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Formation of the Isocyclic Ring of Chlorophyll by Isolated *Clamydomonas reinhardtii* Chloroplasts

David Bollivar
Illinois Wesleyan University, dbolliva@iwu.edu

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Formation of the Isocyclic Ring of Chlorophyll by Isolated *Chlamydomonas reinhardtii* Chloroplasts

David W. Bollivar and Samuel I. Beale

Abstract

Chlamydomonas reinhardtii chloroplasts catalyzed two sequential steps of Chl biosynthesis, *S*-adenosyl-L-methionine:Mg-protoporphyrin IX methyltransferase and Mg-protoporphyrin IX monomethyl ester oxidative cyclase. A double mutant strain of *C. reinhardtii* was constructed which has a cell wall deficiency and is unable to form chlorophyll in the dark. Dark-grown cells were disrupted with a BioNeb nebulizer under conditions which lysed the plasma membrane but not the chloroplast envelope. Chloroplasts were purified by Percoll density gradient centrifugation. The purified chloroplasts were used to define components required for the biosynthesis of Mg-2,4-divinylpheoporphyrin a_5 (divinyl protochlorophyllide) from Mg-protoporphyrin IX. Product formation requires the addition of Mg-protoporphyrin IX, the substrate for *S*-adenosyl-L-methionine:Mg-protoporphyrin IX methyltransferase which produces Mg-protoporphyrin IX monomethyl ester. The Mg-protoporphyrin IX monomethyl ester that is generated in situ is the substrate for Mg-protoporphyrin IX monomethyl ester oxidative cyclase. The reaction product was identified as Mg-2,4-divinylpheoporphyrin a_5 (divinyl protochlorophyllide) by excitation and emission spectrofluorometry and HPLC on ion-paired reverse-phase and polyethylene columns. Mg-2,4-divinylpheoporphyrin a_5 formation by the coupled enzyme system required O_2 and was stimulated by the addition of $NADP^+$, an NADPH regenerating system, and *S*-adenosyl-L-methionine. Product was formed at a relatively steady rate for at least 60 min.

Abbreviations: MgDVP – Mg-2,4-divinylpheoporphyrin a_5 (divinyl protochlorophyllide); SAM – *S*-adenosyl-L-methionine

Introduction

The formation of Chl *a* involves many complex biosynthetic reactions. The steps between Mg-protoporphyrin IX (the first committed Chl precursor) and MgDVP (also known as divinyl Pchlide) consist of Mg-protoporphyrin IX monomethyl ester formation, catalyzed by SAM:Mg-protoporphyrin IX methyltransferase, followed by MgDVP formation, a complex reaction catalyzed by Mg-protoporphyrin IX monomethyl ester oxidative cyclase (Fig. 1). The cyclase reaction has been proposed to involve at least two intermediates (Castelfranco et al. 1994). The first step involves the addition of an O_2-derived oxygen atom to form the 6-methyl-β-hydroxypropionate intermediate, which is then oxidized to the 6-methyl-β-ketopropionate. This is followed by oxidative ring closure and the formation of MgDVP. The evidence for these proposed intermediates was obtained from early studies with *Chlorella* mutants (Ellsworth and Aronoff 1968, 1969) as well as analysis of intermediates accumulated in incubations containing extract of cucumber cotyledon plastids (Wong et al. 1985). In angiosperm etioplasts, MgDVP accumulates, and a portion undergoes reduction of the 4-vinyl group to form Mg-2-vinyl,4-ethyl pheoporphyrin a_5 (also known as Pchlide or monovinyl Pchlide), which accumulates in the dark along with MgDVP. Upon exposure to light,

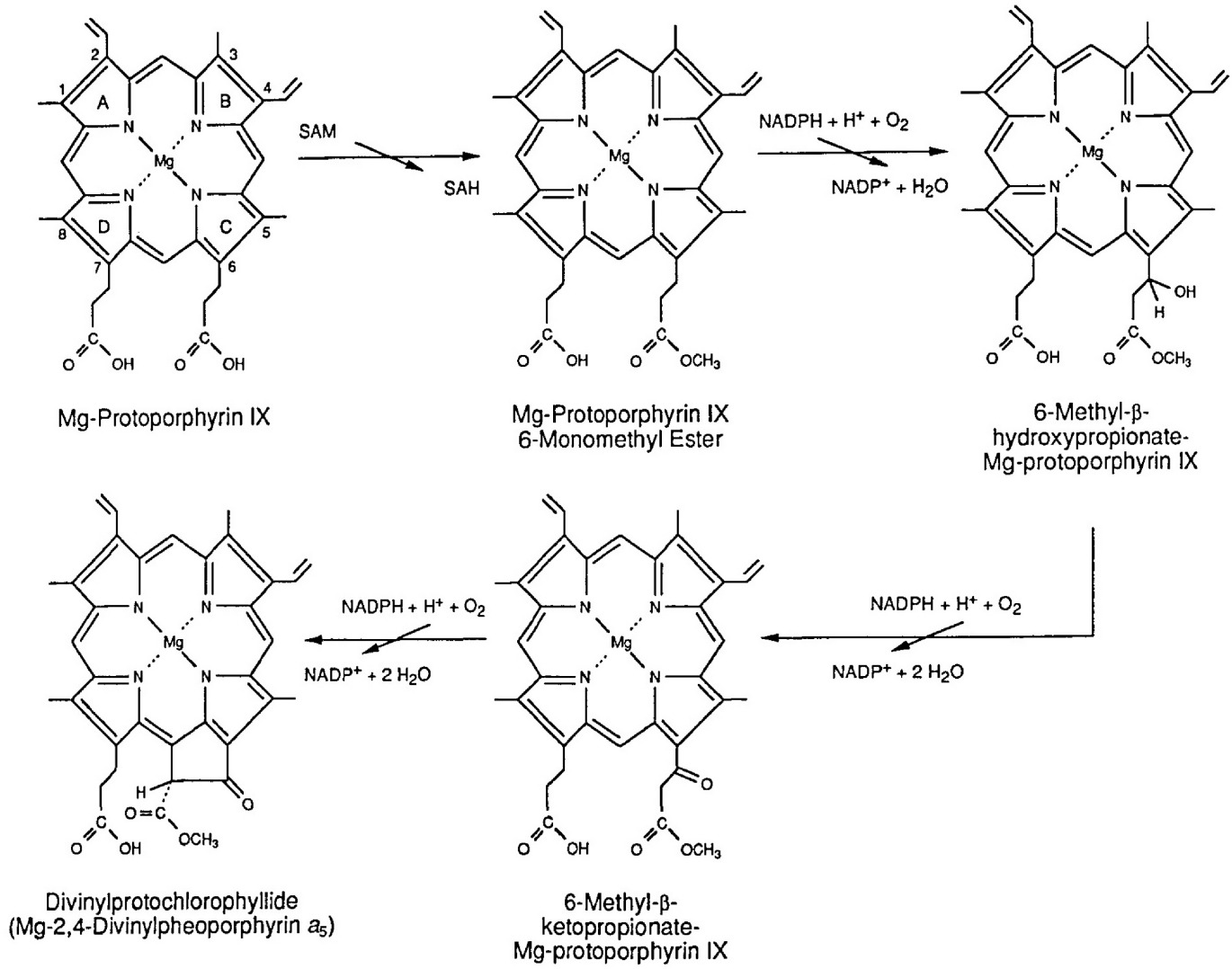

Fig. 1. Proposed biosynthetic pathway from Mg-protoporphyrin IX to MgDVP (Mg-2,4-divinylpheoporphyrin a_5). The Fischer system for tetrapyrrole substituent numbering and the letter designations for the pyrrole rings used in the text are indicated for Mg-protoporphyrin IX. Abbreviation: SAH, *S*-adenosyl-L-homocysteine.

NADPH:Pchlide oxidoreductase-catalyzed reduction of ring D occurs, and any divinyl Chlide that is formed is rapidly converted to (monovinyl) Chlide (Duggan and Rebeiz 1982).

Most studies of the Mg-protoporphyrin IX monomethyl ester oxidative cyclase enzyme system have been done with plastids obtained from cucumber cotyledons (Chereskin and Castelfranco 1982; Chereskin et al. 1982, 1983; Fuesler et al. 1984a,b; Wong and Castelfranco 1984; Walker et al. 1988, 1989, 1991; Whyte et al. 1992; Whyte and Castelfranco 1993), although activity has also been detected in plastids derived from wheat leaves (Ellsworth and Hervish 1975; Nasrulhaq-Boyce et al. 1987). The cyclase was demonstrated to require O_2 as well as a reductant, and both Mg-protoporphyrin IX monomethyl ester and the chemically synthesized proposed intermediate, Mg-protoporphyrin IX 6-methyl-β-ketopropionate, could serve as substrates (Chereskin and Castelfranco 1982; Walker et al. 1988). Incorporation of ^{18}O from $^{18}O_2$ into Pchlide was demonstrated with dark-incubated cucumber cotyledons, indicating that O_2 is the source of the carbonyl oxygen atom formed by the cyclase reaction (Walker et al. 1989). Reconstitution studies with cucumber cotyledon plastid fractions suggest that at least two subplastidic fractions, one soluble and the other membrane-bound, are required for cyclase activity (Wong and Castelfranco 1984). The soluble fraction from cucumber has been partially purified, but attempts to further characterize the membrane-bound fraction were unsuccessful (Walker et al. 1991).

Mutants which accumulate the biosynthetic intermediate Mg-protoporphyrin IX monomethyl ester have been identified in photosynthetic bacteria (Biel and

Marrs 1983; Bollivar et al. 1994b) and in *Chlorella* (Ellsworth and Aronoff 1968, 1969), but a gene associated with the cyclase reaction has been identified only in photosynthetic bacteria (Bollivar et al. 1994a,b). It is unclear whether the reaction occurs by the same mechanism in plants and photosynthetic bacteria, since only one gene has been identified in *Rhodobacter capsulatus* (Biel and Marrs 1983; Bollivar et al. 1994a,b) whereas two cell fractions are required for activity in cucumber, suggesting that at least two genes are required in that system. Additionally, the plant enzyme requires O_2 for activity whereas photosynthetic bacteria synthesize MgDVP under anaerobic conditions.

In this report we describe the use of the BioNeb cell disruptor to obtain chloroplasts from *C. reinhardtii* cells. The chloroplasts were then isolated on a Percoll density gradient by a previously described procedure (Belknap 1983). The purified chloroplasts were demonstrated to catalyze the conversion of Mg-protoporphyrin IX to MgDVP by the coupled reactions catalyzed by SAM:Mg-protoporphyrin IX methyltransferase and Mg-protoporphyrin IX monomethyl ester oxidative cyclase. Product formation was stimulated by addition of components of a NADPH regenerating system, and SAM, and required both Mg-protoporphyrin IX and O_2. A preliminary account of this work has appeared in abstract form (Bollivar and Beale 1994).

Materials and methods

Cell material

C. reinhardtii cell wall defective strain cc406 (*cw-15*, mt−) (Davies and Plaskitt 1971) was obtained from E.H. Harris (*Chlamydomonas* Genetics Center, Duke University, Durham, NC) and mated, using standard procedures (Harris 1988), with a mutant that is defective in light-independent Pchlide reduction (*chlB*, mt+) (Li et al. 1993), obtained from M.P. Timko (University of Virginia, Charlottesville, VA), to create a *cw-15, chlB* double mutant strain which was used for all experiments. The *cw-15, chlB* strain was routinely grown in 500-mL cultures of TAP medium (Gorman and Levine 1965) in the dark at 25 °C to a population density of 0.5–2 × 10^6 cells/mL.

Chloroplast isolation

Chloroplasts were isolated by a modification of the method described by Belknap (1983). The cell cultures were chilled on ice and all subsequent manipulations were done on ice or at 4 °C. Cells were harvested by centrifugation at 4,080 *g* for 8 min. The resulting cell pellet was resuspended to a density of 4–5 × 10^7 cells/mL in chloroplast isolation buffer (300 mM sorbitol, 50 mM Tris, 50 mM Mes, 10 mM $MgCl_2$, 3 mM KH_2PO_4, 2 mM EDTA, 1 mM $MnCl_2$, pH 7.2). The cells were then disrupted with a BioNeb cell disruptor (Glas-Col, Terre Haute, IN), following the procedure described in the instruction manual for the open-drain single cycle mode, with the N_2 gas pressure adjusted to 18 p.s.i. The resulting lysate was sedimented by brief centrifugation at 4,080 *g* and the pellet resuspended in 6 mL of chloroplast isolation buffer. A 3-mL portion of the suspension was layered onto a 20-mL Percoll step gradient, consisting of 10 mL of 45% (v/v) Percoll and 10 mL of 75% (v/v) Percoll in chloroplast isolation buffer, and centrifuged at 7,970 *g* for 20 min in a swinging bucket rotor. The resultant chloroplast-containing band at the 45%/75% interface was removed and diluted into 25 mL of chloroplast isolation buffer, and the chloroplasts were sedimented by brief centrifugation at 4,080 *g*. The chloroplast pellet was resuspended in assay buffer (500 mM sorbitol, 100 mM Tes, 50 mM Hepes, 10 mM $MgCl_2$, 5 mM DTT, 2 mM EDTA, pH 7.7) for incubations. Because very little Chl is present in dark-grown cells, the yield of chloroplasts was estimated by measurement of the carotenoid absorbance at 445 nm in 96% (v/v) aqueous methanol extracts.

Incubation

Standard incubations (0.5 mL) were done for 1 h at 30 °C in the dark with shaking in assay buffer supplemented with 10 mM glucose 6-phosphate, 2 mM SAM, 500 μM $NADP^+$, 20 μM Mg-protoporphyrin IX, 3 units of glucose 6-phosphate dehydrogenase (Sigma Type IX), and *C. reinhardtii* chloroplasts containing 0.1 to 0.4 mg of protein. Protein concentration was determined by the method of Bradford (1976). Reactions were terminated by the addition of acetone (see below).

For incubations containing added SAM:Mg-protoporphyrin IX methyltransferase, the source of this enzyme was a lysate of an *Escherichia coli* strain carrying a plasmid expressing the *R. capsulatus bchM* gene (Bollivar et al. 1994a). Anaerobic incubations were done by placing the chloroplast suspension under

an Ar stream for 20 min in the dark. After Ar flushing, chloroplasts were either incubated aerobically or mixed with Ar-flushed incubation mixture and incubated anaerobically in glass screw-cap tubes sealed with butyl rubber lined caps.

Tetrapyrrole pigment extraction

Extraction of incubation products was done as described previously (Wong et al. 1985) under subdued light conditions. The incubation mixture (0.5 mL) was mixed with 1.5 mL of chilled acetone and centrifuged for 45 s in a tabletop centrifuge to clarify the extract. The supernatant was retained and the pellet was resuspended in 250 μL of 0.125 N NH_4OH, followed by addition of 750 μL of acetone. The suspension was centrifuged as before and the acetone supernatants were combined. The acetone fraction was extracted twice with hexanes (2.5 mL then 1.25 mL) to remove fully esterified tetrapyrroles and other lipophilic material. Residual hexanes were removed from the acetone fraction by extracting with 1 mL of 2-methylbutane and exposing the remaining acetone fraction to a stream of N_2 to remove residual 2-methylbutane. The acetone fraction was then adjusted to pH 6.8 with 0.25 N maleic acid (monosodium salt, pH 5.0), 850 μL of saturated aqueous NaCl was added, and the reaction product was extracted into peroxide-free diethyl ether by two successive 1.5-mL extractions.

Tetrapyrroles in collected fractions from the ion-paired HPLC system described below were extracted into diethyl ether by the addition of 1 mL of ether and a sufficient quantity of water to produce a phase separation. The ether extract was washed with water and stored overnight at −20 °C to allow the residual water to freeze out of solution. The ether fraction was rapidly separated from ice crystals by decantation and used for fluorescence spectroscopy. Pigments in fractions collected from the polyethylene HPLC were adjusted to 80% (v/v) aqueous acetone and the pigments were then extracted into ether as described above.

Product quantitation

Product yield was determined with a Fluorolog fluorescence spectrophotometer (Spex Industries, Metuchen, NJ). Excitation was at 437 nm and the emission at 630 nm was measured. Standard Pchlide was extracted from etiolated cucumber cotyledons as previously described (Chereskin and Castelfranco 1982) and quantitated spectrophotometrically in diethyl ether with a Cary 219 spectrophotometer, using a molar absorption coefficient of 3.56×10^4 at 624 nm, which was derived from the mg-specific absorption coefficient of 39.9 at 623 nm reported for protochlorophyll (probably a mixture of monovinyl and divinyl forms) isolated from barley seedlings (Koski and Smith 1948). The spectrophotometrically-standardized Pchlide solution was diluted for use as a spectrofluorometric standard. Control fluorescence values of nonincubated samples were subtracted from incubated sample values prior to the calculation of product yield. Values reported are the mean and range of two replicates.

Fully-corrected excitation and emission fluorescence spectra were taken with the Fluorolog spectrofluorometer.

HPLC

Ion-paired reverse-phase HPLC was done using the system described by Fuesler et al. (1982). Aliquots of ether extract were diluted into 80 μL in injection solvent consisting of methanol:5 mM tetrabutylammonium phosphate (7:3, v/v) and injected onto an Altex Ultrasphere ODS column (0.46 cm dia × 25 cm long, 5 μm particle size; Beckman Instruments, San Ramon, CA) that was preequilibrated at room temperature in injection solvent. Chromatography was at room temperature and the flow rate was 1 mL/min. Three min after the sample was injected, the solvent was changed to methanol:water (7:3, v/v). Elution was monitored by fluorescence emission using a Fluorochrom flow-through fluorescence detector (Varian Instruments, Palo Alto, CA). Co-injection of reaction product with the Pchlide standard described above was used to test for identical chromatographic behavior.

Polyethylene HPLC was done by the method of Shioi and Beale (1987). The column was constructed by slurry-packing polyethylene powder (Polysciences, Inc., Warrington, PA) into a 0.46 cm dia × 25 cm long chromatography column. The elution solvent was 65% (v/v) aqueous acetone. Chromatography was at room temperature and the solvent flow rate was 1 mL/min. Elution was monitored by fluorescence emission.

Chemicals

Mg-protoporphyrin IX was purchased from Porphyrin Products (Logan, UT). Tetrabutylammonium phosphate was from Altex. All other chemicals were from Fisher (Pittsburgh, PA), Sigma (St. Louis, MO), or Research Organics (Cleveland, OH).

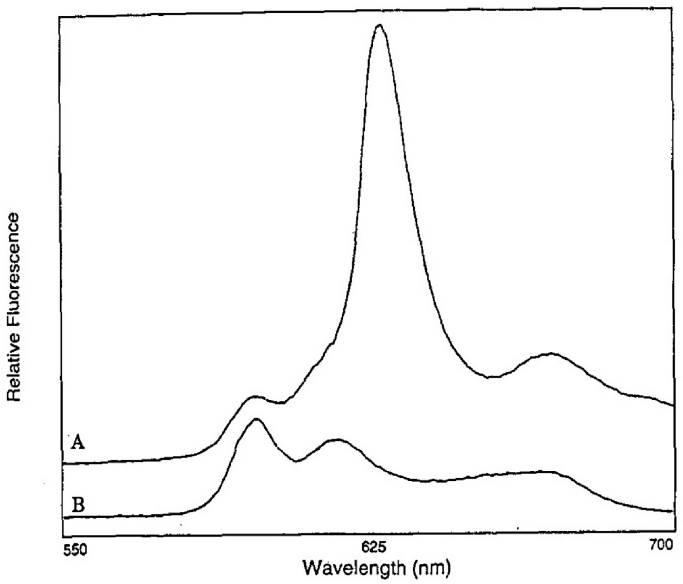

Fig. 2. Fluorescence emission spectra in ether of extracts from a 1-h standard incubation (Trace A) and a nonincubated control sample (Trace B). Excitation was at 437 nm.

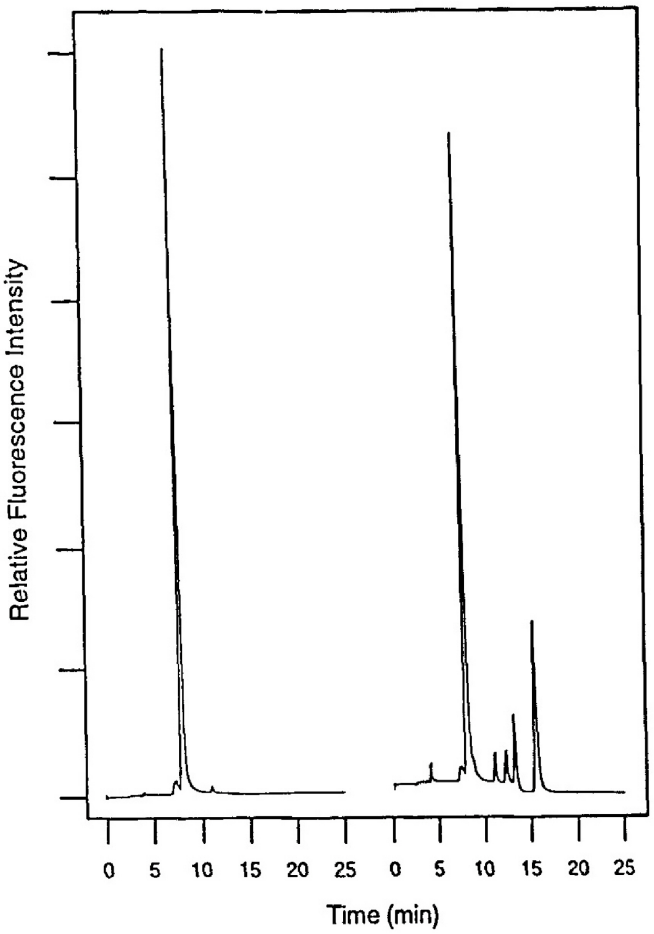

Fig. 3. Ion-paired HPLC fluorescence elution profiles of aliquots from a nonincubated control sample (left trace, 10 μL of ether extract) and a 1-h standard incubation (right trace, 20 μL of ether extract).

Results

Wild-type *C. reinhardtii* cells, unlike angiosperm tissues, are capable of accumulating Chl in darkness as well as in the light. This ability derives from the possession of a chloroplast-encoded light-independent Pchlide reduction system in addition to the light-requiring NADPH:Pchlide oxidoreductase enzyme (Roitgrund and Mets 1990; Suzuki and Bauer 1992; Burke et al. 1993; Li et al. 1993). Because of the potential for interference by Chl and Chlide with the determination of low concentrations of MgDVP, it was desirable to determine MgDVP formation in a strain that could be grown under conditions where these pigments are at low concentrations. Therefore, we used dark-grown cells of a *chlB* strain which is unable to accumulate Chl in the dark because it is defective in one of the components of the light-independent Pchlide reduction system. A *cw-15, chlB* double mutant strain that was created by standard genetic crossing techniques was used for all of the experiments reported here.

Preliminary attempts to obtain Mg-protoporphyrin IX monomethyl ester oxidative cyclase activity from whole cell extracts were unsuccessful (data not shown). Therefore, we attempted to obtain a preparation having a higher concentration of activity by isolating chloroplasts. Although the BioNeb system is capable of lysing wild-type *C. reinhardtii* cells, cell breakage without rupture of the chloroplasts requires the use of a cell wall deficient strain to allow the cells to be broken at low disruption energy. Use of the BioNeb system with cells having normal cell walls resulted in practically no intact chloroplast fraction in the Percoll gradient (data not shown). Chloroplasts were isolated from dark-grown cells of the cell wall deficient, dark-nongreening strain *cw-15, chlB* using the BioNeb disruption system in yields which varied from 25 to 50%, and averaged 35%, on the basis of carotenoid absorbance at 445 nm in 96% (v/v) methanol extracts. To the extent that chloroplasts may not be the sole subcellular site of carotenoids, the calculated yield of intact chloroplasts could be an underestimate. Cell disruption with the BioNeb disruptor produced a significantly greater yield of *C. reinhardtii* chloroplasts than other currently used methods (Katzman et al. 1994).

Chloroplasts isolated using the method described above were approximately 80% intact based on examination by phase-contrast microscopy and were reliably active in Mg-protoporphyrin IX monomethyl ester oxidative cyclase activity as demonstrated by the product accumulation shown in Fig. 2. A fluorescence

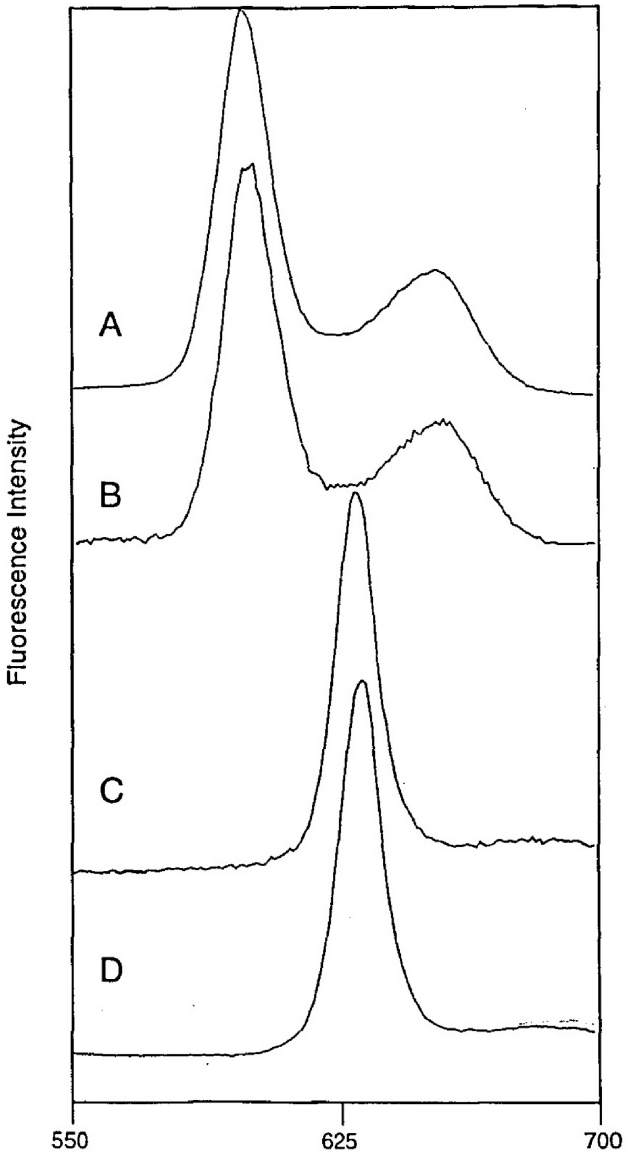

Fig. 4. Fluorescence emission spectra of fractions collected from the HPLC elution shown in Fig. 3 (right trace). Trace A (emission maximum = 595 nm) is the spectrum of standard Mg-protoporphyrin IX which elutes at 7.8 min. Trace B (emission maximum = 599 nm) is the spectrum of a sample collected at 12 min. Trace C (emission maximum = 628 nm) is the spectrum of standard Pchlide extracted from etiolated cucumber cotyledons. Trace D (emission maximum = 629 nm) is the spectrum of the fraction eluting at 13 min. Traces A and B were obtained using an excitation wavelength of 425 nm, and traces C and D with an excitation wavelength of 437 nm.

emission peak at 629 to 630 nm in the incubated control sample indicates the presence of MgDVP, and an emission peak in this region is absent from the non-incubated control sample. The Mg-protoporphyrin IX substrate produced only a low level of fluorescence emission at 595 nm, despite its relatively high concentration (20 μM in the incubation mixture) because the

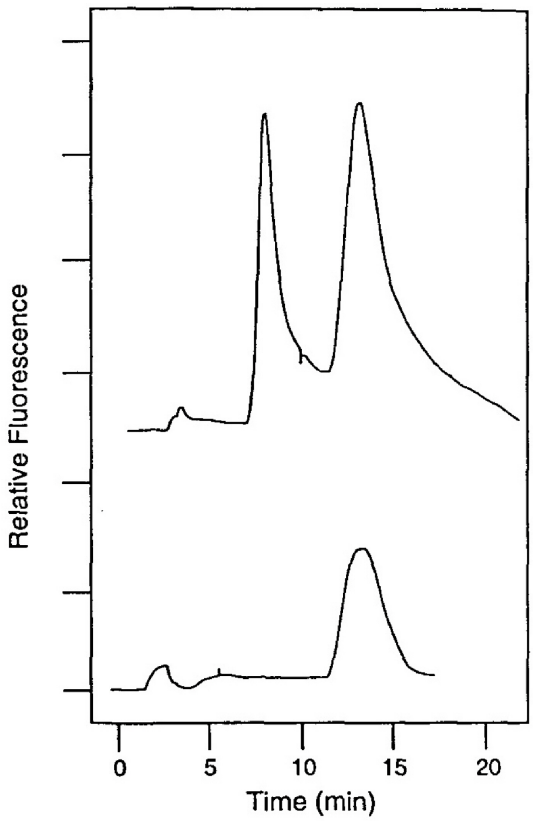

Fig. 5. Polyethylene HPLC fluorescence elution profiles of standard Pchlide (a mixture of MgDVP and monovinyl Pchlide) obtained from etiolated cucumber cotyledons (top trace) and the compound contained in the 13-min fraction in Fig. 3. Samples were injected onto a polyethylene HPLC column equilibrated in 65% (v/v) aqueous acetone (flow rate 1 mL/min).

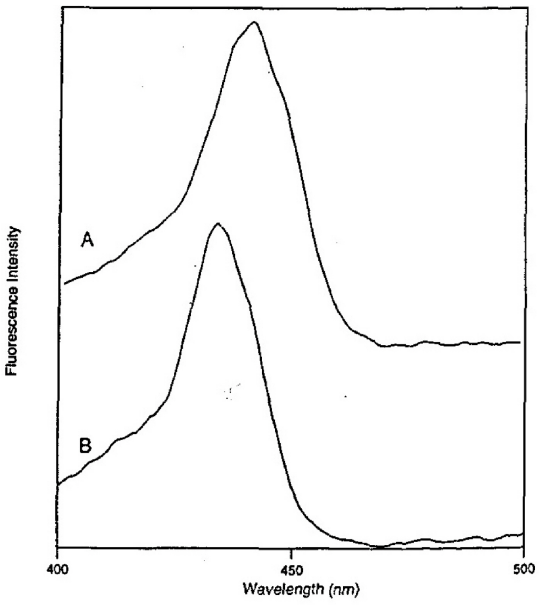

Fig. 6. Fluorescence excitation spectra of fractions collected from the HPLC system described in Fig. 5 using an emission wavelength of 630 nm. Trace A (excitation maximum = 439 nm) is the spectrum of the fraction eluting at 13 min. Trace B (excitation maximum = 433 nm) is the spectrum of the fraction eluting at approximately 8 min.

Table 1. Cofactor requirements for Mg-protoporphyrin IX monomethyl ester cyclase activity

Incubation mixture	Net MgDVP formation (pmol/mg protein)
Experiment 1[a]	
Complete	621 ± 50
– Glucose 6-phosphate dehydrogenase	471 ± 32
– Glucose 6-phosphate	453 ± 29
– Mg-Protoporphyrin IX	38 ± 12
Experiment 2[b]	
Complete	1150 ±106
– SAM	319 ± 19

[a] Protein concentration was 0.68 mg/mL.
[b] Protein concentration was 0.32 mg/mL.

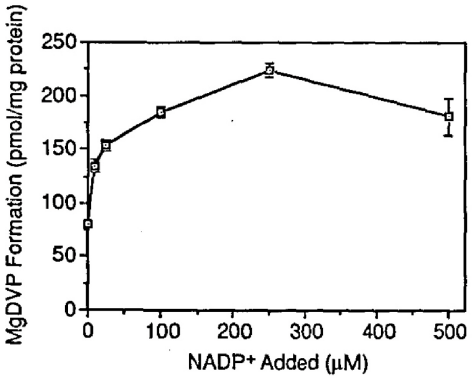

Fig. 7. Reaction dependence on added $NADP^+$. The protein concentration was 0.57 mg/mL.

excitation wavelength used was chosen to be optimal for the product rather than the substrate.

The reaction product co-eluted with standard Pchlide extracted from etiolated cucumber cotyledons (actually a mixture of MgDVP and monovinyl Pchlide) at 13.2 min on a previously described ion-paired HPLC system (Fuesler et al. 1982) (Fig. 3). In the nonincubated control sample, no peak was observed at 13.2 min, and the only peaks present were those attributed to the Mg-protoporphyrin IX substrate at 7.8 min and a small amount of Chlide at 11 min (identified by fluorescence emission spectroscopy, data not shown). The material from the incubated sample that eluted at 13 min was collected and subjected to fluorescence analysis. Its emission spectrum (Fig. 4C) was similar to that of standard Pchlide (Fig. 4D). The accumulated product was determined to be MgDVP, rather than monovinyl Pchlide, by co-migration with authentic MgDVP on

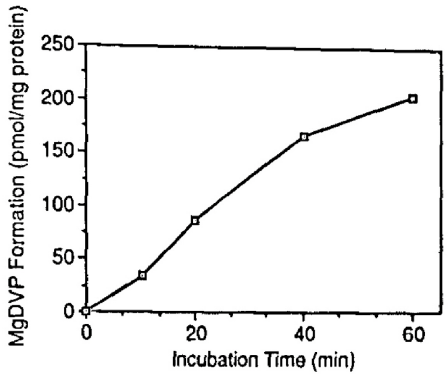

Fig. 8. Time course of product formation. The protein concentration was 0.52 mg/mL.

polyethylene HPLC, a system that effectively separates monovinyl and divinyl forms of Pchlide (Shioi and Beale 1987) (Fig. 5). The compounds that eluted at 8 and 13 min on polyethylene HPLC were identified as monovinyl Pchlide and MgDVP, respectively, by their room temperature fluorescence excitation maxima at 433 nm and 439 nm, respectively (Belanger and Rebeiz 1979; Chereskin et al. 1982) (Fig. 6).

Activity required added Mg-protoporphyrin IX and was stimulated by the addition of glucose 6-phosphate and glucose 6-phosphate dehydrogenase (Table 1). For reasons that are not yet understood, different preparations gave different levels of activity, which required that comparisons be made only for samples obtained from the same preparation (Table 1). Although the reaction did not require added $NADP^+$, supplementation of the reaction with $NADP^+$ increased the product yield, with a maximum stimulation at 250 μM added $NADP^+$ (Fig. 7).

The ability of Mg-protoporphyrin IX to function as the substrate demonstrates that, in addition to cyclase activity, SAM:Mg-protoporphyrin IX methyltransferase activity is also present. This conclusion is confirmed by the presence in incubated extracts of a compound that co-elutes, at 15.6 min on the ion-paired HPLC, with standard Mg-protoporphyrin IX monomethyl ester, prepared by incubation of Mg-protoporphyrin IX with bacterial SAM:Mg-protoporphyrin IX methyltransferase as described in Bollivar et al. (1994a). Added SAM was required for maximal activity, and addition of 2 mM SAM increased measured activity approximately 4 fold over the unsupplemented activity (Table 1). Some incubations were supplemented with *E. coli* extracts containing heterologously expressed recombinant *R. capsulatus* SAM:Mg-protoporphyrin IX methyltransferase (Bollivar et al. 1994a). An increase in the lev-

Table 2. O_2 requirement for Mg-protoporphyrin IX monomethyl ester cyclase activity[a]

Pretreatment	Incubation	Net MgDVP formation (pmol/mg protein)
None	Aerobic	553 ± 32
Ar gas	Anaerobic	−11 ± 5
Ar gas	Aerobic	663 ± 58

[a] Protein concentration was 0.38 mg/mL.

el of Mg-protoporphyrin IX monomethyl ester was observed, but there was no change in the MgDVP accumulation (data not shown). These results are consistent with the interpretation that formation of Mg-protoporphyrin IX monomethyl ester is not the limiting reaction in the standard incubations. Although it is possible that the lack of stimulation of MgDVP accumulation is due to impermeability of the plastids to Mg-protoporphyrin IX monomethyl ester, there is no basis for believing that this compound is less able to enter the chloroplasts than Mg-protoporphyrin IX, which is required for product formation (Table 1).

Although the highest activity was obtained with intact chloroplasts, intactness was not essential for activity. Chloroplasts that were lysed by several freeze-thaw cycles or by nebulization at high pressure yielded approximately 60% of the activity of intact chloroplasts (data not shown).

Mg-protoporphyrin IX monomethyl ester oxidative cyclase activity was time dependent, and the product yield steadily increased over the standard 1-h incubation time (Fig. 8). To test whether the formation of MgDVP was catalyzed by protein components, chloroplast extracts were heated to 95 °C for 5 min and then cooled on ice prior to addition to incubations. No activity was observed in the heat-treated samples, suggesting this reaction is enzymatic in nature.

O_2 was required for activity (Table 2). The complete absence of activity in incubations that were done in an Ar atmosphere is presumed to be due to the lack of O_2. The procedure used to create an anaerobic incubation did not itself destroy enzyme activity, as is shown by the normal level of activity in anaerobic chloroplasts that were placed in aerobic incubation conditions (Table 2).

Experiments were done to determine whether the level of extractable cyclase activity was increased by exposure of the dark-grown *C. reinhardtii* cells to light for periods ranging from 1 to 5 h before the chloroplasts were extracted. The highest activity was obtained from cells that were harvested directly from dark cultures without prior exposure to light (data not shown).

In addition to the reaction products already mentioned, another fluorescent compound was present in samples that were incubated under standard conditions. This compound eluted at 12.3 min in the ion-paired HPLC system (Fig. 3). The fluorescence emission spectrum of this compound in ether was similar to that of Mg-protoporphyrin IX, with an emission maximum at 599 nm compared to 595 nm for the Mg-protoporphyrin IX standard (Fig. 4). With respect to relative HPLC elution time and fluorescence emission, this compound behaved similarly to the 6-methyl-β-hydroxypropionate reaction intermediate described by Wong et al. (1985). An incubation supplemented with excess SAM:Mg-protoporphyrin IX methyltransferase produced a higher concentration of this compound relative to the amount present in the standard incubation (data not shown).

Because one O_2-derived oxygen atom is incorporated into MgDVP in the reaction catalyzed by Mg-protoporphyrin IX monomethyl ester oxidative cyclase (Walker et al. 1989), the reaction appears to be one that is catalyzed by a monooxygenase. Two common classes of monooxygenases are those that contain a flavin prosthetic group (flavin monooxygenases) and those that contain a heme (cytochrome P450-type monooxygenases) (Walsh 1979). Flavin monooxygenases are generally inhibited by quinacrine, a flavin analog which competes for the enzyme flavin-binding site (Haas 1944; Mahler et al. 1954), and cytochrome P450 monooxygenases are generally inhibited by CO, which binds strongly to the heme prosthetic group (Estabrook et al. 1963). Neither the addition of 40 μM quinacrine to the incubation mixture nor preequilibration of the chloroplasts for 15 min with an atmosphere of 100% CO affected the rate of MgDVP formation (data not shown). These results suggest that Mg-protoporphyrin IX monomethyl ester oxidative cyclase is neither a flavin monooxygenase nor a cytochrome P450-type monooxygenase.

Discussion

We have shown that chloroplasts can be obtained from *C. reinhardtii* cells in high yield by lysis of cell wall deficient cells using the BioNeb disruptor, and that these chloroplasts are active in catalyzing the Mg-

protoporphyrin IX monomethyl ester oxidative cyclase reaction of Chl biosynthesis.

Disruption of cell wall deficient cells with the BioNeb instrument yields a high percentage of apparently intact chloroplasts. The two criteria for chloroplast intactness used in this study were their appearance under phase-contrast microscopy and their migration to the appropriate interface in the Percoll gradient centrifugation. Although additional tests for chloroplast intactness and contamination of the isolated chloroplasts with components from other subcellular fractions were not performed, similar Percoll gradient centrifugation procedures have been used for the purification of intact chloroplasts from *C. reinhardtii* cells broken by other means (Belknap 1983), and similar degrees of purity should have been obtained. Because of its rapidity, efficiency, and ability to be operated in continuous flow mode, the BioNeb provides a convenient means of obtaining large quantities of *C. reinhardtii* chloroplasts for studies of biosynthesis and photosynthetic function.

It should be noted that the chloroplasts we obtained from dark-grown mutant cells were deficient in chlorophyll and they may more closely resemble plant etioplasts or proplastids than mature chloroplasts in some ways. For example, the apparent permeability to NADP(H), SAM, and Mg-protoporphyrin IX (also observed with the cyclase reactions in intact plant etioplasts) may be related to their arrested state of development.

The isolated chloroplasts were active in catalyzing two sequential steps of Chl biosynthesis, SAM:Mg-protoporphyrin IX methyltransferase and Mg-protoporphyrin IX monomethyl ester oxidative cyclase. These results are consistent with the plastid location of these activities in cucumber cotyledons (Mattheis and Rebeiz 1977; Fuesler et al. 1984b). The absence of detectable MgDVP formation in whole cell extracts of *C. reinhardtii* has several possible explanations. First, the cyclase activity may depend on chloroplast intactness, as has been reported for wheat plastids (Nasrulhaq-Boyce et al. 1987). This does not appear to be the case for *C. reinhardtii*, as activity can be detected in lysed *C. reinhardtii* chloroplasts (unpublished results). A more likely explanation is that whole cell extracts contain enzyme inhibitors or enzymes (e.g., esterases or Mg dechelatase) that degrade the substrate (Mg-protoporphyrin IX), the esterified intermediate (Mg-protoporphyrin IX monomethyl ester) or the final product (MgDVP) too quickly to allow detectable quantities of product to accumulate. Metabolism of Mg-protoporphyrin IX monomethyl ester has been described by Crawford and Wang (1983). Another contributing factor is that it is possible to obtain a higher final concentration of chloroplast enzymes in the assays using isolated chloroplasts than in assays using whole cell extracts. Because the assay depends on the activity of two sequential enzyme steps, the yield of final product may be highly dependent on the enzyme concentration.

The Mg-protoporphyrin IX monomethyl ester oxidative cyclase activity in *C. reinhardtii* chloroplasts is generally similar to that described in cucumber cotyledon plastids. Added Mg-protoporphyrin IX was required for the reaction. Product accumulation was stimulated by SAM, $NADP^+$, and components of an NADPH regenerating system, which suggests that SAM and NADPH are required for the overall conversion of Mg-protoporphyrin IX to MgDVP. The presence of considerable activity even in the absence of added SAM, $NADP^+$, or components of the NADPH regenerating system is not surprising, since the system was not depleted of endogenous sources of these components. Additionally or alternatively, as has been suggested for the reconstituted cyclase system derived from dialyzed fractions of lysed cucumber cotyledon plastids, protein-bound NADPH may be an effective substrate (Whyte and Castelfranco 1993).

No product accumulated in anaerobic incubations, indicating that O_2 is required for the cyclase reaction. This result complements the previous demonstration with cucumber cotyledons that O_2 is the source of the carbonyl oxygen atom of Chlide (Walker et al. 1989). It is of interest that the cyclase step can occur under strictly anaerobic conditions in photosynthetic bacteria, which suggests that the reaction may occur by a different mechanism in these organisms. In vitro cyclase activity has not been reported for any photosynthetic bacterial extract, although a single *R. capsulatus* gene (*bchE*) has been assigned to this step (Bollivar et al. 1994a,b). A *bchE* homolog has not been reported in organisms that form Chl aerobically.

Because Mg-protoporphyrin IX monomethyl ester oxidative cyclase appears to be a monooxygenase reaction, attempts were made to determine whether the enzyme system involves cofactors common to other monooxygenases. Neither quinacrine (an inhibitor of flavoprotein enzymes) nor CO (an inhibitor of cytochrome P450-type monooxygenases) affected cyclase activity. In their study of the reconstituted cyclase system derived from cucumber cotyledon plastid fractions, Whyte and Castelfranco (1993) showed

that, whereas CO was not inhibitory, other heme ligands such as KCN and NaN_3 inhibited the reaction. It was concluded that these results suggest that the prosthetic group is a ferric-heme. Although our results are consistent with the conclusion of Whyte and Castelfranco, direct identification of the prosthetic group will require analysis of the purified enzyme protein(s).

MgDVP was the sole isocyclic ring-containing product detected in the assays. No monovinyl Pchlide was detected, even though the polyethylene HPLC system could easily resolve these two compounds from extracts of etiolated cucumber cotyledons. These results are consistent with a Chl biosynthetic pathway in which isocyclic ring formation occurs before the vinyl-to-ethyl conversion step, and that insufficient quantities of MgDVP accumulated in the reaction mixture to allow ethyl group formation to occur to a detectable degree. However, an alternative possibility that could not be completely excluded is that vinyl group reduction normally precedes isocyclic ring formation but that some component required for vinyl reduction was missing from the assay mixture, and that the divinyl compound can be accepted as an alternative substrate for the cyclase reaction. Full resolution of this issue will require additional experimentation with isolated enzymes.

Among the incubation products, a compound was detected that has fluorescence and HPLC elution properties consistent with its identification as Mg-protoporphyrin IX 6-methyl-β-hydroxypropionate, a proposed intermediate in the cyclase reaction (Fig. 1). This compound was previously detected among the reaction products of the reconstituted cyclase system from cucumber cotyledon plastids, and the isolated compound was converted to MgDVP when incubated with the cucumber-derived cyclase system (Wong et al. 1985).

The use of *C. reinhardtii* as a system for investigating chlorophyll biosynthesis, as described here, will facilitate the integration of biochemical and molecular genetic approaches to create a better understanding of this complex process, which has been previously studied in systems which were primarily exploited for their genetic or biochemical potentials alone. For example, recently described methods for creating insertionally-tagged nuclear mutants of *C. reinhardtii* (Debuchy et al. 1989; Kindle et al. 1989) will aid in identifying genes associated with specific defects in chlorophyll biosynthesis, and the use of reporter constructs (Davies et al. 1992) will assist in the study of how individual steps of chlorophyll biosynthesis are regulated.

In conclusion, the isocyclic ring-forming step of Chl biosynthesis, catalyzed by Mg-protoporphyrin IX monomethyl ester oxidative cyclase, has been measured in isolated *C. reinhardtii* chloroplasts. Most analyses of chlorophyll biosynthesis have used organisms chosen due to suitability for genetic or biochemical studies. These results demonstrate the feasibility of isolating chloroplasts from *C. reinhardtii*, which will enable the use of this species for detailed biochemical and genetic studies of Chl biosynthesis and its regulation. Moreover, the ability to isolate *C. reinhardtii* chloroplasts in high yield with the BioNeb disruptor indicates that this procedure will be generally useful for studies of photosynthesis and chloroplast development in this genetically amenable species.

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