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The Chlorophyll Biosynthetic Enzyme Mg-Protoporphyrin IX Monomethyl Ester (Oxidative) Cyclase

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The Chlorophyll Biosynthetic Enzyme Mg-Protoporphyrin IX Monomethyl Ester (Oxidative) Cyclase

Characterization and Partia1 Purification from Chlamydomonas reinhardfii and Synechocysfis sp. PCC 6803

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A universal structural feature of chlorophyll molecules is the isocyclic ring. This ring is formed by the action of the enzyme Mg-protoporphyrin IX monomethyl ester (oxidative) cyclase, which catalyzes a complex reaction in which Mg-protoporphyrin IX monomethyl ester is converted to divinyl protochlorophyllide (also called Mg-2,4-divinylpheoporphyrin a_5), with the participation of NADPH and O_2. Cyclase activity was demonstrated in lysed *Chlamydomonas reinhardtii* chloroplasts and extracts of *Synechocystis* sp. PCC 6803. The yield of the reaction product was increased by the addition of catalase and ascorbate or isoascorbate to the incubation mixture. These compounds may act by preventing degradation of the tetrapyrroles by reactive oxygen species. Cyclase activity from *C. reinhardtii* was not inhibited by the flavoprotein inhibitor quinacrine or by the hemoprotein inhibitors CO, KCN, or NaN_3. In contrast, cyclase activity in extracts of *C. reinhardtii* and *Synechocystis* sp. PCC 6803 was inhibited by chelators of Fe, suggesting that nonheme Fe is involved in the reaction. Cyclase in lysed *C. reinhardtii* chloroplasts was associated with the membranes, and attempts to further fractionate or solubilize the activity were unsuccessful. In contrast, cyclase in *Synechocystis* sp. PCC 6803 extracts could be separated into soluble and membrane components, both of which were required for reconstitution of activity. The membrane component retained activity after it was solubilized by the detergent *n*-octyl-β-D-glucopyranoside in the presence of glycerol and Mg^{2+}. The solubilized membrane component was purified further by dye-affinity and ion-exchange chromatography.

Chlorophylls, the light-harvesting and photoactive pigments of all photosynthetic organisms, are members of a large family of tetrapyrrole molecules whose other members include hemes, bilins, and corrins. Chlorophylls are distinguished structurally from these other tetrapyrrole molecules by two features: a centrally chelated Mg atom and a fifth, isocyclic ring, which is derived from the propionic acid group at the 6-position of a precursor porphyrin (Fig. 1).

In the chlorophyll biosynthetic pathway, isocyclic ring formation occurs after the insertion of Mg^{2+} into the macrocycle of protoporphyrin IX and the esterification of the 6-propionate to form Mg-protoporphyrin IX monomethyl ester, the substrate for the isocyclic ring-forming reaction (Beale and Weinstein, 1990). In aerobic chlorophyll-forming organisms, the cyclizing enzyme Mg-protoporphyrin IX monomethyl ester (oxidative) cyclase catalyzes a complex reaction that appears to consist of at least three steps (Wong et al., 1985): hydroxylation of the methylpropionate at the β-carbon atom, oxidation of the hydroxyl group to a carbonyl group, and ligation of the α-carbon of the β-ketomethylpropionate to the γ-meso bridge carbon between the C and D pyrrole rings of the tetrapyrrole (Fig. 1). The reaction product is divinyl protochlorophyllide, which is also known as Mg-2,4-divinylpheoporphyrin a_5 (Chereskin et al., 1983). The carbonyl oxygen atom of divinyl protochlorophyllide has been shown to be derived from O_2 (Walker et al., 1989). Organisms that form (bacterio)chlorophyll anaerobically appear to have a somewhat different isocyclic ring-forming reaction in which the carbonyl oxygen atom is derived from H_2O rather than from O_2 (Porra et al., 1995).

Mg-protoporphyrin IX monomethyl ester (oxidative) cyclase activity has been described in chloroplast extracts of cucumber cotyledons (Chereskin et al., 1982, 1983; Fuesler et al., 1984; Wong and Castelfranco, 1984, 1985; Wong et al., 1985; Walker et al., 1988, 1989, 1991; Whyte et al., 1992; Whyte and Castelfranco, 1993), in intact chloroplasts from wheat leaves (Nasrulhaq-Boyce et al., 1987), and in the unicellular green alga *Chlamydomonas reinhardtii* (Bollivar and Beale, 1995). The most progress has been made with the cucumber cotyledon system, from which cyclase has been extracted from lysed chloroplasts and fractionated into a soluble component and a membrane-bound component (Wong and Castelfranco, 1984; Walker et al., 1991). The soluble component was purified about 40-fold from osmotically-lysed chloroplasts and was shown to have a molecular mass greater than 30 kD (Walker et al., 1991).

Abbreviations: Desferal, desferrioxamine methanesulfonate; PCMBS, *p*-chloromercuribenzene sulfonate.

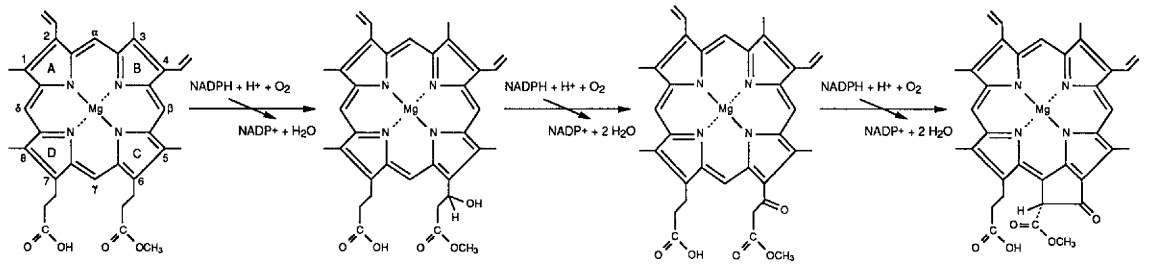

Figure 1. Structures of the substrate, proposed intermediates, and product of the Mg-protoporphyrin monomethyl ester (oxidative) cyclase enzyme. The conventional tetrapyrrole ring and meso bridge letter designations and Fischer numbering system for substituents are shown for the substrate.

The membrane-bound component was more labile than the soluble component (Walker et al., 1991). Cyclase activity in wheat leaf chloroplasts (Nasrulhaq-Boyce et al., 1987) and in cucumber cotyledon chloroplast lysates (Walker et al., 1991) was inhibited by chelators of iron. 8-Hydroxyquinoline, a lipophilic chelator, was more effective than Desferal, a more hydrophilic chelator, suggesting that the target of the chelators is located in a membrane or hydrophobic region of an enzyme.

The isolation of intact chloroplasts from the unicellular green alga *C. reinhardtii* and the preliminary characterization of cyclase activity in the chloroplasts were recently described (Bollivar and Beale, 1995). We now report some properties of the cyclase in lysed *C. reinhardtii* chloroplasts. Because we were unable to recover activity from solubilized chloroplast membranes, we turned to another source of cyclase, the unicellular cyanobacterium *Synechocystis* sp. PCC 6803, from which we were able to obtain and partially purify the solubilized membrane component of the cyclase. A brief report of portions of these results was published in abstract form (Bollivar and Beale, 1996).

MATERIALS AND METHODS

Culture Conditions

A previously described, cell-wall-deficient, yellow-in-the-dark (*cw-15, chlB*) strain of *Chlamydomonas reinhardtii* (Bollivar and Beale, 1995) was grown to the midexponential growth phase (population density 0.5–2 $\times$ 10^6 cells/mL) in 500-mL cultures of TAP medium (Gorman and Levine, 1965) at 25°C with continuous shaking in the dark.

Synechocystis sp. PCC 6803 was grown in BG-11 medium (Rippka et al., 1979) that was supplemented with 50 mM Glc. The cells were grown to the late exponential growth phase in continuous light at an intensity of 32 μE m^{-2} s^{-1} at 25°C with continuous shaking.

Preparation of *C. reinhardtii* Chloroplasts

Chloroplasts were isolated from *C. reinhardtii* cells as previously described (Bollivar and Beale, 1995). Cell cultures were cooled on ice, and all subsequent manipulations were done on ice or at 4°C. Cells were harvested by centrifugation at 4080*g* for 8 min. The sedimented cells were resuspended to a population density of 4 to 5 $\times$ 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 lysed with a cell disruptor (BioNeb 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 cell lysate was sedimented by brief centrifugation at 4080*g*, and the pellet was resuspended in 6 mL of chloroplast isolation buffer. A 3-mL portion of the suspension was layered onto a 20-mL Percoll (Sigma) step gradient, consisting of 10 mL of 45% (v/v) Percoll and 10 mL of 75% (v/v) Percoll in chloroplast isolation buffer, and the gradient was centrifuged at 7970*g* for 20 min in a swinging bucket rotor. The band containing intact chloroplasts at the 45/75% Percoll interface was removed and diluted to 25 mL with chloroplast isolation buffer, and the chloroplasts were sedimented by brief centrifugation at 4080*g*. The chloroplasts were resuspended in assay buffer (100 mM Tes, 50 mM Hepes, 10 mM $MgCl_2$, 5 mM DTT, pH 7.7) supplemented with 500 mM sorbitol and 2 mM EDTA for incubations.

Resuspended chloroplasts were lysed by passage through the bionebulizer with the N_2 gas pressure adjusted to 50 p.s.i. Membranes were separated from the soluble components by centrifugation for 1 h at 285,000*g*, and were then washed by resuspending in assay buffer and re-sedimented by centrifugation for 1 h at 285,000*g*. Salt-washed membranes were prepared from lysed chloroplasts by adding 20 mL of chloroplast lysate to 10 mL of 1.5 M NaCl in assay buffer, the sample was stirred on ice for 20 min, and the membranes were then sedimented by centrifugation for 30 min at 35,000*g*.

Preparation of Cell Extract from *Synechocystis* sp. PCC 6803

Cells from 4 L of culture were harvested by centrifugation at 10,000*g* and 4°C for 10 min and washed with cold lysis buffer (100 mM Tes, 50 mM Hepes, 10 mM $MgCl_2$, 1 M glycerol, pH 7.7). Cells were resuspended in lysis buffer supplemented with 0.004% (w/v) PMSF and disrupted by passage through a French pressure cell (SLM-Aminco, Urbana, IL) twice at 20,000 p.s.i. Cell debris and unlysed cells were removed by centrifugation at 16,000*g* for 15 min to

yield the low-speed supernatant, which was fractionated by centrifugation for 1 h at 285,000*g*. The high-speed supernatant was used directly or chromatographed through Red-agarose (Sigma) prior to use. The high-speed pellet fraction was resuspended in an equal volume of lysis buffer using a tissue homogenizer, *n*-octyl-β-D-glucopyranoside was added to a final concentration of 1% (w/v), and the mixture was centrifuged for 1 h at 285,000*g*. The solubilized membrane component in the supernatant was further purified by chromatography through Red-agarose. The active fraction was eluted from Red-agarose with lysis buffer supplemented with 1% (w/v) *n*-octyl-β-D-glucopyranoside and 1 M NaCl. This fraction was desalted by chromatography through Sephadex G-25 (Sigma) preequilibrated with 30 mM Tes, 15 mM Hepes, 10 mM $MgCl_2$, 1 M glycerol, pH 7.7. The desalted fraction was used directly or purified further by chromatography through DEAE-cellulose (DE-52, Whatman). The active fraction was eluted with 30 mM Tes, 15 mM Hepes, 10 mM $MgCl_2$, 50 mM NaCl, 1 M glycerol, 1% (w/v) *n*-octyl-β-D-glucopyranoside, pH 7.7, and desalted by chromatography through Sephadex G-25 preequilibrated with lysis buffer.

Incubation

Incubations (0.5 mL for *C. reinhardtii*, 1.0 mL for *Synechocystis* sp. PCC 6803) were done for 1 h at 30°C in the dark with shaking, in assay buffer supplemented with 10 mM Glc-6-P, 2 mM *S*-adenosyl-L-Met, 500 μM $NADP^+$, 20 μM Mg-protoporphyrin IX, and 3 units of bakers' yeast Glc-6-P dehydrogenase (EC 1.1.1.49; Sigma type IX). For experiments with intact *C. reinhardtii* chloroplasts, the assay buffer also contained 500 mM sorbitol. In some cases where indicated, the incubation was supplemented with 2 mM EDTA and/or 5 or 1 mM ascorbate or isoascorbate and 0.2 mg/mL bovine liver catalase (EC 1.11.1.6).

The assay relies on in situ generation of the cyclase substrate Mg-protoporphyrin IX monomethyl ester. Intact *C. reinhardtii* chloroplasts contain sufficient *S*-adenosyl-L-Met:Mg-protoporphyrin IX methyltransferase (EC 2.1.1.11) to methylate the supplied Mg-protoporphyrin IX to virtual completion (Bollivar and Beale, 1995). All incubations with *Synechocystis* sp. PCC 6803 extracts, as well as some incubations with lysed *C. reinhardtii* plastids (where indicated), were supplemented with *S*-adenosyl-L-Met:Mg-protoporphyrin IX methyltransferase, which was derived from a lysate of an *Escherichia coli* strain (0.3 mg of *E. coli* extract per mL of incubation) carrying a plasmid expressing the *Rhodobacter capsulatus bchM* gene (Bollivar et al., 1994). For the experiments with *Synechocystis* sp. PCC 6803 extracts, the *S*-adenosyl-L-Met:Mg-protoporphyrin IX methyltransferase was preincubated for 10 min at 30°C in assay buffer with its substrates, Mg-protoporphyrin IX and *S*-adenosyl-L-Met, before the *Synechocystis* sp. PCC 6803 extract and other supplementations were added.

Cyclase reactions were terminated by the addition of acetone (see below) or by freezing at −20°C. Protein concentration was determined by the method of Bradford (1976), with BSA as the standard.

Tetrapyrrole Pigment Extraction

Extraction of incubation products from *C. reinhardtii* incubations was performed as described previously (Wong et al., 1985) under subdued light. 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, the pellet was resuspended in 250 μL of 0.125 N NH_4OH, and then 750 μL of acetone was added with mixing. The suspension was centrifuged as before, and the acetone supernatants were combined. The acetone fraction was extracted twice with hexanes (2.5 mL and 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 transferred to peroxide-free diethyl ether by two successive 1.5-mL extractions.

Extraction of products from incubations with *Synechocystis* sp. PCC 6803 extracts was performed similarly to that for *C. reinhardtii*, but all volumes except the final ether extractions were doubled.

Product Quantitation

Product yield was determined with a fluorescence spectrophotometer (Fluorolog; Spex Industries, Metuchen, NJ). Excitation was at 437 nm, and the emission at 630 nm was measured. Standard protochlorophyllide was extracted from etiolated cucumber cotyledons as previously described (Chereskin and Castelfranco, 1982), and quantitated in diethyl ether with a spectrophotometer (Cary 219, Varian Instruments, Palo Alto, CA), 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 protochlorophyllide solution was diluted for use as a spectrofluorometric standard. Control fluorescence values of unincubated samples were subtracted from incubated sample values prior to the calculation of product yield. Values reported are the means and ranges of two replicates.

Fully-corrected fluorescence excitation and emission spectra at 77K were acquired by cooling samples in liquid N_2.

HPLC

Ion-paired reverse-phase HPLC was done using the system described by Fuesler et al. (1982). Aliquots (200 μL) of ether extract were reduced to 20 μL under a stream of dry N_2 and diluted with 80 μL of injection solvent consisting of methanol:5 mM tetrabutylammonium phosphate (7:3, v/v) ODS and injected onto a column (0.46 cm diameter × 25 cm long, 5-μm particle size; Altex Ultrasphere Beckman Instru-

ments) that was preequilibrated at room temperature with injection solvent. Chromatography was at room temperature and the flow rate was 1 mL/min. Three minutes after the sample was injected, the eluent was changed from an injection solvent to methanol:water (7:3, v/v), and elution was monitored by fluorescence emission using a flow-through fluorescence detector (Fluorochrom, Varian Instruments).

Chemicals

Mg-protoporphyrin IX was from Porphyrin Products (Logan, UT). Red-agarose was from Amicon (Danvers, MA). Tetrabutylammonium phosphate was from Altex (Berkeley, CA). Desferal was from Ciba-Geigy (Tarrytown, NY). All other chemicals were from Sigma, Fisher, or Research Organics (Cleveland, OH).

RESULTS AND DISCUSSION

C. reinhardtii

It was previously shown that intact chloroplasts could be obtained from *C. reinhardtii* cells by nebulization, purified by Percoll gradient centrifugation, and that these chloroplasts had Mg-protoporphyrin IX (oxidative) cyclase activity (Bollivar and Beale, 1995). The cyclase from *C. reinhardtii* chloroplasts, like that from cucumber cotyledon chloroplasts (Chereskin and Castelfranco, 1982; Chereskin et al., 1982; Wong and Castelfranco, 1984), required both NADPH and O_2 for activity. These requirements, together with the demonstration that organisms that form chlorophyll aerobically incorporate an atom from O_2 into the carbonyl group on the isocyclic ring (Walker et al., 1989; Schneegurt and Beale, 1992; Porra et al., 1993), suggest that a monooxygenase is involved in the cyclase reaction.

Two classes of monooxygenase enzymes are hemoproteins and flavoproteins (Walsh, 1979). To investigate whether the cyclase reaction involves a heme- or a flavin-containing enzyme, an inhibitor of each of these classes of enzymes was tested for its effect on cyclase activity in *C. reinhardtii* chloroplasts. No reduction in activity was observed with the addition of quinacrine, a flavin analog that inhibits many flavoprotein enzymes (Izawa and Good, 1972; Dawson et al., 1986), or by pre-exposing the chloroplasts to CO, which binds tightly to hemoprotein oxygenases and inhibits their activity (Walsh, 1979; Dawson et al., 1986) (Table I). The results suggest that neither a heme nor a flavin is a cofactor of the cyclase enzyme. It was previously reported that CO did not inhibit cyclase activity of intact chloroplasts from cucumber cotyledons (Chereskin et al., 1982) and in lysates of these chloroplasts (J.D. Olechno, Y.S. Wong, and P.A. Castelfranco, unpublished results cited in Whyte and Castelfranco, 1993).

The first in vitro studies of cyclase used intact chloroplasts from cucumber cotyledons (Chereskin et al., 1982), wheat leaves (Nasrulhaq-Boyce et al., 1987), and *C. reinhardtii* (Bollivar and Beale, 1995). Attempts to detect activity in lysed chloroplasts were unsuccessful for wheat leaves but did succeed for cucumber cotyledons, in which activity required reconstitution of soluble and membrane-associated chloroplast fractions (Wong and Castelfranco, 1984). Isolated *C. reinhardtii* chloroplasts were lysed by nebulization at high pressure, and the lysed chloroplasts were tested for cyclase activity. The lysed chloroplasts had cyclase activity comparable to that of intact chloroplasts (Table II). The lysed chloroplasts were separated into soluble and membrane fractions by high-speed centrifugation. The soluble fraction had little if any activity, but the membrane fraction had about one-half the activity of the intact chloroplasts. It is interesting that the combined fractions had lower activity than the membrane fraction alone. This result suggests that the soluble fraction contains an inhibitor of cyclase activity. In another experiment, the membrane fraction was washed first with assay buffer and then with assay buffer containing 1 M NaCl; each washing significantly increased the activity. From these results, we conclude that the cyclase activity in *C. reinhardtii* chloroplasts is associated exclusively with the membranes. This result is contrary to the results previously reported for chloroplast extracts from cucumber cotyledons, for which activity required both membrane and soluble fractions (Wong and Castelfranco, 1984; Walker et al., 1991).

Table I. *Effects of carbon monoxide and quinacrine on cyclase activity of intact C. reinhardtii chloroplasts*

For CO-pretreated samples, 100% CO gas was introduced by bubbling the sample for 15 min at 0°C. The samples were then used in standard incubations without explicit removal of the remaining CO. All incubations were supplemented with 2 mM EDTA.

Treatment	Protochlorophyllide Formation	
	pmol mg^{-1} protein	% control
Control	100 ± 6	100
200 μM Quinacrine	112 ± 4	112
CO pretreated	110 ± 4	110

Lysis of the *C. reinhardtii* chloroplasts was assessed by measuring the sensitivity of the cyclase reaction to inhibition by PCMBS, a membrane-impermeable organic mercurial that has been used previously to localize the cyclase enzyme within chloroplasts (Fuesler et al., 1984). More than one-half of the cyclase activity of intact chloroplasts was retained in incubations containing 2.5 mM PCMBS, whereas only 21% of the activity of lysed chloroplasts was retained (Table III). This partial inhibition of intact chloroplasts indicates that they may not be completely intact or that they become partially permeable to PCMBS during the incubation. The 21% residual activity of PCMBS-treated lysed chloroplasts is comparable to the approximately 10% residual activity of PCMBS-treated, reconstituted chloroplast fractions from cucumber cotyledons (Fuesler et al., 1984), and indicates that cyclase may be partially resistant to this inhibitor. In this experiment, NADPH was generated in situ by the reduction of $NADP^+$ through the action of Glc-6-P dehydrogenase and Glc-6-P. Under some conditions, organic mercurials such as PCMBS can inhibit Glc-6-P dehydrogenase. However, the enzyme is protected from this inhibition by the presence of $NADP^+$ at the concentration used in the experiment (Kahn et al., 1972).

Oxygenase reactions can generate reactive oxygen species that are capable of degrading tetrapyrroles. In intact

Table II. *Cyclase activity in C. reinhardtii chloroplast fractions*

Incubations contained extract fractions equivalent to 0.1 g of cells in experiments 1 and 2 and 0.03 g of cells in experiment 3. Incubations were supplemented with 2 mM EDTA.

Fraction	Protochlorophyllide Formation	
	pmol g^{-1} cells	% control
Experiment 1		
Intact chloroplasts	1440 ± 250	100
Lysed chloroplasts	1360 ± 0	94
Experiment 2		
Lysed chloroplasts	1390	100
High-speed supernatant	70	5
High-speed pellet	710	51
High-speed supernatant plus high-speed pellet	470	34
Experiment 3		
Lysed chloroplasts	1600	100
Buffer-washed high-speed pellet	1900	119
Salt-washed high-speed pellet	2033	127

chloroplasts, oxygen radical-scavenging systems exist that can prevent such degradation. However, in lysed chloroplasts these scavenging systems are diluted and may not operate efficiently. Therefore, the effects of two types of reactive oxygen quenchers, catalase and ascorbate or isoascorbate, were tested on the cyclase reaction with salt-washed *C. reinhardtii* chloroplast membranes. The addition of catalase significantly increased the yield of the reaction product (Table IV). In the presence of catalase, ascorbate or isoascorbate further increased the yield of protochlorophyllide. Catalase and isoascorbate were added to all subsequent incubations.

In previous experiments with *C. reinhardtii* chloroplasts, EDTA was added to the isolation and assay buffers to prevent enzyme inactivation by heavy metals that might be present in the preparations. However, EDTA is a strong chelator of Fe^{3+}, and because there is evidence that the cyclase reaction requires iron (see below), the effect of omitting EDTA from the assay buffer was tested with salt-washed chloroplast membranes. The sample without EDTA had significantly higher activity than the control sample (Table IV); therefore, EDTA was omitted from all subsequent incubations. It was previously reported that EDTA inhibited cyclase activity of reconstituted chloroplast fractions from cucumber cotyledons, but this inhibition was attributed to chelation of Mg^{2+} (Whyte et al., 1992).

Table III. *Inhibition of cyclase activity in intact and lysed C. reinhardtii chloroplasts by PCMBS*

Incubations were supplemented with 2 mM EDTA.

Sample	Protochlorophyllide Formation	
	pmol mg^{-1} protein	% control
Intact chloroplasts	143	100
Intact chloroplasts + 2.5 mM PCMBS	80	56
Lysed chloroplasts	136	95
Lysed chloroplasts + 2.5 mM PCMBS	30	21

Although the insensitivity of cyclase activity in intact *C. reinhardtii* chloroplasts to inhibition by CO (Table I) suggested that the reaction does not involve a hemoprotein oxygenase, further attempts were made to inhibit the reaction in salt-washed chloroplast membranes by the hemoprotein inhibitors KCN and NaN_3. Neither compound significantly affected the cyclase activity (Table V). At face value, these results reinforce the conclusion that the cyclase reaction does not involve a hemoprotein oxygenase. However, these results are contrary to those reported for cyclase from cucumber cotyledons, in which the activity in lysed chloroplasts was sensitive to these inhibitors but activity in intact chloroplasts was resistant (Whyte and Castelfranco, 1993). The difference in sensitivity of intact and lysed chloroplasts was attributable to the inability of the inhibitors to penetrate into intact chloroplasts, but this explanation does not seem to be applicable to the cyclase of lysed *C. reinhardtii* chloroplasts. A possible explanation for the difference in the sensitivity of the cyclase in chloroplast lysates from cucumber cotyledons and *C. reinhardtii* to inhibition by KCN and NaN_3 is that the *C. reinhardtii* cyclase, unlike the cucumber cyclase, seems to be associated entirely with the membranes (see above), where it may be inaccessible to the inhibitors.

Irrespective of whether cyclase is a hemoprotein, there is considerable evidence that it requires iron for activity. Iron-deficient plants accumulate Mg-protoporphyrin IX monomethyl ester (Spiller et al., 1982), and cyclase activity in intact wheat leaf chloroplasts (Nasrulhaq-Boyce et al., 1987) and lysates of chloroplasts from cucumber cotyledons (Walker et al., 1991) is inhibited by chelators of iron. We found that cyclase activity in salt-washed *C. reinhardtii* membranes was inhibited by EDTA. Several other iron chelators were tested for their effect on cyclase activity in salt-washed *C. reinhardtii* chloroplast membranes. The Fe^{2+}-chelator α,α'-dipyridyl (Moss and Mellon, 1942) in-

Table IV. *Effect of various changes in the composition of the incubation mixture on cyclase activity of salt-washed chloroplast membranes from C. reinhardtii*

For experiment 1, all incubations were supplemented with 2 mM EDTA, and the control incubation had no added catalase, ascorbate, or isoascorbate. For experiment 2, all incubations were supplemented with 100 μg/mL catalase and 5 mM isoascorbate, and the control incubation had 2 mM EDTA.

Additions to or Deletions from Incubation Mixture	Protochlorophyllide Formation	
	pmol mg^{-1} protein	% control
Experiment 1		
Control	29 ± 0	100
+100 μg/mL catalase	41 ± 5	142
+100 μg/mL catalase + 5 mM ascorbate	80 ± 7	279
+100 μg/mL catalase + 5 mM isoascorbate	95 ± 7	330
Experiment 2		
Control	89 ± 8	100
−EDTA	135 ± 2	152

Table V. *Effect of inhibitors of heme-containing enzymes on cyclase activity of salt-washed C. reinhardtii chloroplast membranes*

Incubations were supplemented with 100 μg/mL catalase and 1 mM isoascorbate.

Treatment	Protochlorophyllide Formation	
	pmol mg^{-1} protein	% control
Control	162 ± 14	100
1 mM KCN	134 ± 14	83
1 mM NaN_3	154 ± 6	95

hibited the reaction, and the inhibition could be partially overcome by adding an equimolar amount of $FeSO_4$ to the incubation (Table VI, experiment 1). However, because $FeSO_4$ itself inhibited the reaction, it was not possible to determine whether the inhibition by α,α′-dipyridyl was caused solely or primarily by its ability to chelate iron. Fe^{2+} was previously reported to inhibit cyclase activity in reconstituted cucumber cotyledon chloroplast fractions, but the inhibition was less than 20% at 500 μM, a concentration that caused more than 50% inhibition of the cyclase in *C. reinhardtii* salt-washed chloroplast membranes.

A second Fe^{2+}-chelating compound, 1,10-phenanthroline (Fortune and Mellon, 1938), also inhibited cyclase activity. As with α,α′-dipyridyl, the addition of $FeSO_4$ to the incubation significantly lessened the inhibition caused by the chelator (Table VI, experiment 2). With this chelator, a greater degree of reversal of the inhibition by added $FeSO_4$ was obtained than with α,α′-dipyridyl. This was possible because the concentration of the chelator required for inhibition was less, and therefore less $FeSO_4$ was required to reverse the inhibition; also, $FeSO_4$ itself was less inhibitory at the lower concentration.

A further test of whether the inhibitory action of 1,10-phenanthroline was caused by its Fe^{2+}-chelating properties was to compare its effects with those of 1,7-phenanthroline, an isomer that does not chelate iron. 1,7-Phenanthroline has been used to distinguish between the effects of 1,10-phenanthroline that are and are not attributable to its chelating ability (Bednarik and Hoober, 1985; Londesborough, 1985; Narasimhan and Miziorko, 1992). The nonchelating compound did not inhibit cyclase at a concentration at which the chelator profoundly inhibited the reaction (Table VI, experiment 3).

Both 1,10-phenanthroline and α,α′-dipyridyl are specific chelators of the Fe^{2+} form of iron (Fortune and Mellon, 1938; Moss and Mellon, 1942). These compounds were more effective than EDTA, which is a chelator of the Fe^{3+} ion (Dawson et al., 1986). However, it is possible that the difference in the inhibitory effectiveness of these compounds is not caused by their differential ability to chelate Fe^{2+} and Fe^{3+} ions, because the two tested Fe^{2+} chelators are more lipophilic than EDTA, which is strongly anionic at physiological pH, and they may be better able to reach the site of the required iron atom within the membranes. Therefore, another iron chelator, Desferal, was tested. This compound, like EDTA, is specific for the Fe^{3+} ion (Dawson et al., 1986), but it is more lipophilic than EDTA. Although Desferal inhibited cyclase to some extent, the degree of inhibition was much less than that caused by much lower concentrations of 1,10-phenanthroline. We interpret these results to indicate that the cyclase enzyme in salt-washed *C. reinhardtii* chloroplasts requires Fe^{2+} ions for activity. The small degree of inhibition by Fe^{3+} chelators may be indirect, possibly caused by strong chelation of the small amounts of Fe^{3+} present in equilibrium with Fe^{2+} in the membranes and eventual depletion of all Fe ions.

Several other metal ions, including Zn^{2+}, Mn^{2+}, Cu^{2+}, and Fe^{3+}, were tested for the ability to reverse the inhibition of the cyclase caused by Fe^{2+} chelators. None of these ions was able even partially to reverse the inhibition (data not shown).

With the improved incubation medium containing catalase and isoascorbate without added EDTA, the cyclase activity obtained with salt-washed *C. reinhardtii* chloroplast membranes routinely was considerably higher than the activity of intact chloroplasts. This increased activity was obtained whether the activity was expressed per equivalent weight of extracted cells or per milligram of protein. However, attempts to further purify or fractionate the chloroplast membranes were unsuccessful. All activity was lost upon the addition of detergents or disintegration of the membranes with the French press (data not shown).

Synechocystis sp. PCC 6803

Because we were unable to solubilize cyclase activity from salt-washed *C. reinhardtii* chloroplast membranes, we explored other cell types as sources of solubilizable cyclase. Cyclase activity was obtained from disrupted cells of the

Table VI. *Effects of iron chelators on cyclase activity of salt-washed C. reinhardtii chloroplast membranes*

Incubations were supplemented with 100 μg/mL catalase, 1 mM isoascorbate, and 0.3 mg/L *E. coli* protein extract containing recombinant *R. capsulatus* *S*-adenosyl-L-methionine:Mg-protoporphyrin IX methyltransferase.

Addition to Incubation Mixture	Protochlorophyllide Formation	
	pmol mg^{-1} protein	% control
Experiment 1		
Control	194 ± 7	100
500 μM α,α′-dipyridyl	46 ± 2	24
500 μM α,α′-dipyridyl and then 500 μM $FeSO_4$	71 ± 3	37
500 μM $FeSO_4$	95 ± 4	49
Experiment 2		
Control	180 ± 6	100
100 μM 1,10-phenanthroline	92 ± 14	51
100 μM 1,10-phenanthroline then 200 μM $FeSO_4$	127 ± 3	71
200 μM $FeSO_4$ and then 100 μM 1,10-phenanthroline	126 ± 1	70
200 μM $FeSO_4$	141 ± 1	78
Experiment 3		
Control	96 ± 2	100
100 μM 1,10-phenanthroline	19 ± 5	20
100 μM 1,7-phenanthroline	108 ± 3	113
500 μM 1,10-phenanthroline	20 ± 1	21
500 μM Desferal	72 ± 4	75

cyanobacterium *Synechocystis* sp. PCC 6803. Fluorescence emission spectra of the unincubated control and a 1-h incubated sample showed the accumulation of a compound that fluoresces at 630 nm, the fluorescence emission maximum of protochlorophyllide (Fig. 2). The HPLC elution profiles confirm that protochlorophyllide was produced. The unincubated control sample contained some protochlorophyllide, as is indicated by the elution peak at 16 min, but the amount of protochlorophyllide dramatically increased during a 1-h incubation (Fig. 3). The elution of standard protochlorophyllide from etiolated cucumber cotyledons at 16 min confirmed that the incubation product was protochlorophyllide. Low-temperature (77K) fluorescence excitation spectra were taken to determine whether the incubation product was monovinyl or divinyl protochlorophyllide. The excitation maximum (443 nm) that was determined for the incubated sample (Fig. 4) indicates that the accumulated product was divinyl protochlorophyllide (Tripathy and Rebeiz, 1985).

The reaction requirements of the enzyme from *Synechocystis* sp. PCC 6803 are generally similar to those determined for *C. reinhardtii*. Both NADPH and Mg-protoporphyrin IX were required for product formation in the incubation system containing *S*-adenosyl-L-Met:Mg-protoporphyrin IX methyltransferase derived from a lysate of an *Escherichia coli* strain carrying a plasmid expressing the *R. capsulatus bchM* gene (Bollivar et al., 1994). All activity was lost when the cell extract was briefly heated to 100°C (data not shown). Substitution of Mg-mesoporphyrin IX for the natural tetrapyrrole substrate resulted in the accumulation of the diethyl analog of protochlorophyllide to approximately 50% of the normal product yield (data not shown), as was previously reported for the cyclase of intact cucumber cotyledon chloroplasts (Walker et al., 1988).

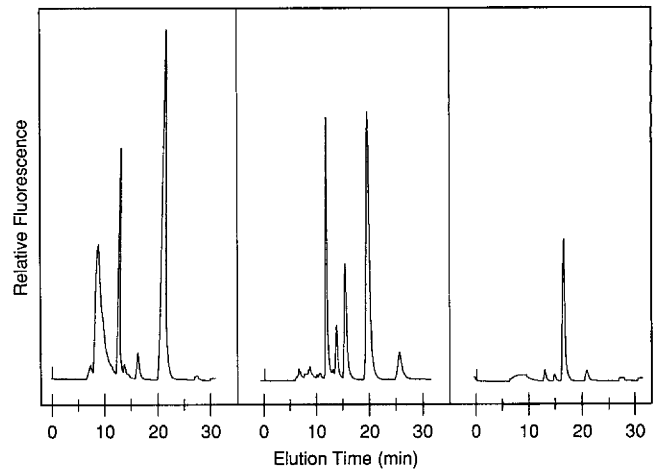

Figure 3. HPLC elution profiles of ether extracts from (left) unincubated control and (middle) 1-h-incubated samples containing low-speed supernatant from disrupted *Synechocystis* sp. PCC 6803 cells and (right) standard protochlorophyllide extracted from etiolated cucumber cotyledons.

As was observed with cyclase derived from *C. reinhardtii* chloroplasts, cyanobacterial cyclase was inhibited by the Fe^{2+} chelator 1,10-phenanthroline and not by the non-chelating isomer 1,7-phenanthroline, and the inhibition by 1,10-phenanthroline was blocked by $FeSO_4$ (Table VII). In contrast to the *C. reinhardtii* cyclase, which was inhibited by $FeSO_4$, the cyanobacterial cyclase was stimulated to levels greater than the control level by $FeSO_4$ when it was added with 1,10-phenanthroline. 3-(2-Pyridyl)-5,6-bis(4-phenylsulfonic acid)-1,2,4-triazine, a hydrophilic Fe^{2+} ion chelator (Stookey, 1970), had relatively little effect on the cyanobac-

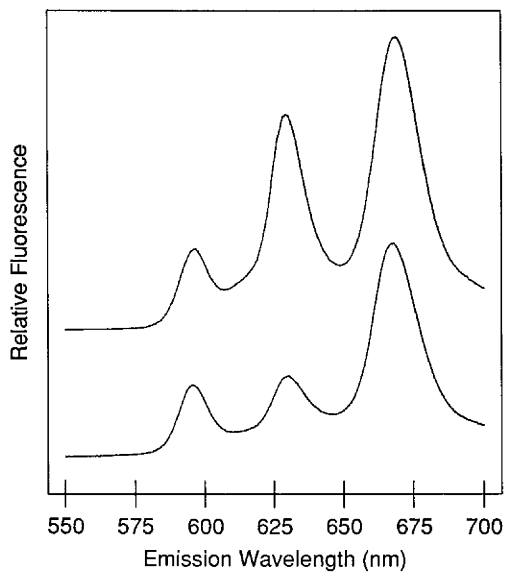

Figure 2. Room-temperature fluorescence emission spectra of ether extracts from (top) 1-h-incubated and (bottom) unincubated control samples containing low-speed supernatant from disrupted *Synechocystis* sp. PCC 6803 cells. Excitation was at 437 nm.

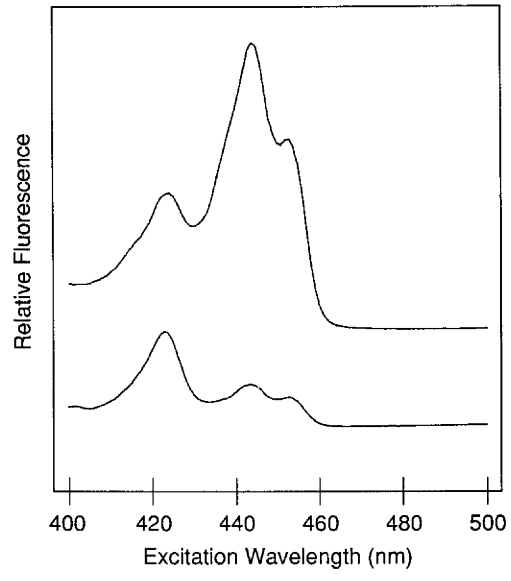

Figure 4. Low-temperature (77K) fluorescence excitation spectra of ether extracts from (top) 1-h-incubated and (bottom) unincubated control samples containing low-speed supernatant from disrupted *Synechocystis* sp. PCC 6803 cells. Emission was measured at 630 nm.

Table VII. *Effects of iron chelators on cyclase activity of Synechocystis sp. PCC 6803 extract*

Incubations contained low-speed supernatant fraction from cell lysate and were supplemented with 100 μg/mL catalase and 1 mM isoascorbate.

Addition to Incubation Mixture	Protochlorophyllide Formation	
	pmol mg^{-1} protein	% control
Experiment 1		
Control	60 ± 1	100
+200 μM 1,10-phenanthroline	32 ± 2	53
+200 μM 1,7-phenanthroline	54 ± 6	90
+200 μM 1,10-phenanthroline then 300 μM $FeSO_4$	87 ± 3	145
+300 μM $FeSO_4$ and then 200 μM 1,10-phenanthroline	105 ± 4	175
Experiment 2		
Control	37 ± 2	100
+200 μM 1,10-phenanthroline	20 ± 1	54
+200 μM 1,10-phenanthroline then 300 μM $FeSO_4$	48 ± 5	130
+500 μM 3-(2-pyridyl)-5,6-bis (4-phenylsulfonic acid)-1,2, 4-triazine	31 ± 3	84

terial cyclase. These results suggest that the cyanobacterial cyclase, like that of *C. reinhardtii*, requires Fe^{2+} for activity and that the Fe^{2+} is located within a hydrophobic region of an enzyme or membrane.

Fractionation, solubilization, and partial purification of the cyanobacterial cyclase were achieved. Activities of the various fractions are shown in Table VIII. The low-speed supernatant was resolved into a membrane fraction and a soluble fraction by ultracentrifugation. Neither fraction had appreciable activity alone, and the residual activities of the individual fractions varied between experiments. This probably reflects the differences in the completeness of separation of the two fractions. When the membrane and soluble fractions were recombined, the specific activity was somewhat higher than that of the starting material. The high-speed supernatant was chromatographed through Red-agarose to remove traces of the membrane material. The active component in the high-speed supernatant was not retained on Red-agarose.

The membrane fraction was solubilized by the addition of 1% (w/v) *n*-octyl-β-D-glucopyranoside in the presence of 1 M glycerol and 10 mM $MgCl_2$. After centrifugation for 1 h at 285,000*g* the membrane-associated component of the cyclase remained in the supernatant, as is shown by the cyclase activity of the supernatant samples reconstituted with the original high-speed supernatant. The pellet fraction of solubilized membranes contained no appreciable activity when reconstituted with the original high-speed supernatant (data not shown). *n*-Octyl-β-D-glucopyranoside was the only tested detergent that solubilized the membrane-bound cyclase component in an active form. The solubilized cyclase component did not retain activity after freezing and thawing, but it was sufficiently stable to permit partial purification, as described below.

The solubilized membrane fraction was chromatographed on Red-agarose, and the active fraction was eluted with lysis buffer containing 1 M NaCl. This step achieved a significant increase in purity, as expressed by the reconstituted activity per milligram of detergent-solubilized protein fraction. Very little activity was detected in the flow-through (unbound) column fraction. It was necessary to remove excess NaCl from the Red-agarose-bound fraction before activity could be measured. During the Sephadex G-25 chromatography step that was used to remove the NaCl, it was necessary to maintain the $MgCl_2$ concentra-

Table VIII. *Purification of cyclase activity from Synechocystis sp. PCC 6803*

In experiment 1, the high-speed supernatant was used directly in the incubations. In experiment 2, the high-speed supernatant was chromatographed through Red-agarose before use in the incubations. All incubations were supplemented with 100 μg/mL catalase and 1 mM isoascorbate. ND, Not determined.

Cell Extract Fraction	Protochlorophyllide Formation	
	pmol mg^{-1} protein	pmol mg^{-1} high-speed pellet protein
Experiment 1		
Unfractionated low-speed supernatant	28 ± 1	
High-speed supernatant	6 ± 0	
High-speed pellet	13 ± 5	
High-speed supernatant plus high-speed pellet	35 ± 2	111 ± 9
Experiment 2		
Unfractionated low-speed supernatant	60 ± 1	
High-speed supernatant	13 ± 1	
High-speed pellet	6 ± 1	
High-speed supernatant plus:		
High-speed pellet	64 ± 0	136 ± 0
Solubilized high-speed pellet	56 ± 6	123 ± 19
Red-agarose-unbound solubilized high-speed pellet	15 ± 6	24 ± 10
Red-agarose-bound solubilized high-speed pellet	50 ± 4	310 ± 30
DEAE-cellulose-unbound fraction	10 ± 0	ND
DEAE-cellulose 50 mM NaCl eluate	34 ± 1	430 ± 35
DEAE-cellulose 150 mM NaCl eluate	2 ± 0	ND

tion at 10 mM or all cyclase activity was lost (data not shown). This result is consistent with the observation by Whyte et al. (1992) that 10 mM $MgCl_2$ is the optimal concentration for cyclase activity in reconstituted chloroplast fractions from cucumber cotyledons.

The desalted Red-agarose-bound fraction was chromatographed on DEAE-cellulose, and the active fraction was eluted with 50 mM NaCl. This step produced another significant increase in purification; further purification steps did not yield reproducible results.

CONCLUSIONS

The results presented here represent a significant advance in the elucidation of the reaction requirements for Mg-protoporphyrin IX monomethyl ester (oxidative) cyclase. The results indicate that Fe^{2+} is required for activity of the cyclase from both eukaryotic and prokaryotic sources and also suggest that the Fe^{2+} ion is associated with the membrane component of the enzyme. A role for Mg^{2+} in stabilizing the enzyme has been established, but the mechanism of this function needs further clarification.

In addition to determining the metal requirements for cyclase, several advances have been made in identifying incubation conditions that favor the formation of protochlorophyllide, including the presence of catalase and a general reducing agent such as ascorbate or isoascorbate. The role of catalase is most likely to decrease the destruction of the product rather than directly stimulate the enzyme, but the role of ascorbate or isoascorbate is not as clear. The observation that either of these reductants is effective suggests that they function in a general capacity to provide reducing conditions rather than by acting at a specific enzyme active site; however, further experiments will be required to verify this conclusion.

The extraction of active cyclase from a prokaryotic organism and the development of a method to solubilize the membrane-associated component of the cyclase are the first such reports to our knowledge. Although the solubilized membrane component appears to be rather labile, it is encouraging that it could be solubilized and then further purified by dye-affinity and ion-exchange chromatography. The use of two column chromatography separations in a series led to a significant degree of purification of the membrane-associated enzyme fraction; however, further work will be required to fully purify the enzyme.

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