PROTOCHLOROPHYLL AND PROTOCHLOROPHYLLIDE AS PRECURSORS FOR CHLOROPHYLL SYNTHESIS IN VITRO

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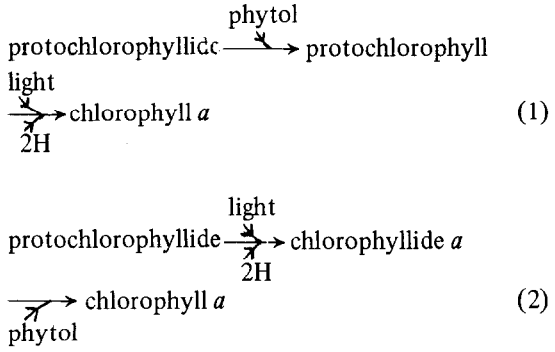
1. Introduction

The final stages of chlorophyll *a* biosynthesis in angiosperms may proceed along one of two alternative paths:



The existence of protochlorophyll (PChl) in etiolated plants would favour the first route but the bulk of the experimental data supports the second alternative. Thus, the phototransformable pigment in the active PChl (ide)-holochrome is PChlide rather than PChl [1]; the photoconvertibility of PChl(ide) in etiolated leaves decreases on ageing-a condition which leads to a higher PChl/PChlide ratio [2], again, photoconversion in etiolated barley leaves is accompanied by a decrease in the PChlide level whereas no such change in the PChl level occurs [3]. However, claims continue to be made for the photoconvertibility of PChl. Thus, it has lately been reported in a carotenoid-less mutant of maize [4] and more recently in cucumber cotyledons by Rebeiz [5]. A cell free system capable of substrate dependent chlorophyll formation could serve to resolve this controversy. The preparation of such a system from etiolated barley shoots is reported here. Experiments are described showing the photoconversion of added PChlide to chlorophyll(ide) in this preparation whereas no such reaction occurs with added PChl.

Abbreviations: PChl, protochlorophyll; PChlide, protochlorophyllide; PChl(ide), protochlorophyll and/or protochlorophyllide; Chl, chlorophyll *a*; Chlide, chlorophyllide *a*; Chl(ide), chlorophyll *a* and/or chlorophyllide *a*; P_{630}, P_{650}, forms of protochlorophyll(ide) occurring in vivo absorbing light maximally at the wavelengths indicated in nm.

2. Materials and methods

2.1. *Preparation of etioplasts and membrane fractions.*

Etioplasts were isolated from seven day old dark grown barley shoots by briefly homogenising in a mediur composed of 0.5 M sucrose, 0.2% (w/v) bovine serum albumin, 5 mM cysteine, 1 mM $MgCl_2$, 1 mM EDTA, 20 mM TES [*N*-tris-(hydroxymethyl)-2-amino-ethanesulphonic acid] and 10 mM HEPES [*N*-2-hydroxy ethyl piperazine *N'*-2-ethanesulphonic acid] adjusted with KOH to pH 7.2. After filtering, ethioplasts were isolated from the homogenate by differential centrifugation as previously described [6,7] and were resuspended in buffer identical to the isolation medium except that cysteine was omitted and 1.5 mM ATP was added [8]. All the operations were carried out under a dim green safe-light and the temperature during the whole procedure maintained as close to 0°C as possible.

Membrane preparations, deactivated membranes, which are inactive in photoconverting PChl(ide) were prepared from the etioplasts by water lysing as already described [6].

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2.2. *Preparation of substrates*

Purified samples of PChl and PChlide were isolated from lipid extracts of etiolated barley leaf tissue. Total lipids were extracted from the tissue by homogenisation in a mixture of acetone/0.1 N NH_4OH, 99/1(v/v), using an Ultra-Turrax homogeniser. The lipid was transferred into ether by adding an equal volume of peroxide-free ether followed by water addition [9]. The ether layer, after backwashing several times with 0.1% (w/v) $MgCO_3$ solution was dried over anhydrous Na_2SO_4 and finally taken to dryness to give an oily lipid preparation. PChl and PChl(ide) in the lipid extract were isolated separately by preparative (0.5 mm) thin layer chromatography using plates coated with adsorbent prepared by mixing 24 g of Kieselgur, 6 g of Kieselgel, 6 g of powdered analytical reagent grade $CaCO_3$, and 36 mg of $Ca(OH)_2$ suspended by shaking vigorously with 69 ml of 5 mM K ascorbate buffer, pH 7.1 to 7.3. The developing solvent was petroleum ether (b.p. 60–80°C): isopropyl alcohol: H_2O (100:5:0.25,v/v) and the separated metalloporphyrins were located on the developed chromatograms by the intense red fluorescence when viewed under long wavelength (360 nm) ultraviolet light. In this system PChl migrated with an Rf of 0.65 and could be eluted off the adsorbent into ether in a spectroscopically fairly pure state. The PChlide however barely moved off the origin during the chromatography (Rf 0.0–0.02) and was scraped off the developed plates as a band heavily contaminated with other coloured pigments. However, these contaminants could readily be eluted from the adsorbent with ether without extracting any of the PChlide. The latter was then eluted free of any spectroscopically identifiable contaminants by extracting the absorbent with methanol.

The purified PChl and PChlide were solubilised for enzymatic studies by evaporating, under nitrogen, a methanolic mixture of the pigment and sodium cholate and finally resuspending the dried residue in 50 mM HEPES pH 7.4 to give a cholate concentration of 0.1% (w/v). Solubilisation in the case of the PChl was facilitated by a brief sonication.

2.3. *Reconstitution of the water lysed membranes and phototransformation*

Incubation of the water lysed membrane preparations in the presence of various substrates was carried out as previously described [6] in a shaking water bath except that the temperature of the incubation was raised to 18°C. Flash illumination was also as before except that the dark time of 15 min between flashes used previously was here reduced to 80 sec. This meant that the incubation period of 10 hr used before could be reduced to 1–2 hr with essentially the same result in terms of chlorophyll(ide) production. At the end of the incubation period the samples were left in darkness at room temperature for about 1 hr to ensure completion of any spectroscopic shifts undergone by the Chl(ide). The samples were then subjected to spectral analysis over the wavelength range 400–725 nm using a sensitive split beam spectrophotometer as previously described [6]. Chlorophyll levels were estimated from the resulting spectra as before [6].

2.4. *Protein assays*

The protein content of the etioplasts and membrane preparations was estimated by the Folin method [10] using bovine serum albumin as standard.

2.5. *Chemicals and enzymes*

Glucose 6-phosphate, ATP and $NADP^+$ were purchased from Boehringer as also was the enzyme glucose 6-phosphate dehydrogenase (EC 1.1.1.37). Sodium cholate was a product of Sigma.

3. Results and discussion

Water lysing of etioplasts has already been shown to result in the conversion of phototransformable PChl(ide) (P_{650}) to inactive PChl(ide) (P_{630}) [6]. The NADPH dependent dark reformation of P_{650} from the inactive P_{630} and its phototransformation into CHl(ide) by an overnight (10hr) flash illumination (1 flash/15 min) has also been previously demonstrated in such 'deactivated' membranes [6]. The same results can be obtained from a considerably shortened incubation by increasing the flashing rate to 1 flash/80 sec (fig. 1). This system has the obvious advantage that Chl(ide) breakdown is negligible over this period. Fig. 1 demonstrates the NADPH dependency of the P_{630} to Chl(ide) phototransformation (compare 1a and 1b). The small amount of chlorophyll formed in the *minus* NADPH incubation (fig. 1a) results from an incomplete conversion of P_{650} to P_{630} on originally water lysing the etioplasts.

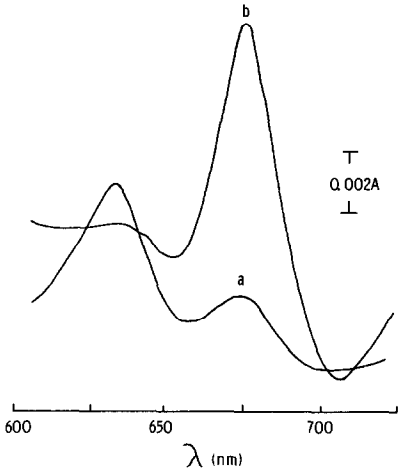

Fig.1. NADPH dependent flash formation of chllorophyll(ide) by membranes from barley etioplasts. Deactivated etioplast membranes were prepared as described in Materials and methods. These were incubated (0.20 mg protein/2.7 ml buffer) at 18°C and given a 1 msec Xenon flash every 80 sec after which spectra of the samples were recorded. Curve (a) incubation with no NADPH; (b) in the presence of an NADPH regenerating system – 0.5 units glucose 6-phosphate dehydrogenase, 5 mM glucose 6-phosphate and 0.5 mM $NADP^+$.

Fig. 2 shows the results of an attempt at getting deactivated etioplast membranes to utilise exogenously added PChl(ide) synthesis. Curve 2a gives the absorption spectrum of the membranes incubated under flash illumination for two hr in the presence of NADPH. The conversion of endogenous PChl(ide) to Chl(ide) absorbing at 673 nm is clearly seen. Addition of PChl, prior to incubation under otherwise similar conditions, failed to give a significant increase in the absorption at 673 nm (fig. 2b), even under conditions when the incubation period was increased to 10 hr (fig. 2c). Thus, this system fails to utilise added PChl for CHl(ide) formation.

When PChlide was added to deactivated etiplast membranes in the presence of NADPH, incubation under flash illumination on resulted in a greatly increased absorbancy at 673 nm (fig. 3b) compared with the absorbancy of a control sample not supplemented with PChlide (fig. 3a). This indicates the ability of this system to utilise added PChlide for Chl(ide) formation which is in marked contrast with the result obtained with added PChl. However, it should be noted that this conversion of added PChlide

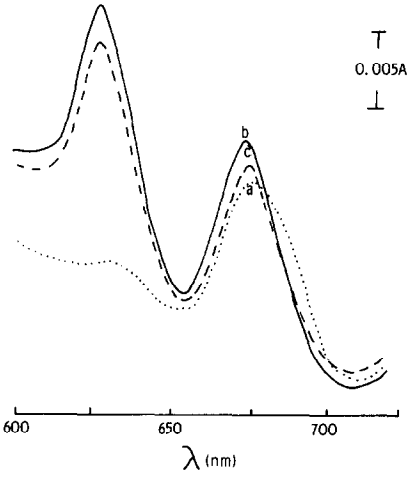

Fig.2. Chlorophyll(ide) synthesis by deactivated etioplast membranes supplemented with protochlorophyll. Deactivated etioplast membranes (0.27 mg protein/2.7 ml) were incubated as in fig. 1, (a) in the presence of an NADPH regenerating system, (b) as in (a) but with the addition of a cholate solubilised PChl solution; (c) as in (b) but with the incubation period extended from two to ten hr.

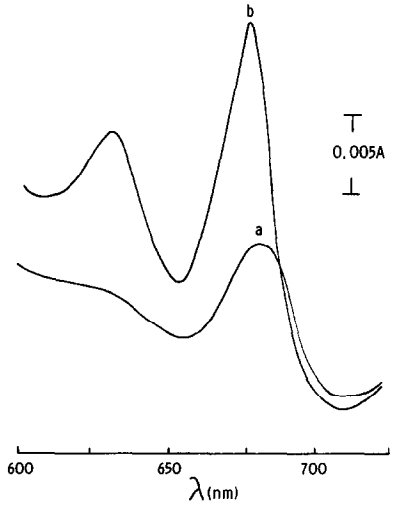

Fig.3. Chlorophyll(ide) synthesis by deactivated etioplasts membranes incubated with added protochlorophyllide. Curve (a) gives the spectrum of deactivated membranes (0.25 mg protein/2.75 ml) incubated as in fig.1. in the presence of an NADPH regenerating system. Curve (b) records the spectrum of a similarly incubated sample supplemented with cholate solubilised solution of PChlide.

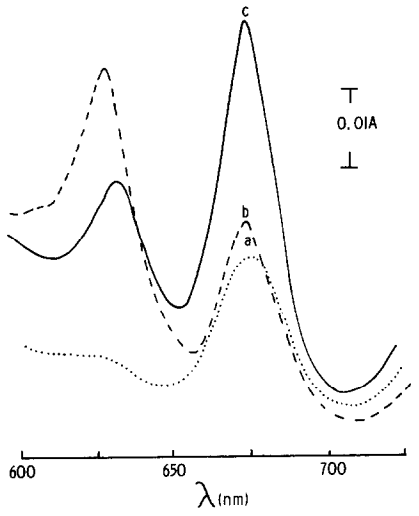

Fig.4. Chlorophyll(ide) synthesis from added substrates by deactivated 'green' etioplast membranes. Deactivated 'green' membranes (0.24 mg protein/2.7 ml) prepared from flashed etioplasts as described in the text were incubated as in fig.1. Curve (a) records the spectrum of the sample incubated with an NADPH regenerating system only. Curve (b) sample incubated as in (a) *plus* added PChl. Curve (c) sample incubated as in (a) *plus* added PChlide.

is not complete and unconverted PChlide absorbing at 632 nm can be seen in the sample (fig. 3b) at the end of the incubation. As before, prolonging the incubation time beyond two hr failed to increase the extent of Chl(ide) synthesis implying that some other component(s) have now become limiting in the system.

Our interpretation of the origin of the Chl(ide) formed during the incubations illustrated in figs. 2 and 3 may be oversimplified since the effect of the added substrates on the endogenous PChl(ide) and its phototransformation was not considered. Membranes devoid of endogenous PChl(ide) were therefore prepared from etioplasts that have been flashed once every 80 secs for 20 min. This procedure converts all the endogenous PChl(ide) to Chl(ide). The absorption spectrum of the resulting 'green' deactivated membranes is shown in fig. 4a which demonstrates the absence of PChl(ide) in the sample, shown by the lack of specific absorption at 630–632 nm. Subjecting this sample to our usual incubation (1 flash/80 sec for 2 hr) in the presence of NADPH failed to produce any additional Chl(ide) confirming the absence of endogenous PChl(ide). Addition of PChl to the sample prior to such an incubation once more failed to increase the chlorophyll(ide) level (fig. 4b) whereas PChlide addition resulted in a greatly increased absorption at 673 nm at the end of the incubation (fig. 4c) indicating Chl(ide) synthesis once more from this added substrate.

Thus, in conclusion, these results describe unequivocally for the first time the cell free synthesis of Chl(ide) from an added specific precursor. Further, the report establishes PChlide but not PChl as the substrate for phototransformation in this system. The suggestion has been made that the failure of PChl to be phototransformed is due to steric hindrance of the actual photoreduction from the phytyl residue [10]. However, we see the inability of PChl to act as a precursor of Chl(ide) as resulting from interference by the bulky phytyl group of the dark formation of a membrane-located, photochemically active, P_{650} complex. Thus, we have shown that PChlide added to 'green' deactivated membranes in the presence of NADPH forms phototransformable P_{650} whereas no such complex is formed from added PChl (Griffiths 1974, in preparation).

Since PChl cannot act as a Chl(ide) precursor its role in the metabolism of etiolated plants is in doubt. It would be tempting to suggest that PChl may be a passive storage form of PChlide, increasing the lipophilic nature of the latter, making it more readily accomodated in such lipid storage structures as osmiophilic globules. Against this view however has to be mentioned the claims that have been made of the existence of separate independent routes for the formation of PChl and PChlide [11,12].

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