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Formation of Mg-Containing Chlorophyll Precursors from Protoporphyrin IX, δ-Aminolevulinic Acid, and Glutamate in Isolated, Photosynthetically Competent, Developing Chloroplasts[1]

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ABSTRACT

Intact developing chloroplasts isolated from greening cucumber (*Cucumis sativus* L. var Beit Alpha) cotyledons were found to contain all the enzymes necessary for the synthesis of chlorophyllide. Glutamate was converted to Mg-protoporphyrin IX (monomethyl ester) and protochlorophyllide. δ-Aminolevulinic acid and protoporphyrin IX were converted to Mg-protoporphyrin IX, Mg-protoporphyrin IX monomethyl ester, protochlorophyllide and chlorophyllide *a*. The conversion of δ-aminolevulinic acid or protoporphyrin IX to Mg-protoporphyrin IX (monomethyl ester) was inhibited by AMP and *p*-chloromercuribenzene sulfonate. Light stimulated the formation of Mg-protoporphyrin IX from all three substrates. In the case of δ-aminolevulinic acid and protoporphyrin IX, light could be replaced by exogenous ATP. In the case of glutamate, both ATP and reducing power were necessary to replace light. With all three substrates, glutamate, δ-aminolevulinic acid, and protoporphyrin IX, the stimulation of Mg-protoporphyrin IX accumulation in the light was abolished by DCMU, and this DCMU block was overcome by added ATP and reducing power.

The path from general, noncommitted metabolites to Chl has been reported to occur entirely within the developing chloroplasts of greening plant cells (19, 23). In higher plants, the Chl biosynthetic scheme begins with the conversion of Glu[2] to ALA (6, 7) which is then further metabolized to pyrrolic and tetrapyrrolic intermediates. In the present paper we describe the isolation of intact, developing chloroplasts from greening cucumber cotyledons. These plastids are able to form Chlide and Chlide precursors using Glu, ALA, Proto, and Mg-Proto Me as substrates. The activity which is responsible for the conversion of Mg-Proto Me to Mg-2,4-divinyl pheoporphyrin a_5 (DV Pchlide) has been studied in isolated developing chloroplasts. Previous publications on this subject include references (10–12). This catalytic activity is referred to in the present paper as 'Mg-Proto Me (oxidative) cyclase' or simply as 'cyclase.' Several aspects of the reaction requirements and the regulation mechanisms are discussed. The general field of chlorophyll biosynthesis has been reviewed recently (6, 7).

MATERIALS AND METHODS

Reagents. ATP, EDTA, Hepes, Tes, Cys, ALA, Glu, SAM, Percoll, PCMBS, Glc-6-P, NADP, NADPH, and NADH were purchased from Sigma. PCMB and BSA (fatty acid poor) were obtained from Calbiochem. DCMU (technical grade) was recrystallized from ethanol. Acetone, Hexanes, and diethyl ether (for anesthesia) were obtained from Mallinckrodt. MB was purchased from Matheson, Coleman, and Bell. Proto (Porphyrin Products, Logan, UT) was purified as previously described (14). Mg-Proto Me was synthesized and purified according to Chereskin *et al.* (12). PCMBS was purified from inorganic mercury by passing a solution of PCMBS through a small column (1 to 2 ml bed volume) of Dowex 50W-X8 (H^+ form). The PCMBS was eluted with water and the concentration determined by measuring the absorbance at 265 nm ($\epsilon_M = 611$ at pH 7.5 [24]).

Plant Material and Chloroplast Isolation. Cucumber seeds (*Cucumis sativus* L. var Beit Alpha), obtained from Moran Seeds, Inc., Modesto, CA, were germinated in complete darkness at room temperature for 5 to 6 d. After 20 h illumination (60 to 80 $\mu E \cdot m^{-2} \cdot s^{-1}$ PAR, at 28 to 30°C), the cotyledons were harvested and developing chloroplasts were isolated as described in "Results."

Incubation Conditions. *Mg-Proto (Me) Accumulation.* Routine incubations contained in 1 ml; 500 μmol sorbitol, 10 μmol Hepes, 20 μmol Tes, 1 μmol EDTA, 1 μmol $MgCl_2$, 2 mg BSA, 8 μmol ATP, and 2 to 4 mg plastid protein as determined by the biuret method. Ten nmol Proto, 6 μmol ALA, or 6 μmol Glu served as substrate for the metalloporphyrin synthesis. Incubations were for 30 min at 29 to 30°C (pH 7.7) and were terminated by freezing at −15°C or by the addition of cold acetone. Further additions to the reaction mixtures are given in figure and table legends.

Mg-Proto Me (Oxidative) Cyclase. The plastid preparations were depleted of endogenous Pchlide by a 5-min light exposure to 40 to 50 $\mu E \cdot m^{-2} \cdot s^{-1}$ PAR (12). Routine incubations contained in 1 ml; 500 μmol sorbitol, 10 μmol Hepes, 20 μmol Tes, 1 μmol EDTA, 1 μmol $MgCl_2$, 10 nmol Mg-Proto Me, 1 μmol SAM, and 6 to 12 mg plastid protein. Incubations were for 1 h at 29 to 30°C (pH 7.7) in complete darkness to prevent phototransformation or photodestruction of newly formed DV-Pchlide. Re-

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[2] Abbreviations: Glu, glutamic acid; ALA, δ-aminolevulinic acid; DV, divinyl; MB, methylene blue; Mg-Proto, Mg-protoporphyrin IX; Mg-Proto Me, Mg-protoporphyrin IX monomethyl ester; Mg-Proto (Me), a mixture of Mg-Proto and Mg-Proto Me of undefined proportion; MV, monovinyl; PCMB, *p*-chloromercuribenzoate; PCMBS, *p*-chloromercuribenzene sulfonate; Proto, protoporphyrin IX; SAM, *S*-adenosylmethionine; Cys, cysteine; Glc-6-P, glucose 6-P.

actions were terminated by freezing at −15°C.

Light Conditions. Incubations in the light were under 40 to 50 $\mu E \cdot m^{-2} \cdot s^{-1}$ PAR. Complete darkness was achieved by wrapping incubation flasks in aluminum foil. All other incubations were conducted under low illumination (1 to 3 $\mu E \cdot m^{-2} \cdot s^{-1}$ PAR).

Pigment Extraction. Routine extraction of metalloporphyrins was described elsewhere (9). Diethyl ether extracts were further purified prior to HPLC analysis as follows: diethyl ether solutions (5 to 7 ml) were placed in 40 ml conical glass centrifuge tubes and shaken with 25 to 35 ml of 50 mM K-phosphate (pH 6.8). The ether phase was withdrawn. The aqueous phase was checked and readjusted to pH 6.8 if necessary, and extracted with 5 ml diethyl ether. The combined ether extracts were washed once with 10 ml H_2O, filtered through Rainin Nylon-66, 0.45 μm pore size filters, and concentrated in a stream of N_2. Chl was determined in 0.4 ml of plastid suspension by extracting with 80% acetone containing 10 mM NH_4OH. The pigment absorbance was determined spectrophotometrically, and the total Chl was calculated from the equations of Anderson and Boardman (1).

HPLC. Pigments were separated using the solvent system of Fuesler *et al.* (13) and a Rainin Microsorb "Short-One" 3 μm C-18 reverse-phase column equipped with a Brownlee 3 cm MPLC Cartridge system and an RP-18 Spheri-5 Guard system. To separate MV and DV Pchlides and Chlides, the method of Hanamoto and Castelfranco was used (16).

RESULTS

Plastids were isolated from cucumber cotyledons by a modification of the procedure of Pardo *et al.* (18). One-half M sorbitol was substituted for 0.5 M sucrose as the osmoticum in the grinding buffer. The 'Pellet 2' obtained from approximately 28 g of cotyledonary tissue was resuspended in 10 ml of grinding buffer (0.5 M sorbitol, 10 mM Hepes, 20 mM Tes, 1 mM EDTA, 1 mM $MgCl_2$, 5 mM Cys, and 0.2% BSA, pH 7.7). Five ml of the suspension was layered over 35 ml of 45% Percoll in the identical grinding buffer, contained in a 50 ml centrifuge tube. For a typical isolation starting with 28 g of plant tissue, two such tubes were used. The tubes were centrifuged in a Sorvall RC-5 centrifuge at 6000*g* for 5 min using an HB-4 rotor. The Chl-containing material was visibly separated into two areas: a wide band near the top of the tube consisting of broken and damaged plastids, and a small pellet consisting of essentially intact plastids. The pellet was resuspended gently in incubation buffer (grinding buffer without Cys). The green upper band was collected with a Pasteur pipette, diluted with 25 ml of grinding buffer without Percoll, and sedimented at 27,000*g* for 5 min. The resulting pellet was resuspended in incubation buffer.

Under transmission electron microscopy, the 'intact chloroplast pellet' appeared to be homogeneous, totally free of mitochondria, largely free of microbodies, and relatively free of unidentifiable debris. The double membrane envelope appeared to be intact, and there was uniform retention of stroma. In these plastids, there appeared to be very few remaining prolamellar bodies.

The two Chl-containing fractions were tested for Mg-chelatase and Mg-Proto Me cyclase activities (Table I). The broken chloroplasts had negligible Mg-chelatase activity and low Mg-Proto Me cyclase activity. The intact chloroplasts were very active in both assays. The specific activity of Mg-Proto Me cyclase was at least as great as that reported previously for sucrose-isolated plastids (12). In the case of Mg-chelatase, the Percoll isolation improved the enzymatic activity 2- to 3-fold (14).

During the course of this investigation, different substrates of the Chl-biosynthetic pathway were incubated with Percoll-isolated plastids, and the accumulation of subsequent intermediates in the Chl-biosynthetic pathway was monitored spectrofluorometrically.

Incubation with Proto. Chloroplasts incubated with 10 μM Proto formed Mg-Proto (Me), Pchlide, or Chlide *a* depending on the incubation conditions (Table II). In the presence of ATP (line 3), Mg-Proto (Me) was the major product. Trace amounts of Pchlide were detected, as well as some Chlide *a* which was probably a breakdown product of the endogenous Chl during the course of incubation and extraction. When SAM was added (line 4), Mg Proto (Me) dropped by more than half, while Pchlide became the dominant product. If MB was added in addition to ATP and SAM, Pchlide was not formed (12) and Mg-Proto (Me) accumulated (line 5). Finally, chloroplasts incubated with Proto, ATP, and SAM in the light (line 6) accumulated Chlide *a*. The three products that were measured spectrofluorometrically [*i.e.* Mg-Proto (Me), Pchlide, and Chlide *a*] can be heterogeneous. Mg-Proto (Me) may consist of Mg-Proto and Mg-Proto Me (13) and Pchlide and Chlide *a* may consist of the DV and MV forms (16). HPLC analysis (data not shown) revealed that the Mg-Proto (Me) formed in the absence of added SAM (line 3) was almost exclusively the dicarboxylic acid (Mg-Proto), while the Mg-Proto (Me) accumulated in the presence of SAM and MB (line 5) was almost exclusively the monomethyl ester (Mg-Proto Me). The Pchlide accumulated in the dark in the presence of ATP and SAM (line 4) consisted of the DV form with only a trace of the MV form, while the Chlide *a* accumulated in the light in the presence of ATP and SAM (line 6) was MV.

Incubation with ALA. Percoll-isolated chloroplasts incubated with 6 mM ALA gave results (Table III) that were entirely comparable with those obtained with 10 μM Proto (Table II). If ATP was added (line 3), the main spectrofluorometrically measured product was Mg-Proto (Me). If the sample was incubated in the dark with ATP and SAM (line 4), Mg-Proto (Me) dropped and Pchlide accumulated. If the sample was incubated with ATP, SAM, and MB (line 5), no Pchlide was formed while Mg-Proto (Me) accumulated. Finally, in the light and in the presence of ATP and SAM (line 6), Chlide *a* accumulated. HPLC analysis revealed the same pattern observed with 10 μM Proto as the substrate (data not shown). In the presence of ATP (line 3), the Mg-Proto (Me) obtained consisted mainly of the dicarboxylic acid, while Mg-Proto Me accumulated in the presence of SAM and MB (line 5). Dark incubation with ATP and SAM (line 4) gave DV Pchlide with only a trace of MV-Pchlide. Light incu-

Table I. *Mg-Chelatase and Mg-Proto Me (Oxidative) Cyclase Activities of Broken and Intact Developing Chloroplast Fractions*

The two fractions were separated by Percoll centrifugation as described in the text. The products measured in the two cases were Mg-Proto and DV-Pchlide, respectively. Proto (10 μM) was used as substrate for Mg-chelatase, and Mg-Proto Me (10 μM) for the cyclase. The entries in all tables give means and experimental ranges.

	Mg-Chelatase		Mg-Proto Me Cyclase	
	pmol/h·μg Chl	*pmol/h·mg protein*	*pmol/h·μg Chl*	*pmol/h·mg protein*
Broken chloroplasts	0.11 ± 0.0	7.3 ± 0.0	0.81 ± 0.17	52 ± 11
Intact chloroplasts	31 ± 0.74	1256 ± 30	12 ± 0.02	465 ± 1.0

Table II. *Accumulation of Mg-Proto (Me), Pchlide, and Chlide from Proto in Intact Plastids*

Experiments 1 and 2 were performed on a different day than experiments 3 to 6. Plastids used in experiments 2 and 4 were given a 5-min light treatment as described in "Materials and Methods". Experiments 3 to 6 included 10 μM Proto. Incubations were in complete darkness, except for experiment 6 which was incubated under 40–50 $\mu E \cdot m^{-2} \cdot s^{-1}$ PAR.

Incubation Conditions	Mg-Proto (Me)	Pchlide	Chlide *a*
	pmol/h·mg protein		
No additions	Trace	77 ± 0	978 ± 96
No additions, light pretreatment	Trace	Trace	873 ± 4
8 mM ATP	1464 ± 119	287 ± 38	503 ± 45
8 mM ATP, 1 mM SAM, light pretreatment	637 ± 17	1205 ± 12	831 ± 76
8 mM ATP, 1 mM SAM, 20 μM MB	1230 ± 16	81 ± 4	657 ± 77
8 mM ATP, 1 mM SAM, light incubation	149 ± 12	152 ± 5	2235 ± 35

Table III. *Accumulation of Mg-Proto (Me), Pchlide, and Chlide from ALA in Intact Plastids*

Conditions for these experiments were exactly as described in Table II, except that in experiments 3 to 6, 6 mM ALA was substitued for 10 μM Proto.

Incubation Conditions	Mg-Proto (Me)	Pchlide	Chlide *a*
	pmol/h·mg protein		
No additions	Trace	83 ± 4	972 ± 20
No additions, light pretreatment	Trace	Trace	947 ± 20
8 mM ATP	1508 ± 58	338 ± 7	528 ± 22
8 mM ATP, 1 mM SAM, light pretreatment	482 ± 46	1147 ± 93	846 ± 46
8 mM ATP, 1 mM SAM, 20 μM MB	1448 ± 32	69 ± 4	592 ± 20
8 mM ATP, 1 mM SAM, light incubation	97 ± 4	139 ± 4	2200 ± 137

Table IV. *Inhibition of Mg-Proto (Me) Accumulation by AMP with ALA or Proto Substrates*

Plastids were incubated either with 6 mM ALA or 10 μM Proto and with or without 10 mM AMP.

Incubation Conditions	Mg-Proto (Me)	Inhibition
	pmol/h·mg protein	%
ALA	1806 ± 31	0
ALA, AMP	581 ± 30	68
Proto	1787 ± 40	0
Proto, AMP	443 ± 11	75

bation with ATP and SAM (line 6) yielded MV-Chlide *a*.

Comparison of ALA and Proto as Substrates for Mg-Proto Formation. The formation of Mg-Proto from ALA was dependent upon exogenous ALA. Maximal activity occurred at about 6 mM. The dependence upon Proto concentration was studied previously (14); maximal activity was observed at 10 μM Proto.

Assuming that in the presence of 6 mM ALA the sequence of reactions between ALA and Proto is not rate limiting, the course of Mg-Proto (Me) formation from ALA should be affected by co-substrates and inhibitors in much the same way as the course of reaction starting with Proto. This is, in fact, what was observed. The ATP concentration dependence of Mg-Proto formation was essentially the same with both substrates. Both activities saturated at 8 mM ATP, a value that is not very different from that reported previously for sucrose-isolated plastids (14). Mg chelation is inhibited by AMP (18). Mg-Proto (Me) formation was inhibited to approximately the same extent regardless of whether the substrate for the reaction was 10 μM Proto or 6 mM ALA (Table

Table V. *Inhibition of Mg-Proto (Me) Accumulation by PCMBS with ALA or Proto Substrates*

Eight mM ATP was included in each incubation, except where mentioned to the contrary. In experiment 1, plastids were incubated either with 6 mM ALA or 10 μM Proto, with or without 1 mM PCMBS, under room illumination. Light conditions in experiment 2 were as in Table II.

Incubation Conditions	Mg-Proto (Me)	Inhibition
	pmol/h·mg protein	%
Experiment 1		
ALA	2404 ± 36	0
ALA, PCMBS	385 ± 30	84
Proto	2400 ± 11	0
Proto, PCMBS	474 ± 4	80
Experiment 2		
Proto, light incubation, no added ATP	1147 ± 56	0
Proto, PCMBS, light incubation, no added ATP	137 ± 17	88
Proto, dark incubation	1127 ± 59	0
Proto, PCMBS, dark incubation	164 ± 55	86

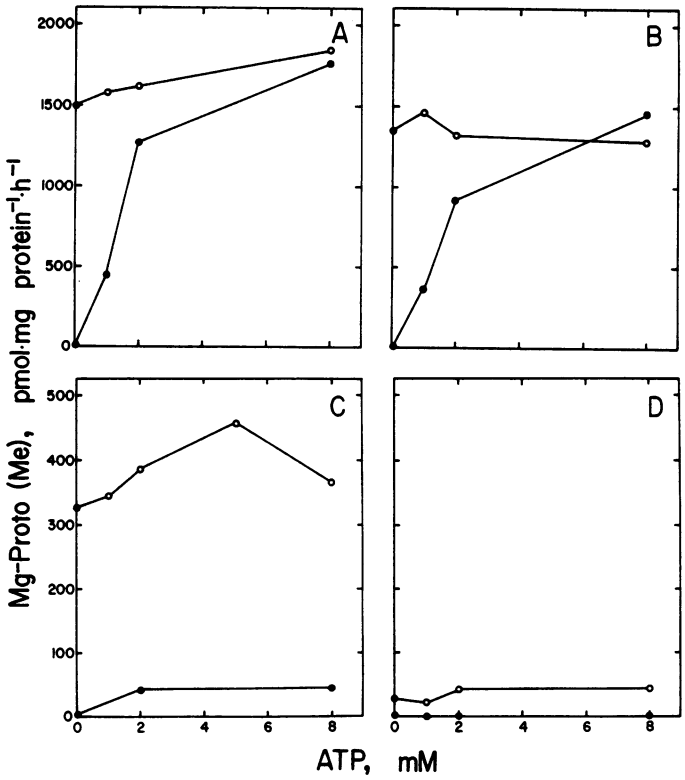

FIG. 1. Effect of light incubation on Mg-Proto (Me) accumulation with different substrates: A, Proto (10 μM); B, ALA (6 mM); C, Glu (6 mM); D, no added substrate. O——O, Light incubations (40 to 50 $\mu E \cdot m^{-2} \cdot s^{-1}$ PAR). ●——●, Dark incubations.

IV). Moreover, Mg-Proto (Me) formation in the uninhibited reaction was the same from both substrates (Table IV).

Sulfhydryl complexing agents like PCMB and *N*-ethylmaleimide inhibit Mg chelatase (Fuesler and Castelfranco, unpublished). The nonpenetrant mercurial PCMBS is also inhibitory, suggesting that some step of Mg chelation occurs at a site that is easily accessible from the medium (24). It was of interest to see whether Mg-Proto (Me) formation was inhibited to the same extent by PCMBS regardless of which of the two substrates was used. One mM PCMBS inhibited Mg-Proto (Me) formation from both substrates to about the same extent (Table V, experiment 1). PCMBS also inhibited the accumulation of Mg-Proto (Me) from Proto in the absence of added ATP, when the incubation was carried out under strong light (Table V, experiment 2). Again, it is worth noting the quantitative agreement of the uninhibited reactions incubated in the light without added ATP, or in the dark with added ATP.

Anaerobiosis did not inhibit Mg-Proto (Me) synthesis from Proto. It was of interest to see whether Mg-Proto (Me) formation from ALA was inhibited under anaerobic conditions because coproporphyrinogenase has been reported to require O_2 (2, 17, 20), while Mg-Proto (Me) accumulation has been observed in plants grown in O_2-poor environments (21). No effect of anaerobiosis was detected on the conversion of ALA to Mg-Proto (Me) when the reaction vessel was flushed with N_2 for 5 min before the substrate was added through a serum cap with a hypodermic syringe. However, if alkaline pyrogallol (21) was added to the center well of the incubation flask and the vessel was flushed with N_2 as before, an 80% inhibition of Mg-Proto (Me) formation from ALA could be demonstrated (data not shown).

Comparison of ALA and Glu as Substrates for Mg-Proto (Me) and Pchlide Formation. A saturating concentration of Glu (6 mM) was about 20% as effective in the accumulation of Mg-Proto (Me) as ALA at the same concentration (data not shown). Addition of α,α'-dipyridyl and NADP gave slight stimulations in the accumulation of Mg-Proto (Me) from Glu, qualitatively confirming a previous observation (8). If 1 mM SAM was added, as well as 8 mM ATP, and the mixture was incubated in the dark, both substrates were converted to DV-Pchlide. Glu was 20% to 30% as effective as ALA in the formation of DV-Pchlide. In the presence of SAM, Glu did not give rise to any accumulation of Mg-Proto (Me); while some Mg-Proto (Me) was accumulated from ALA (data not shown).

Effect of Light on Mg-Proto (Me) Accumulation. Figure 1 shows the effect of light on Mg-Proto (Me) accumulation from various substrates. In the dark, using Proto (Fig. 1A) or ALA (Fig. 1B) as substrate, Mg-Proto (Me) accumulated depending upon the addition of ATP. However, light could replace the requirement for ATP; and if the incubation was carried out in the light, Mg Proto (Me) accumulation did not respond to further addition of ATP.

With Glu as substrate (Fig. 1C), there was very little response to the addition of ATP in the dark. Good activity in the conversion of Glu to Mg-Proto was observed in the dark in the presence of ATP plus reducing power (NADPH and Glc-6-P) (Table VI, line 5). Light did support this conversion (Table VI, line 1), but additional stimulation was observed in the light with added ATP and with added reducing power (Table VI, lines 2, 3, 4). Either ATP alone or reducing power alone in the dark did not support Mg-Proto (Me) synthesis from Glu (data not shown).

DCMU completely inhibited the accumulation of Mg-Proto (Me) if the plastids were incubated in the light with either Proto or ALA (Table VII, experiments 1 and 3). However, DCMU did

Table VI. *Response of Mg Proto Accumulation from Glu to Added Cofactors*

All samples contained 6 mM Glu. The cofactors were added at 5 mM concentration. Light conditions were as in Table II.

Incubation Conditions	Mg-Proto (Me)	Percent of Illuminated Control
	pmol/h·mg protein	%
Light	141 ± 14	100
Light, ATP	235 ± 22	167
Light, NADPH, Glc-6-P	174 ± 3	123
Light, ATP, NADPH, Glc-6-P	297 ± 3	211
Dark, ATP, NADPH, Glc-6-P	220 ± 0	156

Table VII. *Inhibition of Mg-Proto (Me) Accumulation by DCMU with Proto or ALA Substrates*

Eight mM ATP was included where mentioned. In experiments 1 and 2, 10 μM Proto was added, and in experiments 3 and 4, 6 mM ALA was added. DCMU (5 nmol) was added in 25 μl ethanol, and an equal volume of ethanol was added to the controls. Light conditions were as in Table II.

Incubation Conditions	Mg-Proto (Me)
	pmol/h·mg protein
Experiment 1	
Proto, light	1360 ± 33
Proto, light, DCMU	Trace
Proto, ATP, dark	1682 ± 20
Proto, ATP, dark, DCMU	1730 ± 43
Experiment 2	
Proto, light	898 ± 79
Proto, light, DCMU	Trace
Proto, ATP, light	1365 ± 94
Proto, ATP, light, DCMU	1138 ± 25
Experiment 3	
ALA, light	1735 ± 4
ALA, light, DCMU	Trace
ALA, ATP, dark	1549 ± 99
ALA, ATP, dark, DCMU	1755 ± 73
Experiment 4	
ALA, light	910 ± 13
ALA, light, DCMU	0
ALA, ATP, light	708 ± 46
ALA, ATP, light, DCMU	505 ± 18

Table VIII. *Inhibition of Mg-Proto (Me) Accumulation by DCMU with Glu as Substrate*

Five mM ATP, NADPH, and Glc-6-P were included as mentioned. Glu (6 mM) was added to each incubation. DCMU (5 nmol) was added in ethanolic solution and the ethanol was removed by blowing N_2. Light conditions were as in Table II.

Incubation Conditions	Mg-Proto (Me)
	pmol/h·mg protein
Experiment 1	
Light	106 ± 7
Light, DCMU	Trace
Dark, ATP, NADPH, Glc-6-P	267 ± 5
Dark, ATP, NADPH, Glc-6-P, DCMU	288 ± 1
Experiment 2	
Light, ATP	168 ± 1
Light, ATP, DCMU	32 ± 1
Light, NADPH, Glc-6-P	186 ± 2
Light, NADPH, Glc-6-P, DCMU	Trace
Experiment 3	
Light	181 ± 7
Light, DCMU	Trace
Light, ATP, NADPH, Glc-6-P	325 ± 8
Light, ATP, NADPH, Glc-6-P, DCMU	309 ± 0

not inhibit Mg-Proto (Me) accumulation from Proto or ALA in the light if ATP was added (Table VII, experiments 2 and 4). With Glu as the substrate, DCMU again inhibited the accumulation of Mg-Proto (Me) in the light-dependent reaction, but had no effect if the incubation was carried out in the dark (Table VIII, experiment 1). If only one of the two, ATP or reducing power, was added, light was still required for Mg-Proto (Me) formation, and in that case the reaction was sensitive to DCMU (Table VIII, experiment 2). However, DCMU had no effect on Mg-Proto (Me) accumulation in the light if both ATP and reducing power were added (Table VIII, experiment 3).

DISCUSSION

The method of plastid isolation described in this work allows the simple and rapid separation of intact from broken plastids. The intact chloroplast pellet was quite homogeneous, and electron microscopy indicated that the envelope was usually intact. Intactness appears to be required for both the Mg-chelatase and Mg-Proto Me (oxidative) cyclase activities, as the majority of these two activities is found associated with the intact chloroplast fraction (Table I). The absolute requirement of Mg-chelatase for plastid intactness was observed and remains the primary hindrance to enzyme purification (Fuesler and Castelfranco, in preparation). However, the Mg-Proto Me (oxidative) cyclase does not appear to require plastid intactness in the same sense, because a great deal of the activity can be reconstituted from supernatant and pelleted membrane fractions after the disruption of the isolated plastids (Wong and Castelfranco, in preparation).

The full complement of Chl-biosynthetic enzymes was reported to be contained within chloroplasts (19, 23), and our results support this concept. In these experiments, the reactions of Chlide *a* biosynthesis were demonstrated using a highly purified intact plastid preparation: Glu was converted to Mg-Proto and Pchlide; ALA and Proto were converted to Mg-Proto, Mg-Proto Me, DV-Pchlide, and MV-Chlide *a*.

A much higher concentration of ALA (6 mM) was required to saturate the biosynthetic system than of Proto (10 μM). However, either substrate at saturating concentration gave rise to the accumulation of Mg-Proto, Mg-Proto Me, DV-Pchlide, or MV-Chlide *a* to a quantitatively similar extent. The effect of cosubstrates (ATP and SAM), inhibitors (MB, AMP, PCMBS), and incubation conditions (light or dark) on the accumulation of these intermediates was the same regardless of which precursor of the Mg-porphyrin macrocycle was used.

The only condition that appeared to differentiate between ALA and Proto as substrates was anaerobiosis. It was possible to inhibit the accumulation of Mg-Proto (Me) from ALA by rigorous anaerobic conditions achieved by prolonged flushing of the reaction vessels with N_2 prior to the incubation, and also by the presence of alkaline pyrogallol in the center well during the incubation. Nitrogen flushing without pyrogallol was not sufficient to inhibit the process; this might indicate that chloroplast coproporphyrinogenase has a very high affinity for O_2. Coproporphyrin accumulation was looked for but not found in these experiments.

Under our experimental conditions, Mg-Proto (Me) accumulation from Glu never exceeded 30% of the value observed from ALA. The formation of ALA has long been considered as the rate-limiting step in tetrapyrrole biosynthesis (3, 15), and our results are certainly consistent with that hypothesis.

The inhibition of Mg-chelatase by PCMBS (Table V) suggests the participation of a site which is accessible to the medium, presumably in the chloroplast envelope. This site cannot be related to the uptake of Proto because the inhibition persists in the presence of endogenously-formed Proto from ALA. Similarly, this site is not related to ATP penetration because the inhibition persists if Mg-chelatase is driven by ATP recycled within the plastids during a light incubation. The intraplastidic localization of Mg-chelatase and Mg-Proto Me (oxidative) cyclase will form the object of a future communication (in preparation).

The effect of light on Mg-Proto (Me) formation (Fig. 1) is one of the striking features of this system. If Proto (Fig. 1A) or ALA (Fig. 1B) is used as the substrate, light is seen to replace the requirement for exogenous ATP. This observation is easily explained if plastids isolated from dark-grown plant material after 20 h of continuous illumination are able to carry out photophosphorylation.

If Glu is used as the substrate for Mg-Proto (Me) accumulation (Fig. 4C), there is a light requirement that is not replaced by high levels of exogenous ATP in the dark. In the absence of added substrate (Fig. 1D), a small amount of Mg-Proto (Me) was formed in the light, but none in the dark. The addition of both ATP and reducing power (in the form of NADPH plus Glc-6-P) was found to replace the light requirement for Glu conversion to Mg-Proto (Me) (Table VI). In many experiments this combination of exogenous cofactors in the dark produced greater accumulation of Mg-Proto (Me) from Glu than light in the absence of these cofactors. Therefore, under our experimental conditions, the photosynthetic capacity of these plastids does not appear to produce all the ATP and reducing power necessary to support the synthesis of Mg-Proto (Me) from Glu at maximal rates. DCMU inhibited the light-dependent formation of Mg-Proto (Me) from Proto, ALA (Table VII) or Glu (Table VIII). This DCMU inhibition confirms that the light stimulation of Mg-Proto (Me) synthesis from all three substrates (Fig. 1) is photosynthetic in nature. We have not observed any evidence for nonphotosynthetic light effects in the synthesis of Mg-Proto (Me) in isolated developing plastids. However, it should be pointed out that these plastids, after 20 h of exposure to light, had gone a long way in the conversion of etioplasts to chloroplasts. Electron microscopy revealed the presence of grana and the almost total absence of prolamellar bodies. It is possible that nonphotosynthetic light requirements in the reactions of the Chl biosynthetic pathway could be demonstrated in the earlier stages of greening.

It is known that ALA formation *in vivo* requires light, even in cucumber cotyledons that have been previously exposed to continuous light long enough to eliminate the initial lag phase (4, 5). Although this light effect, which persists in the linear phase of Chl accumulation, is not fully understood, it is usually explained as a direct or indirect feedback inhibition of ALA synthesis by Pchlide (6, 7).

Finally, we have confirmed our previous observation (13) that SAM, which is required for the methylation of the 6-propionic acid side chain of Mg-Proto, is not made in the chloroplasts. Similar conclusions about the site of SAM synthesis were reached recently by Wallsgrove *et al.* (22). Therefore, SAM must be supplied from the cytoplasm in order to permit the Chl biosynthetic pathway to proceed beyond Mg-Proto.

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