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Enzymic Conversion of Protoporphyrin IX to Mg-Protoporphyrin IX in a Subplastidic Membrane Fraction of Cucumber Etiochloroplasts

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ABSTRACT

The preparation from Percoll-purified cucumber (*Cucumis sativus*)etiochloroplasts of a subplastidic membrane fraction that is capable of high rates of Mg insertion into protoporphyrin IX is described. The plastid stroma was inactive when used either alone or in combination with the membrane fraction. Successful preparation of the subplastidic membrane fraction required that Mg-protoporphyrin chelatase was first stabilized by its substrate. This was achieved by lysing Percoll-purified plastids in a fortified hypotonic medium containing protoporphyrin IX prior to ultracentrifugation and separation of the stroma from the plastid membranes. Protoporphyrin IX became membrane bound. Other additives needed for enzyme activity fell into two groups: (a) those needed for enzyme stabilization during membrane preparation and (b) those involved in the primary mechanism of Mg insertion into protoporphyrin IX. Ethylenediaminetetraacetate belonged to the first group, magnesium belonged to the second group, and ATP belonged to both groups.

Insertion of Mg into Proto[3] to form Mg-Proto is the first step committed to Chl biosynthesis. Mg-Proto was first detected in x-ray *Chlorella* mutants blocked in their ability to form Pchl (8). Net biosynthetic rates of Mg insertion into exogenous Proto were first demonstrated *in vitro* in cucumber (*Cucumis sativus* L. cv Beit Alpha MR) etiochloroplasts incubated in a fortified medium containing Mg and ATP (19). In this system, the rate of conversion of Proto to Mg-Proto was low, and Mg-Proto formation was accompanied by the formation of Zn-Proto. Significant improvement in the rate of conversion of Proto to Mg-Proto was achieved at higher ATP concentrations (4). Under these conditions, the insertion of Mg into Proto proceeded without Zn-Proto formation. Further progress in understanding the mode of action of Mg-Proto chelatase, its putative monovinyl and divinyl substrate specificity (21), and the role of ATP in Mg chelation has been hindered by the inability to purify the enzyme. Indeed, attempts at rupturing the plastids, a prerequisite for Mg-Proto chelatase purification, resulted invariably in loss of enzyme activity (4, 15, 22).

While this manuscript was in preparation, Walker and Weinstein described a subplastidic system that overcomes the requirement of plastid intactness for Mg-Proto chelatase activity (23). The system was prepared from lysed pea (*Pisum sativum*) chloroplasts and consists of soluble and membrane-bound fractions. Attempts at preparing similar systems from cucumber chloroplasts were not successful (23). In the present work, we describe the preparation from cucumber etiochloroplasts of a stabilized subplastidic membrane fraction that is capable of high rates of Mg insertion into exogenous Proto without addition of a soluble stromal fraction.

MATERIALS AND METHODS

Plant Materials

Cucumber (*Cucumis sativus*) seeds were grown in moist vermiculite in darkness at 28°C for 4 d (12). Seeds were purchased from J. Mollema & Son, Inc. (Grand Rapids, MI).

Chemicals

Disodium Proto and kinetin were purchased from Sigma Chemical Co., and potassium gibberellate was purchased from Calbiochem. All other biochemicals were purchased from Sigma. Organic solvents were obtained from Fisher Scientific.

Pretreatment of Etiolated Seedlings

Four-day-old etiolated cucumber cotyledons were excised with hypocotyl hooks under subdued laboratory light (about 5-foot candles). The excised cotyledons were incubated at 28°C for 20 h in darkness in deep Petri dishes (80 × 100 mm), each containing 3 g of tissue and 9 mL of an aqueous solution composed of 2 mM potassium gibberellate and 0.5 mM kinetin, pH 4.3 (2, 14).

Preparation of Crude and Purified Etiochloroplasts

All procedures were carried out under subdued laboratory light. After removal of the hypocotyl hooks, 20 g of pretreated

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[3] Abbreviations: Proto, protoporphyrin IX; ALA, δ-aminolevulinic acid; G6PDH, gluconate-6-phosphate dehydrogenase; Mg-Proto, magnesium-protoporphyrin IX; Zn-Proto, zinc-protoporphyrin IX.

cotyledons were hand-ground in a cold ceramic mortar containing 75 mL of homogenization medium. The latter consisted of 500 mM sucrose, 15 mM Hepes, 30 mM Tes, 1 mM $MgCl_2$, 1 mM EDTA, 0.2% (w/v) BSA, and 5 mM cysteine at a room-temperature pH of 7.7 (9). The homogenate was filtered through two layers of Miracloth (Calbiochem) and was centrifuged at 200*g* for 5 min in a Beckman JA-20 angle rotor at 1°C. The supernatant was decanted and centrifuged at 1500*g* for 20 min at 1°C. The pelleted crude etiochloroplasts were gently resuspended in 5 mL of homogenization, suspension, or lysing medium using a small paintbrush. The suspension medium was composed of 500 mM sucrose, 200 mM Tris, 20 mM $MgCl_2$, 2.5 mM EDTA, 40 mM NAD^+, 20 mM ATP, 8 mM methionine, 1.25 mM methanol, and 0.1% (w/v) BSA at a room temperature pH of 7.7. Unless otherwise indicated, the lysing medium consisted of 25 mM Tris, 30 mM $MgCl_2$, 7.5 mM EDTA, 40 mM NAD^+, 20 mM ATP, 8 mM methionine, 37.5 mM methanol, and 4.5 mM glutathione at a room temperature pH of 7.7 (9). For further plastid purification, the pelleted crude etiochloroplasts were resuspended in 5 mL of homogenization medium and were purified by Percoll density centrifugation (9). The pelleted, Percoll-purified etiochloroplasts were then resuspended in either suspension or lysing medium.

Preparation of Etiochloroplast Stroma and Membranes

The membrane fraction was separated from the stromal fraction after lysis of the Percoll-purified plastids. Lysis was achieved by addition to the pelleted Percoll-purified plastids (about 15 mg of protein) of 5 mL of lysing buffer. The plastid membranes were suspended to homogeneity at 4°C with a small paintbrush. To stabilize the Mg-Proto chelatase activity, and unless otherwise indicated, 100 nmol Proto/0.33 mL of membrane suspension was added to the lysed plastids immediately after lysis. The stroma and membranes fractions were then resolved following ultracentrifugation at 235,000*g* for 1 h in a Beckman 80 Ti angled rotor at 1°C (9).

Assessment of the Extent of Plastid Lysis

The extent of plastid lysis was determined from the activity of G6PDH (EC 1.1.1.44), a plastid stroma marker, in etiochloroplasts lysed by osmotic shock or by 0.1% Triton X-100. The G6PDH activity of Triton X-100-treated plastids was considered to represent 100% lysis. To measure G6PDH activity, 0.1-mL aliquots of lysed plastids were added to 0.9 mL of reaction medium, which consisted of 300 mM sucrose, 20 mM Tes-NaOH (pH 7.5), 10 mM $MgCl_2$, 0.17 mM $NADP^+$, and 0.2 mM gluconate-6-phosphate (9). A molar extinction coefficient of 6.22×10^3 M^{-1} cm^{-1} at 340 nm was used.

Mg-Proto Chelatase Assay

In a total volume of 1 mL containing 0.33 mL of crude (about 0.7 mg of protein) or purified etiochloroplasts (about 0.5 mg protein), lysed plastids (about 0.6 mg of protein), stroma (about 0.1 mg of protein), or membrane fractions (about 0.6 mg of protein), the reaction mixture consisted of 100 μM Proto, 330 mM sucrose, 200 mM Tris, 20 mM $MgCl_2$, 5 mM EDTA, 27 mM NAD^+, 15 mM ATP, 5 mM methionine, 25 mM methanol, 3 mM glutathione, and 0.1% (w/v) BSA at a room-temperature pH of 7.7. Incubation was in a flat-bottomed glass tube. To each incubation tube was added 0.01 mL of 10 mM Proto (100 nmol) except when the Proto had already been added to the lysed etiochloroplast suspension. The tubes were wrapped in aluminum foil and were incubated at 28°C for 2 h in darkness in a shaking water bath operated at 50 oscillations/min.

Protein Determination

Protein was determined according to Smith *et al.* (17).

Pigment Extraction

Before and after incubation, pigments were extracted by the addition of 5 mL of cold acetone:0.1 N NH_4OH (9:1 v/v)/mL reaction mixture. This was followed by centrifugation at 39,000*g* for 10 min at 1°C. The ammoniacal acetone extract was retained and the pellet was discarded. Chl and other fully esterified tetrapyrroles were transferred from acetone to hexane by extraction with an equal volume of hexane, followed by a second extraction with a one-third volume of hexane. The remaining hexane-extracted acetone residue containing Proto, Mg-Proto, and Pchlide was used for quantitative determination of Mg-Proto by spectrofluorometry (13). The measured Mg-Proto pool consisted of Mg-Proto and smaller amounts of Mg-Proto monoester.

Spectrofluorometry and Mg-Proto Determination

Fluorescence spectra were recorded at room temperature on a high-resolution, fully corrected, photon-counting SLM spectrofluorometer (model 8000C) interfaced with an IBM model XT microcomputer. Determinations of Proto and Mg-Proto were performed on an aliquot of the hexane-extracted acetone fraction in a cylindrical microcell 3 mm in diameter, with a precision of 3 to 6% (12, 13). Minimum detection levels were about 0.2 pmol/mL hexane-extracted acetone extract. All spectra were recorded at emission and excitation bandwidth of 4 nm. The amount of Mg-Proto was determined from its fluorescence amplitude at its emission maximum (595 nm) upon excitation at 420 nm. Fluorescence amplitudes were converted to Mg-Proto concentrations by reference to a standard calibration curve. The digital spectral data were automatically converted by the computer into quantitative values. The amount of Mg-Proto formed after 2 h of incubation was calculated by subtracting the amount of Mg-Proto detected at the beginning of incubation (0 h of control) from the amount of Mg-Proto detected after 2 h of incubation. Because no Mg-Proto peak was detectable at 0 h, the 0 h subtraction amounted to a noise correction at 595 nm. Lack of fluorescence overlap between Proto and Mg-Proto at 595 nm is illustrated in Figure 1.

RESULTS

Determination of the Extent of Plastid Lysis

As shown in Table I, the extent of plastid lysis by osmotic shock as evidenced by G6PDH activity was about 97%. The

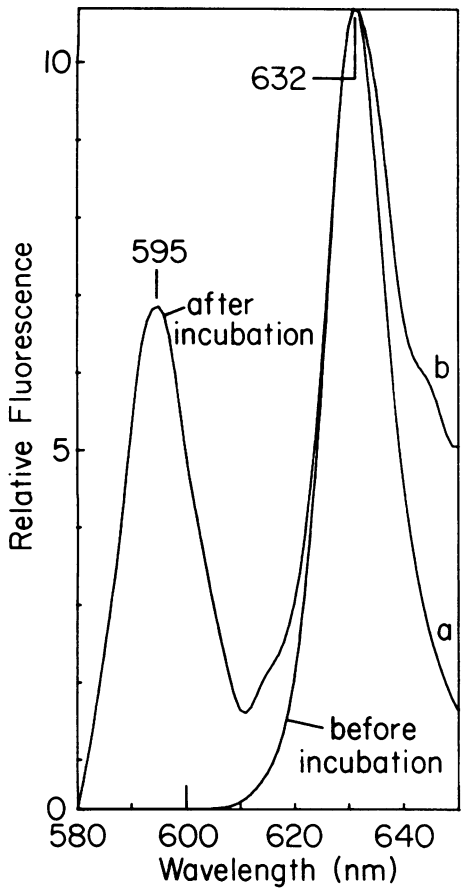

Figure 1. Fluorescence emission spectra in hexane-extracted acetone of lysed, stabilized plastid membranes (a) before incubation and (b) after 2 h of incubation. The emission spectra were elicited by excitation at 420 nm. The two spectra were normalized to the same amplitude at the emission maximum of Proto at 632 nm. The emission maximum at 595 nm is that of Mg-Proto.

Table I. *Comparison of Plastid Lysis in Etiochloroplasts Prepared from Kinetin + Potassium Gibberellate-Treated and Dark/Light-Treated Etiolated Cotyledons*

The extent of plastid lysis was evaluated from the G6PDH activity of plastids lysed by osmotic shock or 0.1% Triton X-100. Values are means of two replications.

Tissue	G6PDH Activity after Lysis by		Plastid Breakage
	Triton X-100[a]	Osmotic shock[b]	
	μmol NADP⁺ reduced/min·mg protein		%
Kinetin + GA_3-treated cotyledons[c]	60.6	58.9	97.2[d]
Dark/light-treated cotyledons	57.1	52.5	91.9

[a] Etiochloroplasts were lysed by addition of 0.1% Triton X-100. [b] Pelleted etiochloroplasts were suspended in the lysing buffer. [c] GA_3 = potassium gibberellate. [d] (100/60.6)58.9 = 97.2.

Table II. *Comparison of Mg-Proto Chelatase Activity Before and After Lysis of Crude and Purified Etiochloroplasts Prepared from Etiolated Cucumber Cotyledons*

Plastid Treatment	Mg-Proto Chelatase Activity	
	Crude etiochloroplasts	Purified etiochloroplasts
	nmol Mg-Proto formed/2 h·100 mg protein	
Unlysed[a]	324.9*[c]	540.2†
Lysed[b]	277.3*	550.5†

[a] Prior to incubation, pelleted etiochloroplasts were suspended in the suspension medium that contained a high concentration of osmoticum. Proto (100 nmol) was added to the reaction mixture just before incubation. [b] Prior to incubation, pelleted etiochloroplasts were suspended in the lysing medium that contained a low concentration of osmoticum. Proto (100 nmol) was added to the reaction mixture just before incubation. [c] Values are means of two replicates. The data were analyzed as a randomized complete block. Partitioning of the means is by LSD at the 5% level of significance. Values followed by different symbols are significantly different.

extent of plastid lysis depended on the pretreatment of the tissue from which the plastids were prepared and on the Tris-HCl concentration in the lysing buffer. For example, lysis of etiochloroplasts prepared from cucumber cotyledons that were subjected to three light (2.5 ms)/dark (60 min) treatments, and which had accumulated significant amounts of Chl *a*, resulted in 92% plastid breakage (Table I). When the Tris-HCl concentration of the lysing buffer was raised 5.8-fold, from 25 to 145 mM, the extent of plastid lysis dropped from 92 to 81%. It was, therefore, considered that the 97% plastid lysis achieved in etiochloroplasts prepared from etiolated cotyledons treated with kinetin plus potassium gibberellate was adequate enough to proceed with further experimentation.

Demonstration of Mg-Proto Chelatase Activity in Ruptured Etiochloroplasts

Mg-Proto chelatase activities before and after plastid disruption are compared in Table II. Spectral changes that accompanied the conversion of Proto to Mg-Proto by stabilized, lysed plastids are depicted in Figure 1. The activities of crude and Percoll-purified etiochloroplasts were 4.50- to 1.36-fold larger than those reported by others for developing cucumber chloroplasts (4–6, 22). They were lower, however, than reported rates for pea plastids measured with the use of Mg-deureroporphyrin, a nonphysiological substrate (23).

No significant differences in Mg-Proto chelatase activity between unlysed and lysed etiochloroplasts were observed, although the activity of purified plastids was significantly higher than that of the crude ones (Table II). It can, therefore, be concluded that the Mg-Proto chelatase activities of either crude or Percoll-purified etiochloroplasts were not altered by plastid rupture. This in turn indicated that in cucumber etiochloroplasts, plastid intactness is not a mandatory requirement for the insertion of Mg^{2+} into Proto by Mg-Proto chelatase. This is at variance with the results of others who

found that any disruption of cucumber chloroplasts resulted in a drastic decrease in Mg-Proto chelatase activity (4, 15, 22, 23).

Stabilization of Mg-Proto Chelatase Activity in a Subplastidic Membrane Fraction

Initial attempts at recovering Mg-Proto chelatase activity in isolated subplastidic fractions met with limited success. Some activity was recovered in unstabilized plastid membranes, and none was found in the plastid stroma (Table III). In other words, although Mg-Proto chelatase activity survived etiochloroplast disruption (Table II), most of the activity was lost upon separating the plastid membranes from stroma by ultracentrifugation. Several attempts were made to stabilize Mg-Proto chelatase activity during ultracentrifugation. Success was achieved when Proto, the natural substrate for Mg-Proto chelatase, was added to lysed plastids immediately after lysis and prior to ultracentrifugation, at a concentration of 100 nmol/0.33 mL of lysed plastid suspension (Table III). It is very likely that protection of Mg-Proto chelatase activity by adsorbed Proto involved stabilization of the enzyme by its substrate, a well-documented phenomenon (3, 16).

After ultracentrifugation, all Mg-Proto chelatase activity was found in the membrane fraction (Table III). The stroma was inactive. The isolated plastid membranes contained the bulk of the added Proto (Table IV). Although the observed membrane-bound Mg-Proto chelatase activity (85.2 nmol/2 h·100 mg protein) was only one-sixth that of purified etiochloroplasts (540.2), it was 145- to 450-fold higher than activities reported by others for cucumber subplastidic preparations (15, 20). It is worth noting that no improvement in Mg-Proto chelatase activity was observed upon recombining stroma and plastid membranes (Table III). On the contrary, the recombination resulted in a statistically significant drop in activity.

Partition of the Exogenous Proto Substrate Between the Membrane and Stromal Fractions

As reported above, exogenous Proto had to be added to lysed etiochloroplasts to stabilize the Mg-Proto chelatase activity during separation of plastid stroma from membranes. After ultracentrifugation, about 80% of the added Proto was found to be associated with the membrane fraction, whereas the remaining 20% was recovered with the stroma (Table IV). To determine whether the adsorbed Proto was loosely or tightly bound to the membranes, the latter were resuspended in the lysing medium and were subjected to a second ultracentrifugation. Almost all the adsorbed Proto was recovered in the membrane fraction, indicating that the Proto was tightly associated with the plastid membranes (Table IV).

An attempt was also made to determine whether the activity of Mg-Proto chelatase, with the Proto substrate already adsorbed to the plastid membranes, would increase upon addition of further exogenous Proto to the incubation medium. No significant differences in Mg-chelatase activities were observed with the addition of various amounts of Proto, ranging from 0 to 20 mmol/mL incubation medium. The highest concentration of added Proto was about fivefold higher than the amount of Proto originally solvated in the membranes. These results indicated that the concentration of Proto adsorbed to the membranes (1.8 μmol/100 mg protein) was high enough to saturate the chelatase activity during 2 h of incubation.

Table III. *Intraplastidic Localization of Mg-Proto Chelatase in the Membrane Fraction of Percoll-Purified Etiochloroplasts*

Treatment	Mg-Proto Chelatase Activity		
	Stroma	Membranes	Reconstituted system
	nmol Mg-Proto formed/2 h·100 mg protein		
Unstabilized[a]	0.0‡[c]	8.2‡	0.00‡
Stabilized[b]	0.1‡	85.2*	59.2†

[a] Lysed etiochloroplasts were subjected to ultracentrifugation in the absence of added Proto. [b] Proto was added to purified etiochloroplasts immediately after lysis and before ultracentrifugation. [c] Values are means of three replicates. The data were analyzed as a randomized complete block. Partitioning of the means is by LSD at the 5% level of significance. Values followed by different symbols are significantly different.

Table IV. *Partition of the Exogenous Proto Substrate Between the Plastid Membranes and Stroma*

Treatment	Proto Content	
	Supernatant	Pellets
	nmol Proto/100 mg protein	
First ultracentrifugation	274.5*[b] (19.7)[c]	1120.4† (80.3)
Second ultracentrifugation[a]	71.4* (6.1)	1092.8† (93.9)

[a] The membrane pellet from the first ultracentrifugation was resuspended in the lysing medium and was subjected to a second ultracentrifugation. [b] Values are means of two replicates. The data were analyzed as a randomized complete block. Partitioning of the means is by LSD at the 5% level of significance. Values followed by different symbols are significantly different. [c] Values in parentheses represent percent of total Proto content.

ATP Requirement for Subplastidic Membrane-Bound Mg-Proto Chelatase Activity

In intact cucumber etiochloroplasts, Mg-Proto chelatase activity became saturated at about 10 mM ATP (4). Two sets of experiments were designed to determine whether similar ATP concentrations are required for optimal activity of the subplastidic membrane-bound chelatase. In one set, ATP was omitted from the lysing medium, whereas in the other, ATP was included in the lysing medium.

When ATP was omitted from the lysing medium prior to separating plastid membranes from stroma, Mg-Proto chelatase activity was lost irrespective of the amount of ATP subsequently added to the incubation medium (Fig. 2). This indicated that ATP was required for enzyme stabilization during lysis and ultracentrifugation. When ATP was included

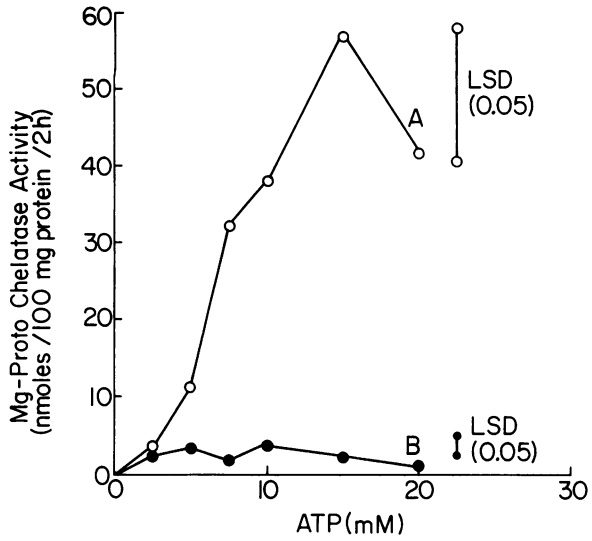

Figure 2. Effect of ATP addition to the incubation medium on the activity of membrane-bound Mg-Proto chelatase prepared from Percoll-purified etiochloroplasts in two different ways. A, ATP (20 mM) was added to the lysed plastids immediately after lysis and prior to ultracentrifugation. B, ATP was omitted from the lysed plastid suspension, and ultracentrifugation was performed in the absence of added ATP. The amounts of ATP shown on the abcissa were added to the reaction medium just before incubation. Mg-chelatase activity is reported in nmol Mg-Proto formed/2 h·100 mg protein. Values are means of four replicates. Partitioning of the means is by LSD at the 5% level of significance.

in the lysing medium, membrane-bound Mg-Proto chelatase responded to addition of further ATP to the incubation medium in a manner similar to whole etiochloroplasts (Fig. 2). It exhibited optimum activity at about 15 mM ATP.

Mg Requirement for Subplastidic Membrane-Bound Mg-Proto Chelatase Activity

As reported for intact etiochloroplasts (6), added Mg^{2+} was also required for Mg-Proto chelatase activity. Washing with EDTA prior to demonstrating the Mg^{2+} requirement, which was reported by others to be necessary when studying intact plastids (6), was not necessary. After an initial lag phase, which was overcome at concentrations of $MgCl_2$ higher than 5 mM, the activity of Mg-Proto reached a maximum at 10 mM $MgCl_2$ (Fig. 3). Higher $MgCl_2$ concentrations were inhibitory. We conjecture that higher Mg chelation rates may have been observed throughout if 10 mM instead of 20 mM $MgCl_2$ concentrations had been used during incubation.

EDTA Requirement for Subplastidic Membrane-Bound Mg-Proto Chelatase Activity

It has been our experience that optimal conversion of ALA to Proto, Mg-porphyrins, and Pchlide requires the presence of EDTA in both the homogenization medium of greening tissues and the incubation medium of isolated etiochloroplasts (10). When EDTA was omitted from the homogenization, lysing, and incubation media, Mg-Proto chelatase activity was lost and membrane-bound Proto was converted to Zn-Proto (emission maximum at 590 nm in hexane-extracted acetone at room temperature) instead of being converted to Mg-Proto (emission maximum at 595 nm). The fluorescence properties of Mg-Proto and Zn-Proto have been described elsewhere (11, 13, 18). Addition of 2.5 to 20 mM EDTA to the incubated plastids did not restore the Mg-Proto chelatase activity, but suppressed Zn-Proto formation. When EDTA was included in the homogenization and lysing media, addition of EDTA (2.5–10 mM) to the incubation medium did not affect Mg-Proto chelatase activity, which appeared to proceed normally (data not shown). However, higher concentrations of added EDTA (15–20 mM) severely inhibited (74–83%) Mg-Proto formation, probably as a consequence of Mg^{2+} chelation.

Lack of Effect of Other Additives on Mg-Proto Chelatase Activity

The lysing medium also contained NAD^+, glutathione, methanol, and methionine, and the effect of these substances upon Mg-Proto chelatase activity had not been determined. Percoll-purified plastids were therefore lysed in the normal lysing medium, which contained NAD^+, glutathione, methanol, and methionine, or in a lysing medium that lacked the above additives. The separated membranes were then incubated in the presence and absence of the aforementioned

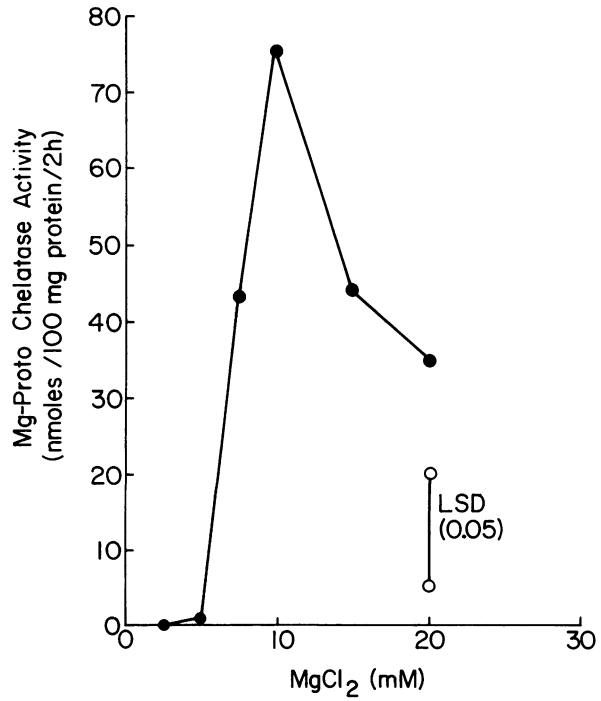

Figure 3. Effect of $MgCl_2$ addition to the incubation medium on the activity of membrane-bound Mg-Proto chelatase prepared from Percoll-purified etiochloroplasts. $MgCl_2$ was not added to the lysed plastids and ultracentrifugation was performed in the absence of added Mg^{2+}. Values are means of four replicates. Partitioning of the means is by LSD at the 5% level of significance.

pooled additives. These additives exhibited no measurable effect on Mg-Proto chelatase activity (Table V).

DISCUSSION

This is the first report of a subplastidic membrane-bound enzyme system capable of inserting Mg into exogenous Proto at high rates without the addition of a soluble stroma fraction. Although Walker and Weinstein also reported high rates of Mg insertion into exogenous Proto by a subplastidic pea chloroplast preparation, the latter required both a membrane-bound and a soluble fraction (23). Attempts to assay Mg-Proto chelatase activity in broken cucumber chloroplasts were unsuccessful (23). Furthermore, recombination of pellet and supernatant fractions of cucumber chloroplasts did not restore activity (23).

We have reported elsewhere that the separation of plastid stroma from plastid membranes may result in the solubilization of membrane components if appropriate precautions are overlooked (9). It is possible, therefore, that the coordinated action of two membrane-bound proteins may be needed for the insertion of Mg into Proto (as reported in ref. 23), but that one of the two proteins is more loosely bound to the plastid membranes and may be dissociated readily under certain preparative conditions. A similar dissociation of the loosely bound enzymes that catalyzed the conversion of ALA to Proto has been reported elsewhere (9). A thorough understanding of the mechanism of action of Mg insertion into Proto may therefore require a knowledge of how the two putative protein fractions are functionally and structurally related to each other and to the plastid membranes. The availability of highly active Mg-Proto chelatase preparations made up of one membrane fraction (this work) or of membrane-bound and soluble fractions (23) may help in this undertaking.

EDTA is a better chelator of Zn^{2+} than Mg^{2+}. It was originally used to decrease Zn^{2+} concentrations during the conversion of Proto to Mg-Proto monomethyl ester by *Rhodopseudomonas spheroides* (7). The complex effects of EDTA on Mg-Proto chelatase activity observed in this work argue, however, for a more involved role for EDTA than as a simple chelator of Zn^{2+}. For example, EDTA appears to be somehow involved in the stabilization of Mg-Proto chelatase activity during experimental manipulations, as evidenced by the necessity of adding EDTA to the homogenization and lysing media. Its Zn^{2+} complexation role also appears to be corroborated by the suppression of Zn-Proto formation when EDTA was added to incubated membranes prepared in the absence of added EDTA. However, (a) the inability of added EDTA to restore Mg-Proto chelatase activity in membranes prepared in the absence of added EDTA and (b) the inability of added EDTA to increase the rate of Mg insertion into Proto in membranes prepared in the presence of EDTA suggest that although this chelator is involved in the stabilization of Mg-Proto chelatase, it is not likely to be involved in the primary reaction mechanism of the enzyme.

In contrast to EDTA, ATP appears to be involved in both the stabilization of Mg-Proto chelatase activity and the primary mechanism of Mg^{2+} insertion into Proto. For example, in the absence of added ATP in the lysing medium, Mg-Proto chelatase activity was lost and could not be restored by the addition of ATP to the incubation medium. This suggested that irreversible inactivation of Mg-Proto chelatase occurred during membrane isolation in the absence of added ATP. On the other hand, Mg-Proto chelatase activity of membranes prepared when ATP was present in the lysing medium increased considerably in response to the addition of ATP to the incubation medium. This in turn suggested a second role for ATP, one involved in the primary reaction mechanism of Mg insertion into Proto.

In contrast to ATP and EDTA, Mg^{2+} did not appear to be involved in the stabilization of Mg-Proto chelatase activity. Indeed, the omission of Mg^{2+} from the homogenization and lysing media had no detectable effects on enzyme activity. However, Mg-Proto chelatase activity increased as increasing amounts of Mg^{2+} were added to the incubation medium (Fig. 3). This suggested that Mg^{2+} was probably somehow involved in the primary reaction mechanism of the enzyme.

It has been suggested that the role of ATP in Mg^{2+} chelation is to squeeze out the water of hydration surrounding the Mg^{2+} ion in aqueous solution (1). Walker and Weinstein provided evidence that ATP hydrolysis may be required for chelation (22). Obviously, the exact role of Mg^{2+} and ATP in the enzymic insertion of Mg^{2+} into Proto must await further investigations of the mode of action of the enzyme with purified enzyme preparations.

Table V. *Lack of Effect of Other Additives on Membrane-Bound Mg-Proto Chelatase*

Other Additives[a]	Mg-Proto Chelatase Activity
	nmol Mg-Proto formed/2 h·100 mg protein
Absent	60.2*[b]
Present	62.1*

[a] NAD^+ + methanol + methionine + glutathione. [b] Values are means of two replicates. Partitioning of the means is by LSD at the 5% level of significance. Values followed by the same symbol are not significantly different.

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