate of the protochlorophyllide lipoprotein complex (dry PLC).

calcium chloride; they were kept in the box at −15° for one week.

After that time, the fractions became pasty. Each fraction was then deposited on a little plastic sheet and squashed into a 100 μ thick film using a micro rolling-mill (in darkness at +2°). The films were placed in a dark box on anhydrous calcium chloride and kept at −15° (dry PLC films).

Fluorescence emission and excitation spectra at +77°K

The 77°K fluorescence spectra were registered with the apparatus described in Sironval et al. [4] equipped with an EMI 9558 B photomultiplier (S20 response). The method for obtaining the fluorescence excitation spectra at +77°K was described by Brouers et al. [5].

Absorption spectra at +77°K

The absorption spectra were recorded at 77°K using a Cary 17R spectrophotometer. The sample was placed in a transparent Dewar with two plane optical glass faces behind a 5 x 15 mm window cut in an adjustable copper plate, whose base was immersed in liquid nitrogen. In some cases a milk suspension in water was used as a blank.

RESULTS

Restoration of $P_{657-647}$ in vitro

The 77°K absorption of a fresh PLC precipitate showed a red maximum at 638 nm. In most cases the red band was a little enlarged around 650 nm (Fig. 1, curve a). After drying a film of this precipitate the 77°K absorption exhibited a second red maximum at 649 nm in addition to the original one at 638 nm (Fig. 1, curve b).

An absorption band around 625 nm was also generally suspected. After exposure to light (a 1 msec flash) at room temperature, the 638 and 649 nm absorption disappeared and a new absorption was seen around 676—678 nm (at 77° K).

Kinetics of $P_{657-647}$ restoration

Curve 6a, Fig. 2 shows the red absorption at +77° K of a small fraction of a fresh PLC precipitate which was kept for 6 days in darkness at −15° in normal wet air and was then squashed into a 100 μ thick film for registration of absorption.

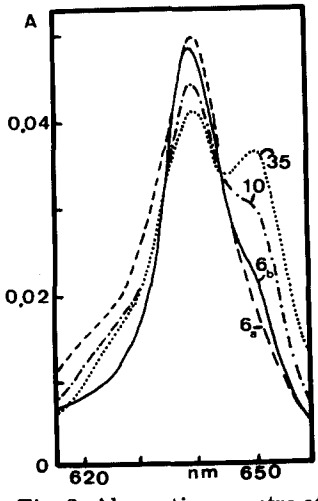
Curve 6b, Fig. 2 shows the red absorption at +77° K of a small fraction of the same precipitate which remained for 6 days in darkness at −15°, in dry air, *i.e.* on anhydrous calcium chloride and was squashed into a 100 μ thick PLC film for registration of the absorption spectrum. Drying caused an increase of the absorbance around 650 nm obviously linked to a decrease of the absorbance at 638 nm. Curves 10 and 35, Fig. 2, are spectra of films kept under dry air for 10 and 35 days respectively. The absorbance at 650 nm of the 35-day film was nearly equal to its absorbance at 638 nm, The 650 nm absorption did not increase further for times longer than 30 days.

Energy transfer to restored $P_{657-647}$

The 77° K fluorescence spectrum of a dry PLC film excited with 436 nm light is shown in curve E_{436}, Fig. 3B. This spectrum was similar to the corresponding absorption shown in curve b, Fig. 1. A 3 to 4 nm Stokes shift was observed for the three red transitions (*A* 625–*F* 628; *A* 638–*F* 642; *A* 649–*F* 653 nm). Assuming an equal fluorescence yield for both $P_{657-647}$ and $P_{645-638}$ PLC complexes, the efficiency of the energy transfer to restored $P_{657-647}$ in the dried PLC films is low. On the other hand, when excited with 436 nm light at +77° K the fresh PLC precipitate emitted more light around 655 nm than expected on the basis of its absorbance at 650 nm (compare curve a in Fig. 1 to curve E_{436} in Fig. 3A). This led us to suspect an energy transfer from $P_{645-638}$ towards the few $P_{657-647}$ molecules present in the fresh precipitate, in agreement with the results of Kahn et al. [6].

Fluorescence emission action spectra of restored $P_{657-647}$

Using dry PLC films, it was possible to distinguish in the blue the fluorescence excitation of $P_{645-638}$ from that of $P_{657-647}$. Fig. 4 shows that exciting



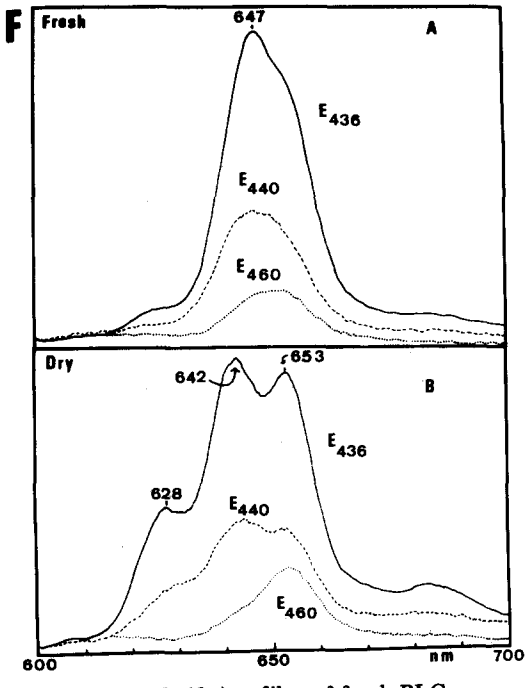


Fig. 2. Absorption spectra at liquid nitrogen temperature of: (6a), a film of fresh PLC precipitate after 6 days in normal wet air at −15°; (6b), 6 days dried PLC film, after 6 days in dried air at −15°; (10), 10 days dried PLC film; (35), 35 days dried PLC film.

Fig. 3. Fluorescence spectrum at liquid nitrogen temperature of fresh (A) and dried (B) preparations of the PLC. Subscripts under E refer to excitation wavelengths. The spectra are not corrected for the photomultiplier response.

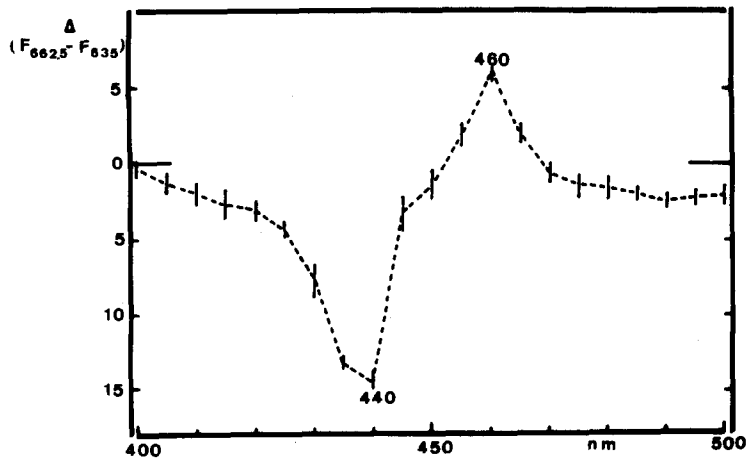


Fig. 4. Difference fluorescence emission action spectrum of a dry PLC film: △, action spectrum of $P_{657-647}$ obtained by looking at the fluorescence at 662.5 nm — action spectrum of $P_{645-639}$ obtained by looking at the fluorescence at 635 nm. In order to avoid as far as possible the contribution from $P_{645-639}$, the fluorescence at 662.5 nm is taken as a measure of the fluorescence of $P_{657-647}$. The fluorescence at 635 nm is taken as a measure of the fluorescence of $P_{645-639}$.

a dry PLC film with light around 440 nm favoured the emission at 635 nm due to $P_{645-638}$. On the contrary exciting with light around 460 nm favoured the emission at 662.5 nm essentially due to $P_{657-647}$.

The fluorescence of a fresh PLC preparation was similar for both excitations, with 440 or with 460 nm light (curves E_{440} and E_{460}, Fig. 3A) although the 460 nm excitation somewhat increased the emission of the 650—657 nm band. Excitation of a dry PLC film either with 440, or with 436 nm light gave emission spectra of identical shapes, showing both the 640—645 and the 650—657 main emission bands (curves E_{436} and E_{440}, Fig. 3B), but excitation with 460 nm light clearly selected the emission of the 650—657 nm band (curve E_{460}, Fig. 3B).

Blue absorption band of restored $P_{657-647}$

Absorption of dry PLC films at 77° K showed a shoulder around 460 nm which was lacking in the fresh PLC precipitate.

Fig. 5 is a difference spectrum obtained by recording at 77° K the absorption of a dry PLC preparation against a reference containing the same preparation previously kept at +45° in the dark for 20 min.

Positive absorption differences were observed in the red at 639 and 649 nm and in the blue at 460 nm. Negative differences were observed at 628 and 438 nm. The changes are due to a decrease in the reference of the amount of the $P_{645-638}$ and $P_{657-647}$ correlated with an increase in the amount of the $P_{633-628}$, as a result of thermal denaturation of the former complexes [1].

Absorption difference spectra of the same kind were obtained using intact and denaturated etiolated bean leaves instead of the dry PLC preparations.

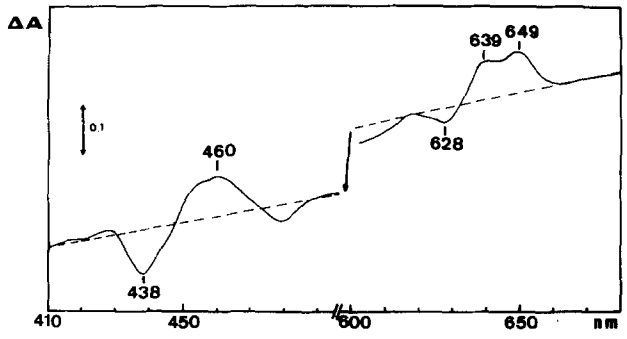


Fig. 5. Difference absorption spectrum at liquid nitrogen temperature: ΔA, absorbance of a dry PLC film — absorbance of this film after denaturation. Denaturation was obtained by warming in the dark at +45° for 20 min. The dashed line corresponds to an approximated base line.

DISCUSSION AND CONCLUSIONS

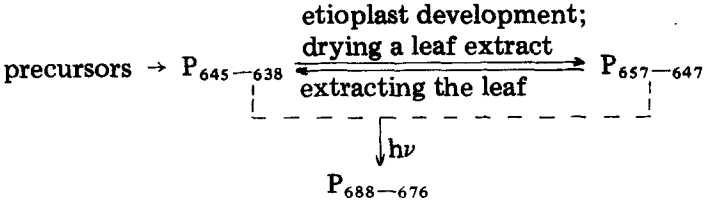
The Soret band of restored $P_{657-647}$ in dry PLC films differs from that of $P_{645-638}$ in fresh PLC precipitate; it is located at 460 nm (Figs. 3, 4 and 5).

A Soret band around 470 nm together with a red absorption around 650—655 nm characterizes aggregates of pure protochlorophyllide formed in dried non-polar solvents [7, 8]. In such aggregates the major red and blue absorption maxima are at 650 and 465 nm respectively. Similar forms were obtained in a solid film of vinylprotochlorophyll [9]. The similarities between the properties of these aggregates and those of the restored $P_{657-647}$ in dry PLC films are striking.

In the etiolated leaf $P_{657-647}$ appears to be the major PLC form. When extracting the leaf it is transformed into $P_{645-638}$. We now find that a $P_{657-647}$ pigment form is reconstituted at the expense of the $P_{645-638}$ when drying the extracts. This is to be compared with the observation that in the bean leaf $P_{657-647}$ appears later and faster than $P_{645-638}$ during the development of etioplasts in darkness [10].

Kahn and Nielsen [11] think that in the leaf the 635 (638 in this work) and 650 nm (647 in this work) absorption bands belong either to a single species of protein bound protochlorophyllide molecules or to two species in dynamic equilibrium, a possibility that they cannot rule out.

The facts presented here and the observation of Klein and Schiff [10] do not support the first term of this alternative. They are in favour of the occurrence of an equilibrium between two distinct species in vivo and in vitro:



The protein pigment links of the in vivo $P_{657-647}$ are possibly preserved for a major part in the in vitro restored $P_{657-647}$ as suggested by the phototransformability of the latter. Nevertheless this does not imply the reconstitution of those links which are involved in the maintenance of energy transfer units as shown by comparing Fig. 1 curve b, to Fig. 3B, curve E_{436}. It remains to be seen in what extent the in vivo $P_{657-647}$ PLC is identical to the restored $P_{657-647}$ in dry PLC films.

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