MAGNESIUM 2,4-DIVINYLPHAEOPORPHYRIN a_5 AS A SUBSTRATE FOR CHLOROPHYLL BIOSYNTHESIS IN VITRO

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1. Introduction

Progress in determining the sequence of intermediates in the biosynthesis of plant chlorophylls has been slow, largely because of the difficulty in isolating, identifying and synthesising the magnesium tetrapyrroles that may be involved and because the enzymes involved appear particularly unstable in vitro. In consequence there is great uncertainty about the pathway leading from magnesium protoporphyrin IX monomethyl ester to protochlorophyllide (magnesium 2-vinyl, 4-ethyl phaeoporphyrin a_5 monomethyl ester, formula II). Protochlorophyllide accumulates in the etioplasts of the leaves of dark-grown plants and is rapidly transformed to chlorophyllide by illumination; magnesium protoporphyrin monomethyl ester accumulates in chlorophyll-less mutants of *Chlorella* and barley [1] and its enzymic formation may be assayed in vitro, using preparations derived from photosynthetic bacteria [2] or chloroplasts [3]. Magnesium 2,4-divinylphaeoporphyrin a_5 monomethyl ester (MgDVP) (formula I) has been postulated as an intermediate between these two compounds [4]: it accumulates in photosynthetic bacteria where chlorophyll synthesis has been blocked by inhibitors [5] or by mutation [6,7] and has been found in inner seed coats of *Cucurbitaceae* [7–9] and in a mutant of *Chlorella* [10]. In this communication we present evidence that MgDVP is converted, in vitro, to chlorophyll(ide) *a* by an etioplast preparation from dark-grown barley. This greatly strengthens its claim to a place in the normal biosynthetic sequence leading to the production of chlorophyll *a*.

2. Materials and methods

2.1. *Isolation of etioplasts*

Seven-day-old dark grown barley shoots were briefly homogenised (10 sec) using an Ato-Mix homogeniser in a medium 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-aminoethane sulphonic acid) and 10 mM HEPES (*N*-2-hydroxyethylpiperazine *N'*-2-ethane sulphonic acid) adjusted with KOH to pH 7.2. Etioplasts were isolated from the filtered homogenate and were resuspended in cysteine-free isolation medium supplemented with 1.5 mM ATP as previously described [11,12].

Membrane preparations, prolamellar body membranes, were prepared from etioplasts by water lysing followed by centrifugation as before [12]. 'Dark' prolamellar bodies were derived from unilluminated etioplasts. 'Green' prolamellar bodies were prepared from etioplasts that had been given an initial saturating illumination for 90 sec from a 100 W tungsten lamp followed by a flash illumination with 1 msec xenon flashes from a photographic lamp (Mecablitz) given once every 80 sec for 30 min. During the flash illumination an NADPH regenerating system –0.5 mM NADPH, 5 mM glucose 6-phosphate and 0.5 units glucose 6-phosphate dehydrogenase – was included, and the samples were gently shaken at 0°C.

2.2. *Preparation of substrates*

Protochlorophyllide was isolated by thin-layer

chromatography (TLC) from a total lipid extract of etiolated barley leaf tissue by methods already described [13]. An additional thin-layer chromatographic step was included, using cellulose as adsorbent with dried petroleum ether (B.P. 60–80°C): acetone: *n*-propanol (90:10:0.45, v/v) as developing solvent (Dr P. A. Castelfranco, personnel communication). In this system protochlorophyllide migrated with an R*f* of 0.25. The spectral characteristics of the eluted sample in ether were identical with previously published data for protochlorophyllide [8].

MgDVP was isolated from a *Rhodopseudomonas spheroides* mutant. The mutant, V-3, which is blocked in bacteriochlorophyll synthesis and which accumulates and excretes MgDVP, was a gift from Dr Venetia A. Saunders. It was prepared by treating cells with *N*-methyl-*N*-nitroso-*N'*-nitroguanidine. Cells were grown in 8 l batches with gentle aeration in the medium of Sistrom [14]. At the end of the log phase of growth, cells were removed by centrifugation and the green supernatant extracted with diethyl ether. The extract, after several washings with water, was dried and concentrated. MgDVP in the extract was purified by TLC on cellulose plates developed with methanol, dichloromethane and water (100:85:25, v/v) [15] and its absorption spectrum recorded in ether.

The purified protochlorophyllide and MgDVP were solubilised for enzymatic studies using sodium cholate, as previously described [13].

2.3. *Reconstitution of light dependent chlorophyllide formation in prolamellar body membranes by added substrates*

Incubation of the prolamellar body membranes in the presence of various substrates was carried out in a shaking water bath maintained at 18°C. Chlorophyll formation during the 2 hr incubation was activated by 1 msec xenon flashes given once every 80 sec. Spectra of the samples were then recorded over the wavelength range 400–725 nm using a sensitive split-beam spectrophotometer as previously described [11].

2.4. *Protein assays*

These were carried out by the Folin method [16] 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). The TLC adsorbents used were, MN-Kieselgel and MN-Kieselgur G from Machray, Nagel and Co. and cellulose powder (CC 41) from Whatman.

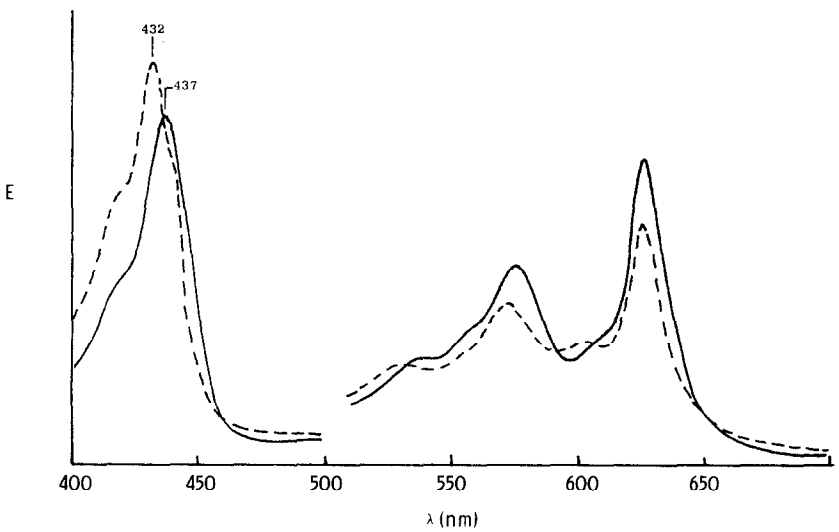


Fig.1. Absorption spectra of purified protochlorophyllide and MgDVP. Samples of protochlorophyllide and MgDVP isolated and purified as described in the text were dissolved in anhydrous diethyl ether and their absorption spectra recorded using an Unicam S.P. 800 recording spectrophotometer. (– – – –); protochlorophyllide from barley. (———); MgDVP form *Rsp. spheroides* mutant V-3.

3. Results and discussion

The pigment excreted by mutant V-3 was identified as MgDVP from its absorption spectrum, band ratios, partition properties and characteristic changes in absorption spectra following acid treatment to convert the magnesium complex to a phaeporphyrin [4]. Because of the presence of an additional vinyl group conjugated to the ring the absorption maxima in ether are at slightly longer wavelengths than those of protochlorophyllide (see fig.1). The medium from which V-3 was harvested proved to be a rich source of easily purified MgDVP.

When 'dark' prolamellar bodies were incubated in a flashing regime some chlorophyllide, absorbing at around 680 nm, was formed form the trace of endogenous protochlorophyllide that remains in these lysed and washed membranes (fig.2). When NADPH was present in the incubation mixture the yield of chlorophyllide was several times greater. This is due to the activity of NADPH in the reaction that generates protochlorophyllide absorbing at 650 nm, from non-convertible protochlorophyllide were added to the incubation containing prolamellar bodies, the yield of chlorophyllide was even further increased, since the added protochlorophyllide was used in the regeneration of the photoconvertible complex [13].

A similar series of experiments were performed to demonstrate the utilisation of MgDVP for chlorophyllide synthesis (fig.3). 'Greened' prolamellar bodies were incubated in a flashing light with either NADPH or MgDVP. In neither case was there any further synthesis of chlorophyllide over and above

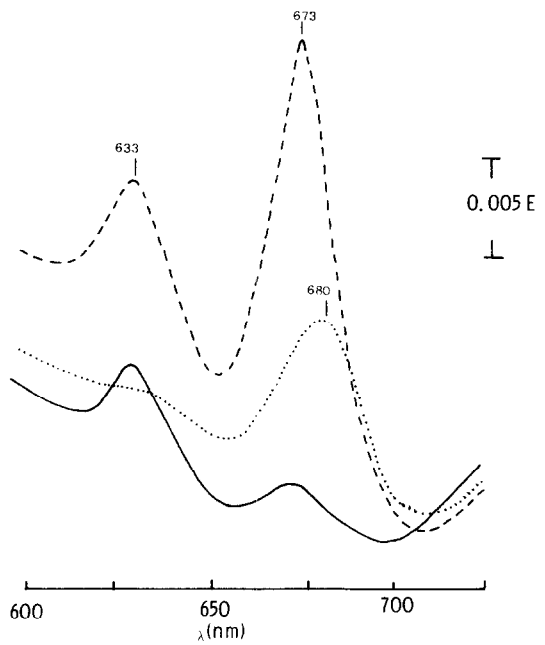


Fig.2. Illumination induced chlorophyllide formation from added protochlorophyllide and NADPH by membrane preparations from barley etioplasts. 'Dark' prolamellar body membranes isolated from non-illuminated barley etioplasts (200 μg protein in 2.75 ml) were incubated in the presence of different compounds at 18°C whilst illuminated with a 1 msec xenon flash every 80 sec for 2 hr. At the end of the incubation absorption spectra of the samples were recorded on a sensitive split-beam spectrophotometer. (———); incubation with no NADPH regenerating system. (.....); in the presence of an NADPH regenerating system. (– – – –); in the presence of an NADPH regenerating system and added protochlorophyllide.

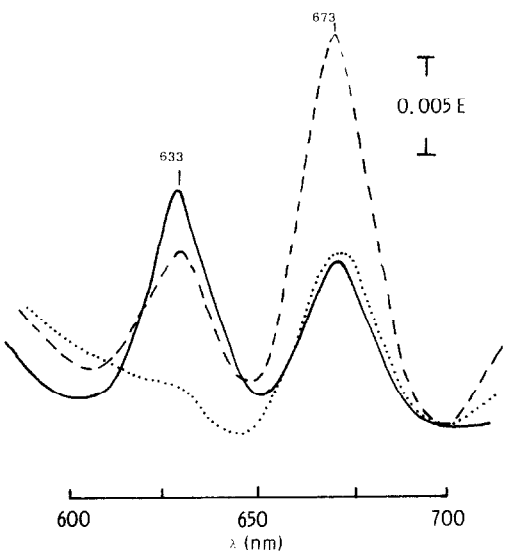
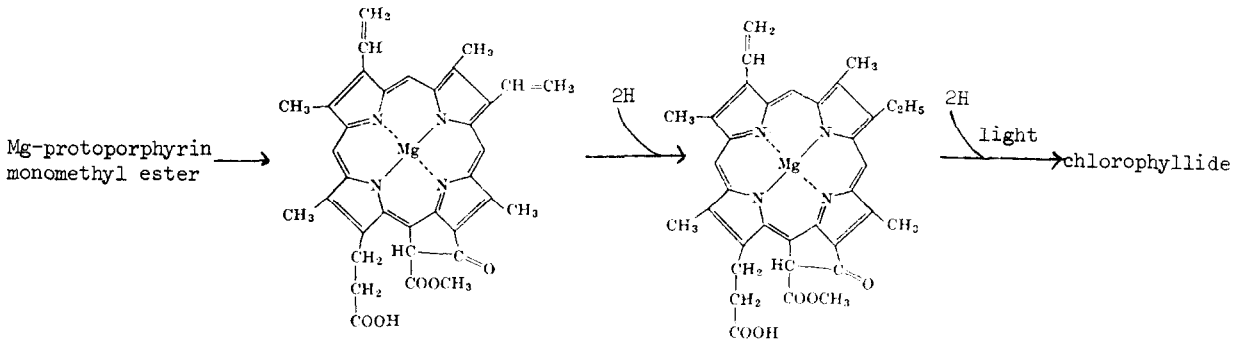


Fig.3. Chlorophyllide formation form added MgDVP and NADPH by membrane preparations from barley etioplasts. 'Green' prolamellar body membranes were isolated as described in fig.2 except that the etioplasts used were first flashed at 1 flash/80 sec for 30 min to deplete them of their endogenous photoconvertible protochlorophyllide by its conversion to chlorophyllide. Incubations (430 μg protein in 2.75 ml) were carried out at 18°C whilst illuminated with 1 msec xenon flashes every 80 sec for 2 hr. Spectra of the samples were then recorded as in fig.2. (.....); incubation with added NADPH regenerating system. (———); incubation with added MgDVP. (– – – –); incubation with added MgDVP and NADPH regenerating system.



I. Mg-2,4-divinylphaeoporphyrin a_5 monomethylester

II. Protochlorophyllide

that already present. When both NADPH and MgDVP were added to the prolamellar bodies there was considerable synthesis of chlorophyllide (see the increased absorption at 673 nm in fig.3). That is, in the presence of NADPH and a flashing light, etioplast membranes catalyse the conversion of MgDVP to chlorophyllide. Since NADPH is necessary for the conversion of protochlorophyllide itself (see above) it is not possible to decide from these experiments if the reduction of MgDVP is itself an NADPH requiring reaction. However these experiments provide substantial support for a sequence of intermediates in chlorophyllide synthesis as shown in Scheme 1. Reduction of the 4-vinyl group is postulated to be the last reaction in the biosynthesis of protochlorophyllide.

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