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Further Characterization of the Magnesium Chelatase in Isolated Developing Cucumber Chloroplasts[1]

Substrate Specificity, Regulation, Intactness, and ATP Requirements

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ABSTRACT

Mg-chelatase catalyzes the first step unique to the chlorophyll branch of tetrapyrrole biosynthesis, namely the insertion of Mg into protoporphyrin IX (Proto). Mg-chelatase was assayed in intact chloroplasts from semi-green cucumber (*Cucumis sativus*, cv Sumter) cotyledons. In the presence of Proto and MgATP, enzyme activity was linear for 50 minutes. Plastid intactness was directly related to (and necessary for) Mg-chelatase activity. Uncouplers and ionophores did not inhibit Mg-chelatase in the presence of ATP. The nonhydrolyzable ATP analogs, β,γ-methylene ATP and adenylylimidodiphosphate, could not sustain Mg-chelatase activity alone and were inhibitory in the presence of ATP (I_{50} 10 and 3 millimolar, respectively). Mg-chelatase was also inhibited by *N*-ethylmaleimide (I_{50}, 50 micromolar) and the metal ion chelators 2,2′-dipyridyl and 1,10 phenanthroline (but not to the same degree by their nonchelating analogs). In addition to Proto, the following porphyrins acted as Mg-chelatase substrates, giving comparable specific activities: deuteroporphyrin, mesoporphyrin, 2-ethyl, 4-vinyl Proto and 2-vinyl, 4-ethyl Proto. Mg-chelatase activity and freely exchangeable heme levels increased steadily with greening, reaching a maximum and leveling off after 15 hours in the light. Exogenous protochlorophyllide, chlorophyllide, heme, and Mg-Proto had no measurable effect on Mg-chelatase activity. The potent ferrochelatase inhibitors, *N*-methylmesoporphyrin and *N*-methylprotoporphyrin, inhibited Mg-chelatase at micromolar concentrations.

Chl and heme synthesis share a common pathway up to the level of Proto[2]. At this point, iron insertion commits the porphyrin to heme synthesis and magnesium insertion commits it to Chl biosynthesis. Thus, magnesium insertion is the first step unique to Chl synthesis and is at the branch point of two major biosynthetic routes in the tetrapyrrole pathway.

The enzyme which catalyzes magnesium insertion, Mg-chelatase, has been assayed in plastids derived from semigreen cucumber (*Cucumis sativus*, cv Sumter) cotyledons by Castelfranco and coworkers (7, 14, 16, 17). They found that the activity required magnesium, ATP, and Proto as substrates and could be inhibited by the organo-mercurial sulfhydryl reagents, PCMB and PCMBS: the poor ability of PCMBS to cross lipid bilayers implied that the enzyme was located in the envelope (16). They found that Mg-chelatase activity could only be measured in intact chloroplasts; any disruption of the plastids caused a complete loss of activity.

In an attempt to simplify the Mg-chelatase assay system, Richter and Rienits (25) ruptured cucumber plastids by osmotic lysis and removed the soluble intraplastid contents by buffer washes. They were able to measure low levels of Mg-chelatase activity by using a [^{14}C]Proto substrate; however, this specific activity was only 2.5% of the activity of intact chloroplasts. Clearly, some factor or condition was being lost during the lysis, but, unfortunately, they could not resolve this problem. Again, the substrate requirements for the enzyme were magnesium, ATP, and Proto.

Over the last 5 years there has been no progress made on elucidating either the mechanism or regulation of this key enzyme in the Chl biosynthetic pathway. In the work reported in this paper we have continued the characterization of Mg-chelatase activity in cucumber chloroplasts. Due to the lower activities in the ruptured chloroplast procedure, as well as the possibility that Mg-chelatase might behave differently in a system where a cofactor may have been lost, we assayed the enzyme in intact chloroplasts. The method reported by Castelfranco and coworkers (7, 14, 16, 17, 23) was modified to decrease both the assay time and the time required for the pigment extraction procedure. Using this technique, we have extended our knowledge of both the enzymology and regulation of magnesium insertion.

MATERIALS AND METHODS

Materials

Tricine and DTT were purchased from Research Organics. Proto, Mg-Proto, NMPP, NMMP, deuteroporphyrin, and mesoporphyrin were obtained from Porphyrin Products. Pchlide was the gift of Dr. B. J. Whyte. Monovinyl Proto and Mg-Deuteroporphyrin were the gift of Dr. I. N. Rezzano and Dr. K. M. Smith (University of California, Davis). Cucumber seeds (*Cucumis sativus*, cv Sumter) were purchased from Asgrow Seeds Co. BSA was obtained from Calbiochem. All

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[2] Abbreviations: Proto, protoporphyrin IX; PCMB(S), *p*-chloromercuribenzoate (sulfonate); NMMP, *N*-methylmesoporphyrin IX; NMPP, *N*-methylprotoporphyrin IX; FCCP, carbonyl cyanide *p*-trifluoromethoxyphenylhydrazone; AMP-PCP, β,γ-methylene ATP; AMP-PNP, adenylylimidodiphosphate.

other biochemicals were purchased from Sigma. Organic solvents were obtained from Fisher.

Chloroplast Isolation

Excess Captan fungicide was removed by extensive rinsing, then the cucumber seeds were imbibed with water for 20 min, and germinated in the dark at 25°C for 6 to 7 d (18). Seedlings were greened under white fluorescent light (23 $\mu E \cdot m^{-2} \cdot sec^{-1}$, PAR) for 24 h.

Intact chloroplasts were isolated from greening cucumber cotyledons as previously described (26), except that the final pellet was resuspended in grinding buffer (0.5 M sorbitol, 50 mM Tricine, 1 mM DTT, 1 mM EDTA, 1 mM $MgCl_2$, 0.1% (w/v) BSA [pH 7.8]). The intact plastids were adjusted to give a final protein concentration of 1.0 mg/mL in the Mg-chelatase assay incubation. Plastid intactness was assessed by the latency of 6-phosphogluconate dehydrogenase activity (22).

Mg-Chelatase Assay

Mg-chelatase activity was measured by an adaptation of the method described by Fuesler *et al.* (14). All manipulations were performed under dim lighting to prevent porphyrin-mediated photooxidative damage. Chloroplast incubations were carried out in flat bottomed glass vials (1.5 cm diameter, 4.4 cm high) placed in a heating block set to 30°C and covered with two layers aluminum foil. In a routine assay, chloroplasts (1.0 mg protein/mL) were incubated in a total volume of 250 μL grinding buffer containing 1.5 μM Proto and 4.0 mM MgATP (final concentration of Mg^{2+} in the assay was 5.0 mM) in a regenerating system (20 mM phospho*enol*pyruvate, 5.4 units/mL pyruvate kinase). Reactions were started by the addition of plastids and allowed to proceed for 20 min with constant shaking (100 rpm on an orbital shaker) before termination by chilling on ice. Any deviations from the standard incubation conditions are indicated in the text.

Pigment Extraction and Quantitation

The entire contents of each incubation vial were added to 750 μL ice-cold acetone in a 1.5 mL microcentrifuge tube. The samples were vortexed and centrifuged (13,000*g*) for 2 min in the microcentrifuge to sediment precipitated proteins. The green supernatant was reserved, and the pellet resuspended in 250 μL of 0.12 N NH_4OH. The proteins and starch were, again, precipitated by adding 750 μL cold acetone and centrifuging for two min. The supernatants were combined and 4 mL 75% (v/v) acetone added. Esterified pigments were removed by two washes with hexanes (4 mL then 2 mL).

The product, Mg-Proto, in the acetone phase was quantitated on a Perkin-Elmer 650–40 fluorometer calibrated with an authentic standard (excitation 419 ± 5 nm). The emission peak of the product was at 595 nm. In experiments where deuteroporphyrin was the substrate, the fluorometer was calibrated with Mg-deuteroporphyrin (excitation 404 ± 5 nm); the same calibration curve was used for quantitating Mg-mesoporphyrin (excitation 406 ± 5 nm). No attempt was made to separate Mg-Proto from Mg-Proto monomethyl ester, and the fluorometric assay cannot distinguish between these two products. Thus, the product we call Mg-Proto may contain a certain amount of the monomethyl ester. However, in plastids from greening cucumbers, conversion of Mg-Proto to the monomethyl ester requires the addition of exogenous *S*-adenosylmethionine (15), and it is likely that our product contains little, if any, of the methyl ester.

Porphyrin Solutions

Proto, deuteroporphyrin, and mesoporphyrin were purified before use by addition of the porphyrin in a 95% (v/v) ethanolic solution to a DEAE-Sepharose (acetate form) column (26). The porphyrins bound to the column which was then washed successively with 95% (v/v) ethanol, 1-butanol:ethanol (1:1, v/v), and 95% (v/v) ethanol. The porphyrins were eluted in 66.5% ethanol/17% glacial acetic acid/16.5% water (v/v/v) and extracted into diethyl ether. Aliquots of the ether extract were evaporated and dissolved in DMSO for addition to the Mg-chelatase assay.

Chlorophyllide was generated by the degradation of Chl after the endogenous chlorophyllase was activated by treatment of the chloroplasts with 40% (v/v) acetone (4). The chlorophyllide was recovered by extraction into ether (7).

Porphyrin concentrations were determined spectrophotometrically with the following wavelengths and millimolar extinction coefficients: Proto (2.7 N HCl) 554 nm, 13.5 (1); deuteroporphyrin (0.1 N HCl) 548 nm, 13.7 (1); mesoporphyrin (0.1 N HCl) 547 nm, 15.1 (1); heme (66.5% ethanol/17% glacial acetic acid/16.5% water [v/v]) 398 nm, 144 (30); Pchlide (diethyl ether) 432 nm, 289.7 (21); Chlide (acetone) 663 nm, 75.05 (21); NMPP ($CHCl_3$) 418 nm, 126 (6); NMMP (0.1 N HCl) 406 nm, 455 (2); 2-ethyl, 4-vinyl Proto and 4-ethyl, 2-vinyl Proto (2.7 N HCl) 552, 13.5. Standard solutions of Mg-porphyrins, other than Chlide and Pchlide, were measured as their metal-free derivatives, as above. All porphyrins were added to the plastid incubations in DMSO.

Other Procedures

Plastid protein was determined by the method of Bradford (5) using BSA as a standard. Freely exchangeable heme was measured by reconstitution with horseradish apo-peroxidase (26). Chl content was measured by extraction of the plastids with 80% (v/v) acetone and quantitation using published extinction coefficients (21). All absorbance measurements were made on a Cary 219 spectrophotometer.

RESULTS

Optimization of the Mg-Chelatase Assay

In previous assays, the accumulated product was extracted from the aqueous acetone phase into diethyl ether before measuring the fluorescence (7, 14, 17, 23). In the present report this step has been omitted, and the Mg-Proto fluorescence was read directly in the hexane-washed aqueous acetone (27). Although some sensitivity is lost due to the lower fluorescence yield of Mg-Proto in the aqueous solvent, the activities were sufficiently large that the loss of sensitivity was not a limitation. The emission maximum (595 nm) and fluores-

cence intensity of Mg-Proto in the solvent mixture from a chloroplast extract was not different from that of the solvent mixture plus buffer control. Thus, there was no significant quenching from the solvent soluble components of the chloroplast. Although the measured activity from different preparations of chloroplasts sometimes varied (usually not more than 30%), duplicate assays within a single preparation rarely varied by more than 5%.

The Mg-chelatase assay was optimized for ATP, magnesium, and Proto concentrations (4.0 mM, 5.0 mM, and 1.5 μM, respectively; data not shown). Since these optimal concentrations appeared to vary with the amount of plastid protein in the assay, all incubations were carried out at a protein concentration of 1 mg/mL. Under these conditions, Mg-chelatase activity was linear for 50 min (Fig. 1), and the specific activities measured were comparable with those previously reported (14, 17). In our standard assay, a 20 min incubation time was chosen to ensure that Mg-chelatase activity was measured within this linear phase.

Activity Requires Plastid Intactness

Plastid intactness was necessary for Mg-chelatase activity (Fig. 2). Under iso-osmotic conditions (0.5 M sorbitol), only 18% of plastid intactness was lost after a 30 min incubation period, and Mg-chelatase activity was high. In contrast, in a hypotonic buffer of 0.25 M sorbitol, the plastids lost 60% of their intactness during the incubation and 50% of the Mg-chelatase activity. In plastids incubated in 0.1 M sorbitol, there was a complete loss of intactness and no Mg-chelatase activity was measurable.

It was possible that the intactness requirement for Mg-chelatase activity might be linked to a membrane potential or ionic gradient, generated by ATP hydrolysis. However, treatment with either uncouplers (FCCP) and/or ionophores (valinomycin or gramicidin), which should collapse such a gradient, did not inhibit Mg-chelatase (Table I). The nonhydrolyzable ATP analogs, AMP-PCP and AMP-PNP, inhibited Mg-chelatase in the presence of ATP (Table I); neither of these two compounds was able to support Mg-chelatase activity in the absence of ATP (data not shown), which suggested that the ATP requirement was for hydrolyzable ATP. AMP also inhibited Mg-chelatase activity (Table I), confirming previous observations (23).

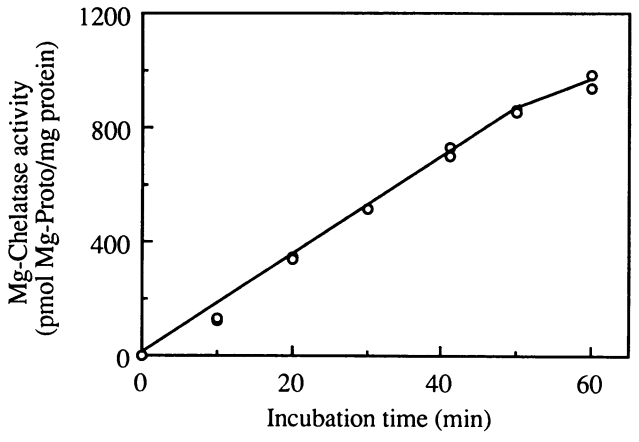

Figure 1. Time course of Mg-Proto accumulation from added Proto in isolated, intact, developing chloroplasts. Chloroplasts (1.1 mg/mL plastid protein) were incubated under standard assay conditions (see "Materials and Methods") for various lengths of time up to 60 min. The product, Mg-Proto, was extracted and quantitated as described in "Materials and Methods." All time points shown in the figure are duplicates.

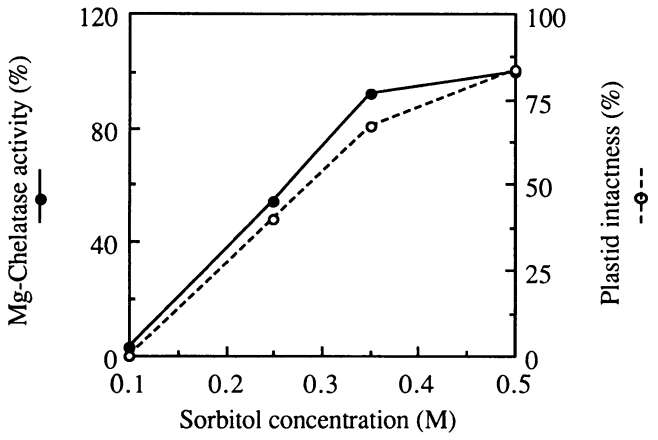

Figure 2. Effect of reduced osmoticum on Mg-chelatase activity. Chloroplasts were assayed for Mg-chelatase activity (●) under standard conditions and at differing hypotonic sorbitol concentrations. The Mg-chelatase assay time was extended to 30 min and at the end of this time, samples of each incubation were also assayed for plastid intactness (○). The sample incubated under iso-osmotic conditions (0.5 M sorbitol) was the control, and Mg-chelatase activities are expressed relative to this amount (100% = 722 pmol Mg-Proto/30 min/mg protein).

Activity is Sensitive to Sulfhydryl Reagents and Metal Ion Chelators

Mg-chelatase activity was very sensitive to the sulfhydryl modifying reagents, *N*-ethyl maleimide, PCMB, and PCMBS (Table II), confirming the presence of essential cysteine residues (14, 17). When tested at concentrations as high as 10.0 mM, butanedione and methylacetimidate, which modify ly-

Table I. *ATP Requirement for Mg-Chelatase Activity*

Mg-chelatase activity was assayed in chloroplasts under standard conditions, which included 4.0 mM ATP and ATP regenerating system. The potential inhibitors were tested over a broad concentration range. Data for compounds which inhibited are presented as I_{50} values. For those compounds that had no effect, the highest concentration tested is given in parentheses.

Additions to the Incubation	I_{50}
	mM
AMP	1.8
AMP-PNP	3.0
AMP-PCP	10.0
Gramicidin (20 μM)	No effect
Valinomycin (20 μM) + FCCP (40 μM) + KCl (2 mM)	No effect

Table II. *Sensitivity of Mg-Chelatase to Protein-Modifying Reagents and Metal Ion Chelators*

Mg-chelatase activity was assayed under standard conditions with the addition of the compounds indicated below. In all cases, several concentrations of the compounds were tested, and the data are summarized in the form of I_{50} measurements. For those compounds that had no inhibitory effect, the highest concentration tested is given in parentheses.

Addition to Incubation	I_{50}
	mM
Protein-modifying reagents	
NEM[a]	0.050
PCMB[a]	0.050
PCMBS[a]	0.10
2,3-Butanedione (10 mM)[b]	No effect
Methyl acetimidate (10 mM)[b]	No effect
Metal ion chelators and analogs	
2,2-Dipyridyl	2.0
4,4′-Dipyridyl (2 mM)	No effect
1,10-Phenanthroline	0.60
1,7-Phenanthroline (0.6 mM)	Slight inhibition (10%)

[a] The chloroplasts were washed and incubated in grinding buffer without DTT. [b]Measured in the absence of borate.

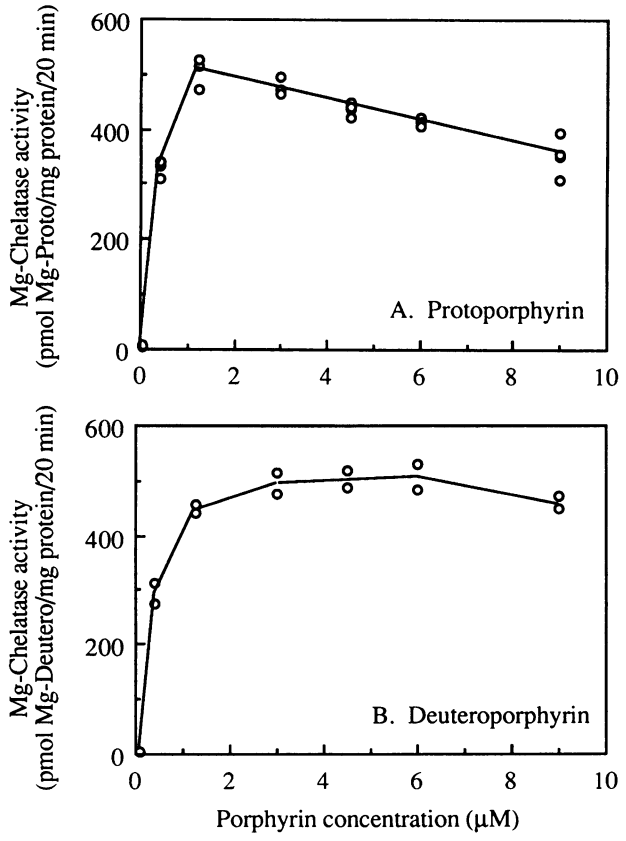

Figure 3. Comparison of Proto and deuteroporphyrin as substrates for Mg-chelatase. Intact, developing chloroplasts were incubated under standard conditions except that the porphyrin concentration was varied. Panel A, Proto; panel B, deuteroporphyrin.

Table III. *Ability of Proto Analogs to Act as Mg-Chelatase Substrates*

Chloroplasts were incubated under standard conditions except that the porphyrin substrate was varied as indicated. Each porphyrin was tested at concentrations between 0.5 and 9.0 μM, and the V_{max} determined from resulting substrate curves.

Porphyrin Substrate	Porphyrin Substituent		Specific Activity	Porphyrin Concentration
	−2 position	−4 position		
			pmol Mg-porphyrin/20 min/mg protein	*μM*
Experiment 1				
Proto	Vinyl	Vinyl	488	1.5
Monovinyl Proto	Ethyl	Vinyl	593	2.5
Monovinyl Proto	Vinyl	Ethyl	567	2.5
Experiment 2				
Proto	Vinyl	Vinyl	526	1.5
Mesoporphyrin	Ethyl	Ethyl	577	4.0
Deuteroporphyrin	H	H	430	5.0

sine and arginine residues, did not inhibit in our standard assay (Table II). However, both these compounds require the presence of a borate buffer to form a stable covalent adduct (13). Unfortunately, borate was strongly inhibitory to Mg-chelatase, possibly indicating the importance of vicinal hydroxyl groups in some component of the assay system (10 mM borate inhibits over 90% of the activity, data not shown), so optimal conditions for these reagents could not be tested.

We also observed inhibition by 2,2′-dipyridyl (Table II and ref. 25). The nonchelating analog of this compound, 4,4′-dipyridyl, had no measurable effect on Mg-chelatase activity, when tested at a concentration of 2.0 mM. A similar pattern was seen with the metal ion chelator, 1,10-phenanthroline. This compound inhibited 50% of the Mg-chelatase activity at 0.6 mM, whereas its nonchelating analog, 1,7-phenanthroline, was only slightly inhibitory at this concentration. However, 1,7-phenanthroline did inhibit Mg-chelatase activity by about 40% at 2 mM (data not shown). It should be emphasized that although 2,2′-dipyridyl and 1,10-phenanthroline have an affinity for Mg^{2+}, this affinity is low, and the high Mg^{2+} concentrations in the incubation (5 mM) make inhibition of the Mg-chelatase by actual sequestering of Mg^{2+} improbable.

Alternative Porphyrin Substrates

Several porphyrins could act as Mg-chelatase substrates. Mg-chelatase activity was optimal at 1.5 μM Proto, and above this concentration, it became inhibitory (30% inhibition at 9 μM, compared to 1.5 μM; Fig. 3) In contrast, the substrate curve for deuteroporphyrin shows that maximal Mg-chelatase activity was attained at 5.0 μM porphyrin, and inhibition at higher concentrations was less pronounced (10% at 9.0 μM). Both forms of monovinyl Proto (2-ethyl, 4-vinyl and 2-vinyl, 4-ethyl) as well as mesoporphyrin could act as Mg-chelatase substrates (Table III). The specific activity of Mg-chelatase with all the alternative porphyrin substrates were comparable with Proto, but the porphyrin concentration at which maximal activity was observed varied between 1.5 and 5 μM (Table III).

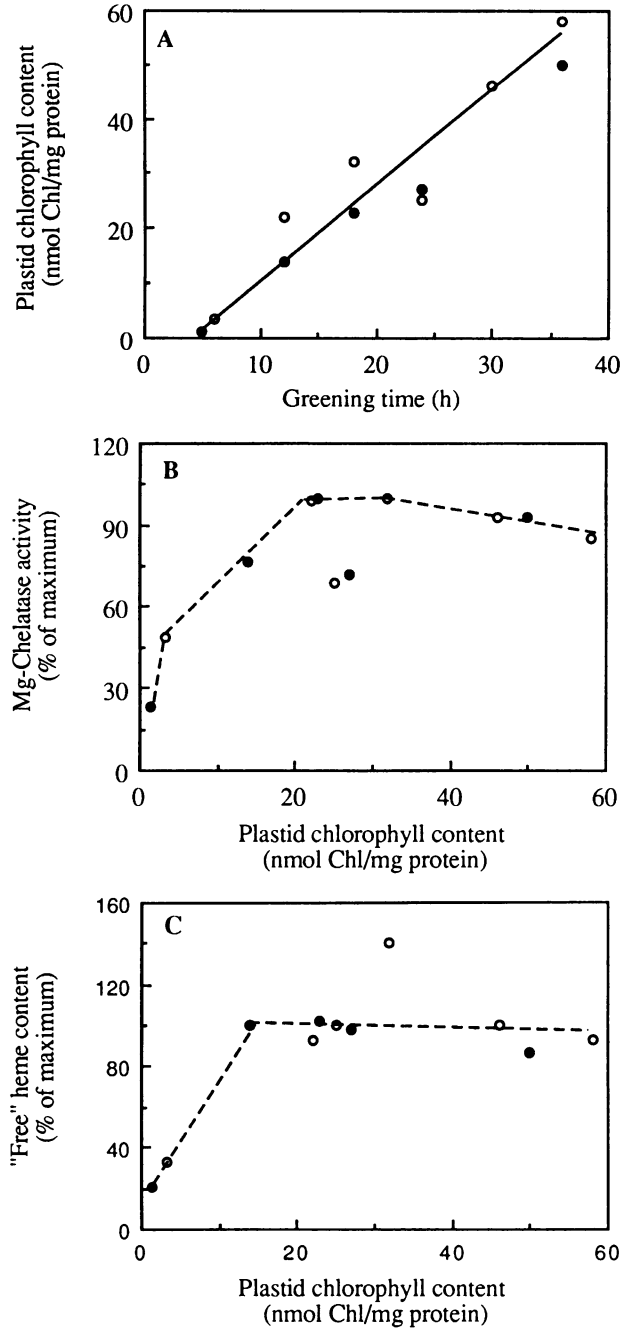

Figure 4. Effect of greening on Chl accumulation, Mg-chelatase activity, and free heme levels. Intact chloroplasts were prepared from seedlings which were grown in the dark and exposed to light for various lengths of time. Panel A: Chl content of the isolated plastids. Panel B: each preparation was assayed for Mg-chelatase activity at several substrate concentrations between 0.5 and 5 μM Proto, and the maximum activity for each preparation is shown (100% activity = 660 ± 90 pmol Mg-Proto/20 min/mg protein; average and deviation for two separate experiments). Panel C: freely exchangeable heme levels in each preparation were determined in triplicate, and the average of these measurements is shown (100% heme level = 40 pmol heme/mg protein). The open and closed circles represent two individual experiments.

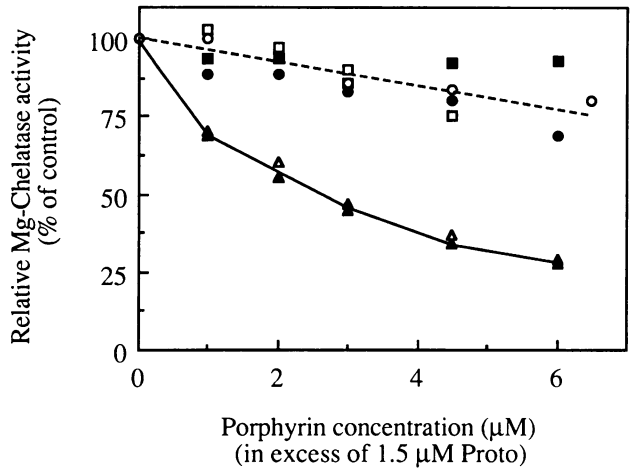

Figure 5. Effect of exogenously added porphyrins and metalloporphyrins on Mg-chelatase activity. Mg-chelatase was assayed under standard conditions in the presence of increasing concentrations of the following porphyrins: Proto (○); Chlide (□); Pchlide (●); heme (■); NMMP (△); NMPP (▲). Proto (1.5 μM) was present as a substrate in all samples; thus "1 μM porphyrin" on the graph indicates that 1.5 μM Proto is present in addition to 1 μM of the porphyrin being tested.

Regulation of Mg-Chelatase Activity by Greening and Putative Feedback Inhibitors

When etiolated cucumber seedlings were brought into the light, Chl accumulation was linear after an initial lag (Fig. 4, panel A). Under our temperature and light conditions, Chl accumulation was 2.0 nmol/h/mg plastid protein. To allow for an easy comparison of our data with other work, we have expressed the results in terms of Chl accumulation rather than greening time. Plastids prepared from plants at various stages of greening were assayed for Mg-chelatase activity. In etiolated tissue there was little Mg-chelatase activity, but this activity increased with greening, reaching a maximum when the Chl content was 30 nmol/mg plastid protein (Fig. 4, panel B). Continued greening beyond this point resulted in a slight decrease in Mg-chelatase activity. Activity in plastids isolated from seedlings grown for 7 d under a diurnal light/dark cycle was about the same as in plastids isolated from greening cotyledons (data not shown). Freely exchangeable heme (26) content was also measured in these plastids (Fig. 4. panel C). Again, etiolated tissue contained only low levels of "free" heme, but with greening these levels increased from 6.0 to 35.0 pmol heme/mg plastid protein when the Chl content reached 20 nmol/mg plastid protein, at which point the free heme concentration became constant.

The possible regulation of Mg-chelatase by other stable intermediates or endproducts of the tetrapyrrole pathway was examined by the addition of exogenous porphyrins to the assay system (Fig. 5). Neither Chlide, Pchlide nor heme significantly inhibited Mg-chelatase when compared to the Proto control. The potent ferrochelatase inhibitors, NMMP and NMPP, inhibited Mg-chelatase at micromolar concentrations (I_{50}, 2.0 μM).

The possibility that the product of the Mg-chelatase reaction (Mg-Proto) inhibited Mg-chelatase, was tested by supple-

menting assays with known amounts of Mg-Proto (Fig. 6). If the background of exogenously added Mg-Proto was subtracted from the total Mg-Proto recovered after the incubation period (solid circles), no significant inhibition of Mg-chelatase was observed up to 2 μM Mg-Proto (corrected line, open circles). Higher concentrations of Mg-Proto were not tested due to the technical difficulties associated with measuring comparatively small increases in Mg-Proto (due to Mg-chelatase activity) over a high background of exogenously added Mg-Proto. However, 2.0 μM Mg-Proto does represent at least twice the Mg-Proto concentration typically present at the end of an incubation.

DISCUSSION

Our Mg-chelatase assay has the advantage that the sample size has been reduced, allowing the handling of multiple samples in relatively short spaces of time. In our hands, 24 samples may be extracted in about 90 min. The assay also differs from previous techniques (7, 14, 17, 23) in that a shorter incubation time of 20 min has been used. Since Mg-chelatase activity is only linear for 50 min (Fig. 1), our incubation time ensures that the reaction is within its linear phase and that we are measuring rates rather than total activities. The activities we measure, typically 400 to 600 pmol/20 min/mg plastid protein, are comparable with those in the literature (7, 14, 17, 23), and come close to accounting for the rate of Chl accumulation in intact tissue during greening (approximately 660 pmol Chl/mg plastid protein/20 min, Fig. 4A).

The enzyme catalyzing the insertion of iron into Proto, ferrochelatase, has been well characterized in bacterial (11, 19), mammalian (8–10), and to some extent in plant systems (20). The reaction is clearly very similar to that catalyzed by Mg-chelatase since both enzymes incorporate a divalent metal ion into Proto. However, ferrochelatase does not require ATP or any other high energy compound for catalysis, raising the question of why ATP is required in the Mg-chelatase reaction. Conceivably, ATP could act as an allosteric activator, or ATP might serve to bind the magnesium in such a way that when MgATP binds to the active site of the enzyme, the magnesium is precisely placed for insertion into Proto. In both cases, ATP hydrolysis would not necessarily be required. However, our data suggest that ATP is actually being hydrolyzed during the reaction: the two nonhydrolyzable analogs of ATP could not sustain Mg-chelatase activity alone (data not shown) and, in the presence of ATP, were actually inhibitory to the reaction (Table I). While we have shown that ATP hydrolysis is an integral part of the reaction, the reason for this requirement is unclear. However, we have eliminated the possibility that ATP hydrolysis is connected with the generation of an ionic gradient or membrane potential, because the presence of ionophores and uncouplers had no effect on the reaction (Table I).

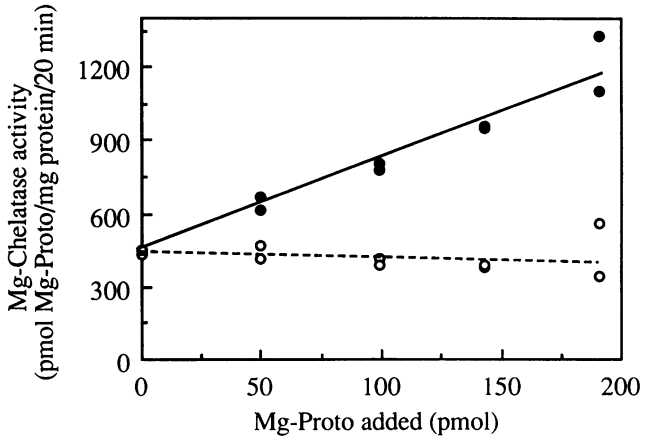

Figure 6. Effect of exogenously added Mg-Proto on Mg-chelatase activity. Intact chloroplasts (1.0 mg protein/mL) were incubated with substrates, supplemented with various amounts of Mg-Proto, and assayed for Mg-chelatase activity. The total amount of Mg-Proto extracted at the end of a 20 min incubation (as a function of added Mg-Proto) is shown on the figure (●). The open circles (○) represent the theoretical amount of Mg-chelatase activity present in each sample, and was calculated by subtracting the exogenous Mg-Proto. All data points are shown in duplicate.

Like ferrochelatase, Mg-chelatase activity is inhibited by the sulfhydryl reagents PCMB and PCMBS (Table II and ref. 16) and is very sensitive to N-ethyl maleimide. However, since these studies were performed on intact chloroplasts, it is still possible that these compounds might inhibit by an indirect mechanism. As yet, we have seen no protection from sulfhydryl reagents by MgATP or Proto (data not shown), and the role of these sulfhydryl groups remains unclear.

The sensitivity of Mg-chelatase to 2,2′-dipyridyl was unexpected (Table II) since there have been reports of *in vivo* accumulation of Mg-porphyrins in the presence of this chelator (12, 29). The discrepancy between our results and these data may reflect the differences between *in vivo* and *in vitro* work. There was also a possibility that metal ion chelators might be inhibiting by nonspecific means unconnected with their metal-binding properties. However, the nonchelating analogs of these compounds were far less effective at inhibiting Mg-chelatase. These results suggest that the metal-binding abilities of the chelators were the source of their inhibitory action, and that there may indeed be a metal ion requirement for Mg-chelatase activity in addition to the magnesium requirement. Preliminary experiments have shown that exogenous $FeSO_4$ (50 μM) has no effect on Mg-chelatase activity in isolated plastids (data not shown).

Although the physiological substrate for Mg-chelatase is Proto, deuteroporphyrin (which lacks both 2- and 4-vinyl groups) could substitute for Proto in the reaction and gave slightly higher maximal activities (Fig. 3, Table III). Various other porphyrins, differing in their substituents at the 2- and 4- positions, could also act as Mg-chelatase substrates (Table III), giving specific activities comparable to Proto. This lack of specificity at the 2- and 4- positions of the porphyrin is common in enzymes in this portion of the pathway. The methyl transferase, the Mg-Proto monomethyl ester cyclase (28), Pchlide reductase (CJ Walker, PA Castelfranco, personal observation) as well as ferrochelatase (10, 11, 20) have all been shown to share this flexible specificity to some extent. The implication of this work is that probably none of these enzymes absolutely require the 2- and 4-vinyl groups for substrate binding. In addition, the ability of the 2-vinyl, 4-

ethyl porphyrins to serve as substrates for these enzymes might be of physiological significance if 4-vinyl reduction can occur at several levels in the pathway, as has been suggested by Rebeiz and coworkers (24, 27).

The position of Mg-chelatase at the branch point between Chl and heme synthesis makes the regulation of this enzyme particularly interesting. In greening cucumber seedlings, the level of Mg-chelatase activity rises during greening up to a certain point and then remains constant (Fig. 4). Thus, Mg-chelatase levels are regulated, presumably in relation to changing demands for Chl synthesis. The product of the iron branch of the pathway, measured as steady-state free or easily exchangeable heme, also increases with greening and again remains constant when it has reached a certain level. Since Mg-chelatase and ferrochelatase utilize the same substrate, and, considering that the relative demand for heme and Chl probably differs during the lifetime of the chloroplast, Mg-chelatase and ferrochelatase activities must be coordinated. It has been suggested that either Chlide or Pchlide might inhibit Mg-chelatase. Therefore, a build-up of these compounds would effectively cause a shut-down of the Mg-branch of the pathway (recently reviewed [3]). In our assay we tested for such inhibition. Interestingly, we did not detect inhibition of Mg-chelatase by Pchlide or Chlide over and above the general phenomenon of low-level, nonspecific porphyrin inhibition. In addition, neither Mg-Proto nor heme inhibited Mg-chelatase activity (Fig. 5). These data reopen the question of how Mg-chelatase is regulated in the chloroplast. Possibly, Mg-chelatase is regulated by AMP, which inhibits at millimolar concentrations (Table I), and the enzyme responds to the "energy charge" of the cell as proposed by Pardo *et al.* (23). Demonstration of the mechanism of Mg-chelatase and ferrochelatase regulation must ultimately involve assaying both these enzymes in the same preparation. To date, such a study has not been done, and we are currently adapting our assay system to do these experiments.

Surprisingly, the only porphyrins which were found to inhibit Mg-chelatase were NMPP and NMMP (Fig. 5) which, up to this point, have been considered to be specific ferrochelatase inhibitors (10, 11). Although these compounds have been reported to have no effect on Chl synthesis (and presumably Mg-chelatase) *in vivo* (2, 6, 19), *in vitro* studies have shown that only nanomolar concentrations of these compounds are required to inhibit ferrochelatase. This thousandfold difference in potency might point to a different mechanism of inhibition. However, due to the difficulty of conducting meaningful kinetic studies in intact chloroplasts, we have not determined whether the inhibition of Mg-chelatase by NMMP or NMPP is of the competitive type, as was seen for ferrochelatase (10).

Although Mg-chelatase activity can be measured in chloroplasts, the major obstacle to unraveling the mechanism of this enzyme is the requirement for plastid intactness. Loss of intactness under hypotonic conditions (Fig. 2) is paralleled by a decrease in Mg-chelatase activity, indicating that the two factors are closely related. We have assayed Mg-chelatase activity in osmotically lysed and freeze/thawed plastids and have observed very low activities, comparable with those reported by Richter and Reinits (25). Attempts to stabilize this activity in our preparations have been unsuccessful. The reason for the plastid intactness requirement is still unclear, and efforts to isolate an organelle-free, high activity Mg-chelatase preparation are continuing in this laboratory.

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