Plant Physiol. (1980) 65, 956–960
0032-0889/80/65/0956/05/$00.50/0

ATP Requirement for Mg Chelatase in Developing Chloroplasts[1]

Received for publication October 19, 1979 and in revised form December 14, 1979

ANTHONY D. PARDO, BARBARA M. CHERESKIN, PAUL A. CASTELFRANCO[2], VINCENT R. FRANCESCHI, AND BARBARA E. WEZELMAN
Department of Botany, University of California, Davis, California 95616

ABSTRACT

The synthesis of Mg-protoporphyrin-IX from exogenous protoporphyrin-IX, in a crude plastid pellet extracted from greening cucumber cotyledons was found to require L-glutamate as a cofactor. It has now been shown that glutamate acts in the presence of contaminating mitochondria to provide an ATP regenerating system. With purified plastids, Mg chelatase is not stimulated by glutamate; instead, it requires a high concentration of ATP and is greatly stimulated by added phosphoenolpyruvate and pyruvate kinase. GTP, UTP, CTP, and ITP will not substitute for ATP. ADP in the absence of an ATP generating system is completely ineffective, whereas it is slightly inhibitory in the presence of 10 mM ATP. AMP is strongly inhibitory in the reaction; 50% inhibition is obtained at approximately 3.5 mM AMP in the presence of 10 mM ATP.

In our previous communications (1, 2) we reported the synthesis of Mg-Proto[3] in chloroplast preparations isolated from greening cucumber cotyledons. These particles could utilize exogenous Proto as the substrate for Mg chelatase, but could also synthesize Proto from ALA or Glu. Regardless of the substrate added to the incubation, Mg-Proto synthesis required ATP and Glu. We concluded that Glu had a double role in Mg-Proto biosynthesis: (*a*) it was a precursor of ALA and, therefore, of Proto; and (*b*) it played a more specific role in Mg chelation. The nature of the latter role was completely unknown at the time of our last contribution. Our research has now progressed further, and the role of Glu in Mg chelation is no longer a mystery: Glu interacts with the mitochondria present in the crude plastid preparation to constitute an ATP regenerating system. In the absence of Glu, Mg chelatase activity can still be seen in the presence of high ATP concentrations. A preliminary account of these findings has appeared elsewhere (3). The experimental evidence for the identity of the reaction product as a mixture of Mg-Proto and Mg-Proto-Me was presented previously (2).

[1] This research was supported by National Science Foundation Grants PCM-75 10957 and PCM-78 13250.

[2] To whom correspondence should be sent.

[3] Abbreviations: Mg-Proto: Mg-protoporphyrin-IX; Proto: protoporphyrin-IX; ALA: δ-aminolevulinic acid; Glu: L-glutamic acid; Mg-Proto-Me: Mg-protoporphyrin-IX monomethyl ester; GSH: reduced glutathione; DTNB: 5,5′-dithiobis(2-nitrobenzoic acid); PEP: phosphoenolpyruvate; PK: pyruvate kinase; Cys: cysteine; DCPIP: 2,6-dichlorophenol indophenol; PMS: phenazine methosulphate; Mg-Proto-Me_2: Mg-protoporphyrin-IX dimethyl ester; MS: malate synthase; SDH: succinic dehydrogenase; IL: isocitrate lyase; α-KG: α-ketoglutaric acid; OAA: oxaloacetic acid. "MPE equivalents" is a term used by C. A. Rebeiz and co-workers to indicate the sum of Mg-Proto, Mg-Proto-Me, and the various biosynthetic intermediates between the latter and protochlorophillide.

MATERIALS AND METHODS

The following materials were obtained from Sigma: EDTA, GSH, Glu, CTP (Na salt), ITP (Na salt), GTP (Na salt), UTP (Na salt), AMP, CoA, Na_2 succinate, Fiske-Subbarow reducer, DTNB, phenylhydrazine HCl, PEP (Na_3 salt, hydrate), PK (rabbit muscle, type III).

Hepes, Tes, Cys, BSA (fatty acid-poor), ATP, ADP, DCPIP (Na salt), PMS, Na_2 malate were obtained from Calbiochem. Disodium malonate was purchased from MC&B and NAD from Nutritional Biochemicals.

Uranyl acetate ($UO_2Ac_2 \cdot 2H_2O$) and $(NH_4)_2MoO_4$ were purchased from Fisher Scientific; Pb citrate and glutaraldehyde (25% aqueous solution) were obtained from Eastman Kodak; OsO_4 from Stevens Metallurgical Corporation, 342 Madison Ave, New York, N.Y., and Spurr's resin from Polysciences Inc., Paul Valley Industrial Park, Warrington, Pa.

Proto was obtained from Porphyrin Products, Logan, Utah; Mg-Proto-Me_2 was a gift of Dr. Kevin Smith (Chemistry Department, University of California, Davis) and cucumber seed (*Cucumis sativus* L. var. Alpha Green) was a gift of the Niagara Chemical Division, FMC Corporation, Modesto, Calif.

Germination and Incubation Conditions. Cucumber seeds were germinated in the dark at approximately 25 C for 5.5 days as previously described (8). The cotyledons were harvested after greening for 4–5 h under 83 μE m^{-2} s^{-1}, at room temperature (22–26 C). Routine incubations contained in 1 ml: 500 μmol sucrose, 1 μmol $MgCl_2$, 1 μmol EDTA, 20 μmol Tes, 10 μmol Hepes, 4.0 μmol GSH, 0.6 μmol NAD, 2.0 mg BSA, varying amounts of ATP and Proto, and 3.3–11 mg plastid protein as determined by the biuret method. Incubations were for 1 or 2 h, at 28 or 30 C, under ordinary laboratory illumination (15–30 μE m^{-2} s^{-1} of white fluorescent light) and were terminated by freezing. Modifications and additions to this standard protocol are given in table and figure legends.

Routine Extraction and Determination of Porphyrins. Porphyrins and metalloporphyrins were extracted into ether by a previously described procedure (2). The ether extract was read in a Perkin-Elmer MPF 44-A fluorescence spectrophotometer with the excitation set at 410 ± 10 nm. Emission peak height for Mg-Proto was measured at 594 ± 3 nm. The concentration was determined by reference to standard Mg-Proto-Me_2 (17). In our experiments the samples were run in duplicates. The tabulated values indicate the mean and the experimental range.

Chloroplast Isolation. Developing chloroplasts were isolated initially as a crude pellet by a previously published procedure (17). During the course of this study, a fast but effective purification scheme was developed, which removes essentially all mitochondrial contamination. This procedure (adapted from Leese *et al.* [11]) is shown in Figure 2.

Enzyme Assays. SDH activity was determined by a modification of Hiatt's procedure (9). Our fractions were incubated for 5 min before reading a decrease in *A* at 600 nm using a Gilford spectrophotometer equipped with a Corning 840 recorder. This preincu-

bation eliminated an initial, rapid decrease in *A* that was not dependent upon the addition of succinate. IL was measured according to Dixon and Kornberg (5). MS was measured using a modification of Hock and Beevers' procedure (10). The assay contained, in a total volume of 1.0 ml, 50 μmol K-phosphate buffer (pH 8.0), 1.0 μmol DTNB, 7.5 μmol $MgCl_2$, 1.0 μmol acetyl CoA (prepared just prior to use by the method of Stadtman [15]), and 0.1 ml plastid suspension. The reaction was initiated by the addition of 1 μmol Na-glyoxylate and the increase in *A* at 412 nm followed. For SDH, IL, and MS determinations the plastid suspension did not include cysteine HCl and was sonicated for 15 s prior to use.

ATPase. Phosphate release was measured under our standard incubation conditions using the method of Fiske and Subbarow (6) and the Fiske-Subbarow reducer (Sigma).

Electron Microscopy. Pellets were resuspended in isolation medium and fixed by addition of an equal volume of 7% glutaraldehyde in isolation medium. Fixation was for 12 h at 4 C and was followed by three 20-min rinses with 0.1 M phosphate buffer (pH 7.2, 4 C). Samples from each pellet were transferred to small conical capsules, pelleted, then postfixed at room temperature for 4 h with 2% OsO_4 in phosphate buffer (0.1 M, pH 7.2). The samples were given four 20-min phosphate buffer rinses, dehydrated with acetone and infiltrated with Spurr's resin (14). Thin sections were stained with uranyl acetate (16) and lead citrate (12) and viewed in a JEOL JEM 100S transmission electron microscope.

RESULTS

Mg chelatase activity in crude plastid pellets was inhibited by anaerobiosis and malonate (Table I). Mg chelatase activity was supported by 5 mM citrate, α-KG, succinate, and malate, as well as 5 mM Glu. Acetate and OAA were inactive. Malonate inhibited Mg-Proto synthesis in the presence of succinate, but not in the presence of malate (Table II).

Using the same crude plastid preparation, the ATP dependence of Mg chelatase was investigated (Fig. 1). Curve 1 shows the ATP dependence in the presence of 5 mM Glu; curve 2 shows the ATP dependence in the absence of Glu. In the presence of Glu, the curve starts out with a positive slope and becomes saturated around 4 mM ATP. In the absence of Glu, the curve starts out with a zero slope and does not become saturated at any ATP concentration tested. When the ATP concentration was raised to 10 mM, reasonable Mg chelatase activity was obtained even in the absence of Glu (Fig. 1, Table III). Under these conditions, anaerobiosis was no longer inhibitory, while the malonate inhibition was reduced to 17%, which is at the limit of the experimental error (Table III). With this crude plastid preparation, Glu caused an increase in Mg chelatase activity even in the presence of 10 mM ATP.

A rapid procedure was developed to obtain purified plastids free of mitochondrial contamination (Fig. 2). In the course of this purification there was a progressive enrichment of the Mg chelatase activity (Table IV). There was also a decrease in glyoxysomal contamination (followed by means of the glyoxysomal markers, II, and MS) and a sharp decline in mitochondrial contamination (followed by means of the mitochondrial marker, SDH). ATPase

Table I. *Inhibition of Mg Chelatase Activity in Crude Plastids by Malonate and Anaerobiosis*

All samples contained 5 nmol Proto and 3.3 mg plastid protein/ml incubation mixture. Other concentrations were: ATP, 1.5 mM; Glu, 5 mM; malonate, 20 mM. The anaerobic sample was flushed with N_2 for 5 min prior to incubation. Incubation was for 1 h at 28 C.

Additions to Standard System	Mg-Proto
	pmol
Control	15.0 ± 0.3[a]
Glu	398 ± 37
Glu + N_2	22.0 ± 2.2
Glu + malonate	5.6 ± 0.2

[a] In this and subsequent tables, this notation indicates the range between duplicates, not the SD.

Table II. *Inhibition of Mg Chelatase Activity in Crude Plastids by Malonate in Presence of Succinate but Not in Presence of Malate*

Experiment 1 contained 4.6 mg plastid protein and 10 nmol Proto/ml incubation mixture; experiment 2 contained 6.4 mg plastid protein and 20 nmol Proto. Other concentrations were: ATP, 1.5 mM; succinate, 5 mM; malate, 5 mM; malonate, 20 mM. Incubations were for 1 h at 28 C.

Additions to Standard System	Mg-Proto
	pmol
Experiment 1	
Proto	8.5 ± 1.5
Succinate	13.0 ± 3.0
Proto + succinate	163 ± 8
Proto + succinate + malonate	13.6 ± 0.3
Experiment 2	
Proto	10.1 ± 1.4
Malate	3.0 ± 0.2
Proto + malate	224 ± 20
Proto + malate + malonate	248 ± 16

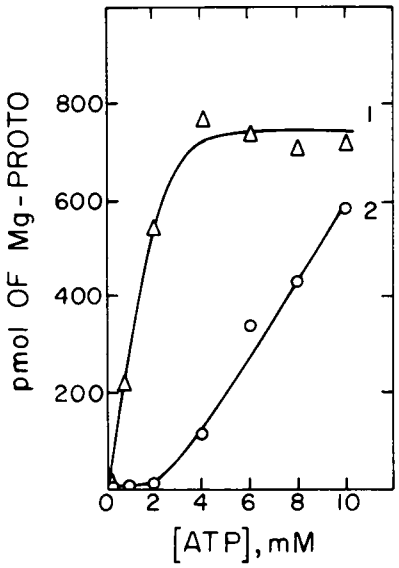

FIG. 1. ATP dependence of Mg chelatase activity in crude plastid pellets. Incubations for curve 1 contained 9.0 mg protein and 5.0 μmol of Glu in 1.0 ml total. Incubations for curve 2 contained 7.0 mg plastid protein and no Glu. All samples contained 10 nmol Proto and were incubated for 2 h at 28 C.

Table III. *Lack of Inhibition of Mg Chelatase Activity in Crude Plastids by Anaerobiosis and Malonate in Presence of 10 mM ATP*

Each flask contained 8.1 mg plastid protein in 1.0 ml reaction mixture. Concentrations were: ATP, 10 mM; Proto, 10 μM; Glu, 5 mM; malonate, 20 mM. The anaerobic sample was flushed for 5 min prior to incubation. Incubation was for 2 h at 28 C.

Additions to Standard System	Mg-Proto
	pmol
Control	271 ± 30
N_2	323 ± 32
Malonate	224 ± 2
Glu	450 ± 110

Table IV. *Comparison of Mg Chelatase, ATPase, and Various Organelle Markers during Plastid Purification*

The pellets were isolated as shown in Figure 2 and resuspended in the standard incubation medium. Mg chelatase and ATPase assays contained, in addition to the components of our standard assay system, 10 mM ATP and 10 μM Proto. For SDH, IL, and MS, Cys was omitted in the resuspension of the pellets; for the incubation conditions see under "Materials and Methods."

	Mg Chelatase	ATPase	SDH	IL	MS
	pmol Mg-Proto synthesized mg^{-1} protein h^{-1}	*μmol Pi released mg^{-1} protein h^{-1}*	*μmol mg^{-1} protein h^{-1}*		
Pellet 1	7.5	0.75	0.131	2.29	4.44
Pellet 2	14.8	0.95	0.00	1.15	4.75
Pellet 3	45.8	0.66	0.00	0.53	1.95
Reference		(6)	(9)	(5)	(10)

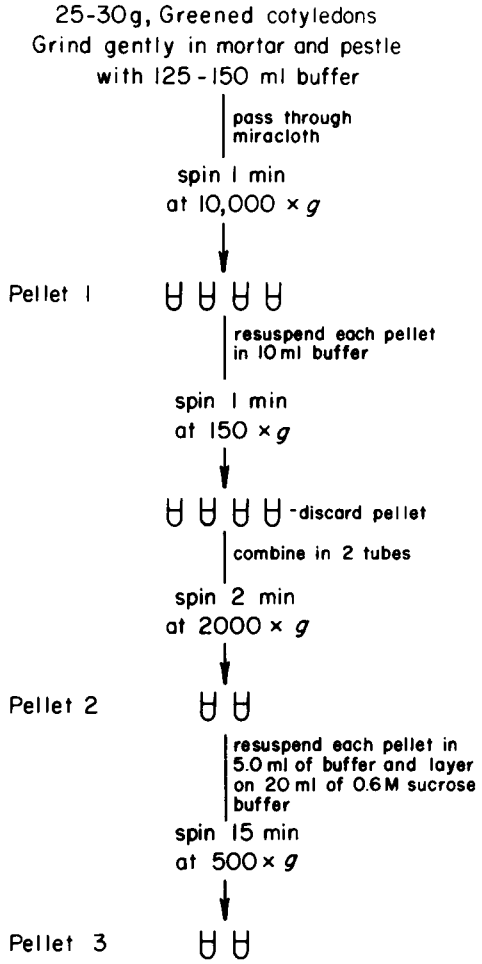

FIG. 2. Protocol for the purification of developing chloroplasts adapted from (11). Grinding buffer contained 0.5 M sucrose, 1 mM $MgCl_2$, 1 mM EDTA, 20 mM Tes, 10 mM Hepes, 5 mM cysteine, and 0.2% BSA (pH 7.7) (17). The last centrifugation was done through 20 ml grinding buffer in which the sucrose concentration was raised from 0.5 to 0.6 M.

activity, however, did not appear to decrease during plastid purification. The crude plastid pellet isolated according to the old method (17) and the initial and final pellets which were obtained during plastid purification (Fig. 2) were examined by electron microscopy (Fig. 3).

The crude plastid pellet (Fig. 3A) contained a variety of organelles and membranous constituents including developing chloroplasts and mitochondria. In pellet 1 of the plastid purification procedure, the developing chloroplasts were contaminated with mitochondria, numerous microbodies, starch grains, broken plastids, and other debris (Fig. 3B). Pellet 3, the final purified pellet of our scheme (Fig. 2) was still contaminated with microbodies and starch grains (Fig. 3C). Mitochondria were eliminated, chloroplasts were largely intact (Fig. 3C and 3D) and the population of small debris was greatly reduced. These electron microscope observations agreed well with the results of enzyme assays (Table IV).

The purified plastid pellet (pellet 3 of Fig. 2) no longer responded to the addition of Glu, as can be seen from the ATP dependence of Mg chelatase activity in the presence and absence of Glu (Fig. 4). The two curves are superimposable, starting out with zero slope and becoming linear around 3 mM ATP. There was no indication of either curve approaching saturation even at the highest ATP concentration tested (15 mM).

The ATP regenerating system, PEP plus PK, caused a pronounced stimulation of Mg chelatase activity in the purified plastid suspension, particularly at the lower ATP concentration (Table V).

The probable products of the ATPase activity were tested for the inhibition of Mg chelatase. In the presence of 10 mM ATP, 15 mM Pi gave no inhibition, 15 mM ADP gave only a slight inhibition (about 20%), whereas 15 mM AMP was strongly inhibitory. The inhibition of Mg chelatase at various AMP concentrations is shown in Figure 5; 50% inhibition was obtained at approximately 3.5 mM AMP.

ATP specificity seems to be absolute, since none of the other trinucleotides tested showed any activity in our system (Table VI). Likewise, ADP at twice the concentration did not replace ATP (Table VI).

Other properties of the Mg chelatase system in the purified organelle preparation were investigated. The pH optimum was 8.0–8.1. The temperature optimum was 29–30 C, and the Mg chelatase activity was saturated with respect to Proto around 7–10 μM. The system did not seem to respond to variations in the concentration of added $MgCl_2$. There did not seem to be any barrier to the penetration of Proto and ATP since gentle lysing of the organelles always resulted in a decrease in the observed Mg chelatase activity, never in an increase.

DISCUSSION

The stimulation of Mg chelatase in the crude plastid pellet was clearly dependent upon mitochondrial tricarboxylic acid cycle activity (Table I and II). However, it could proceed in the absence of tricarboxylic acid cycle substrates if the ATP concentration was high enough (Fig. 1). In the presence of 10 mM ATP, Mg chelatase was no longer inhibited by malonate and anaerobiosis (Table III).

When the developing chloroplasts were purified by differential centrifugation (Fig. 2) the mitochondrial contamination was essentially eliminated (Fig. 3C and Table IV) and the Mg chelatase in the purified plastid pellet no longer responded to Glu (Fig. 4). Mg chelatase activity in the purified plastid pellet was greatly stimulated by the ATP regenerating system, PEP plus PK. In the presence of the latter, good chelatase activity was obtained with

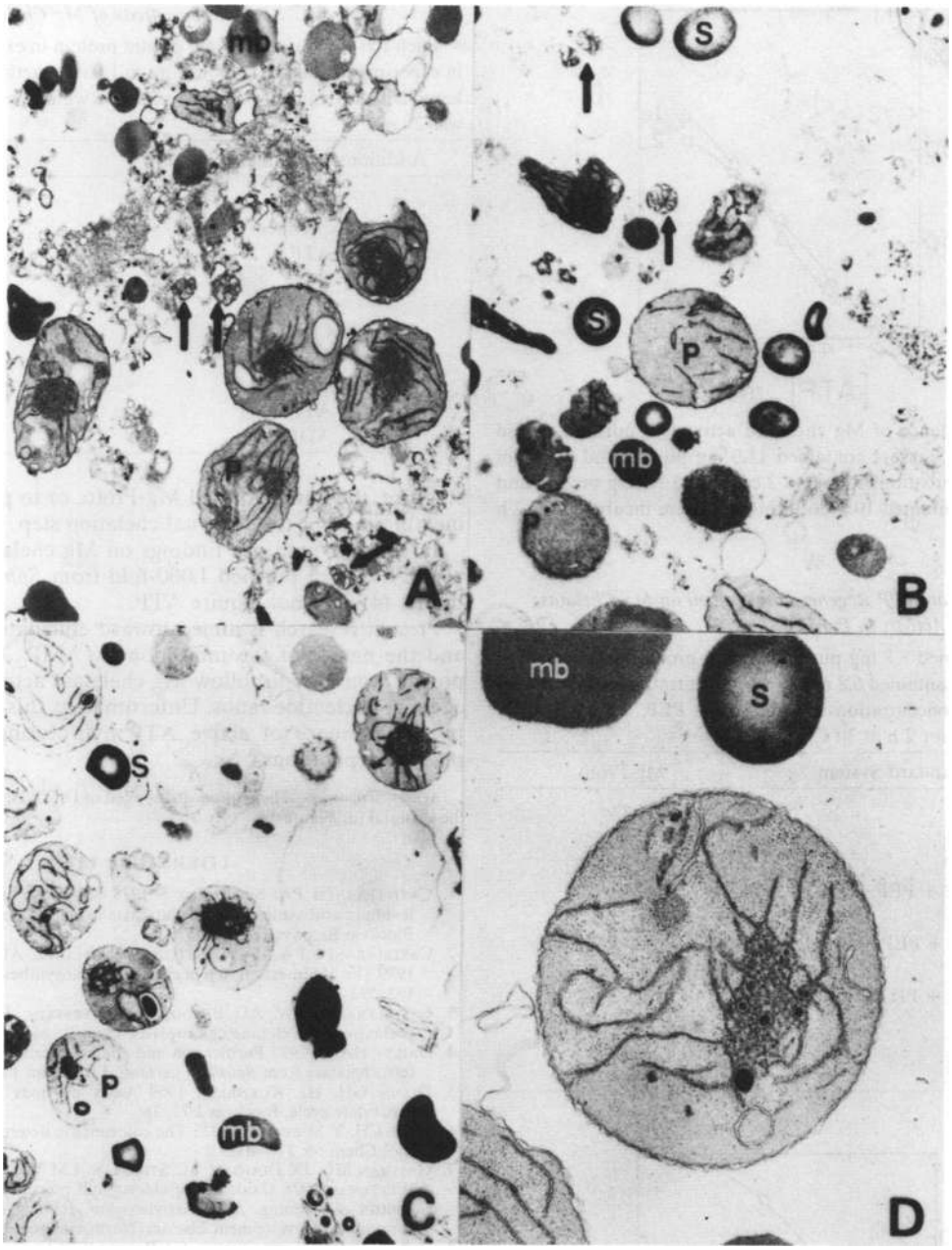

FIG. 3. Electron micrographs of portions of plastid pellets: mb, microbody; P, developing chloroplast; S, starch grain; →, mitochondrion. A: crude plastid pellet isolated according to (17) (× 7,560). B: pellet 1, isolated according to Figure 2 (× 7,438). C: pellet 3, isolated according to Figure 2 (× 4,550). D: developing chloroplast from pellet 3 (× 21,000).

1.5 mM ATP (Table V).

The ATP requirement for the chelatase activity was shown to be highly specific. GTP, UTP, CTP, ITP, and ADP were unable to replace ATP in the chelatase system (Table VI).

With our purified particulate system, a high ATP concentration is needed for Mg chelatase activity. In fact, we have not yet achieved ATP saturation, even at the highest concentration tested. The reason that such a high ATP concentration is required is probably complex. First, our purified plastid preparation has been shown to release Pi from added ATP at a very high rate (Table IV); second, the products of ATP hydrolysis, ADP, and particularly AMP, inhibit the Mg chelation reaction in the presence of 10 mM ATP (Fig. 5). It is easy to see that the combined effect of these two factors must be to make a high initial ATP concentration necessary to obtain measurable Mg chelatase activity. When the products of ATP hydrolysis are not permitted to accumulate, as in the presence of PEP plus PK, 1.5 mM added ATP is quite sufficient to support the chelatase reaction (Table V). At this time, the nature of the ATPase and the mechanism of the chelatase inhibition by AMP are not known.

The role of ATP in the Mg chelatase reaction could be quite indirect. For instance, Smith and Rebeiz (13) have recently reported that while the synthesis of Proto from added ALA occurs in the stroma, endogenous Proto, "MPE-equivalents", and protochlorophyllide are membrane-bound. Likewise, Gassman *et al.* (17) have inferred that Mg-Proto is membrane-bound from *in vivo* measurements of Mg-Proto decay, and preliminary measurements by Castelfranco and Schwarcz (unpublished) also indicate that the Mg-Proto synthesized in our system is probably membrane-bound. Therefore, the ATP could be required to bind, and therefore

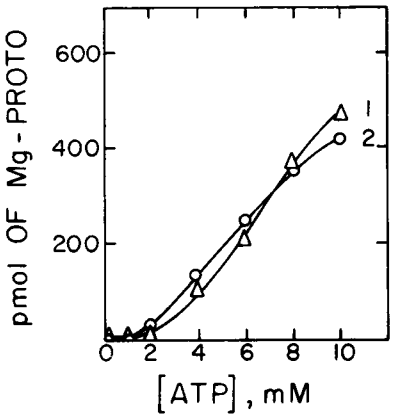

FIG. 4. ATP dependence of Mg chelatase activity in purified plastid pellets. Incubations for curve 1 contained 11.0 mg protein and 5.0 μmol Glu in 1.0 ml total. Incubations for curve 2 contained 7.1 mg protein and no Glu. All samples contained 10 nmol Proto and were incubated for 2 h at 30 C.

Table V. *Effect of an ATP Regenerating System on Mg Chelatase Activity in Purified Plastids*

Experiment 1 contained 8.8 mg purified plastid protein/ml incubation mixture; experiment 2 contained 6.8 mg. ATP concentration in experiment 2 was 1.5 mM. Other concentrations: Proto, 10 μM; PEP, 20 mM; PK, 20 μg/ml. Incubation was for 2 h at 30 C.

Additions to Standard System	Mg-Proto
	pmol
Experiment 1	
1.5 mM ATP	3.5 ± 0.2
1.5 mM ATP + PEP + PK	820 ± 68
10 mM ATP	625 ± 5
10 mM ATP + PEP + PK	922 ± 20
Experiment 2	
ATP + PEP + PK	566 ± 66
PEP + PK	6.4 ± 0.8
ATP + PK	1.5 ± 0.1
ATP + PEP	65.6 ± 7

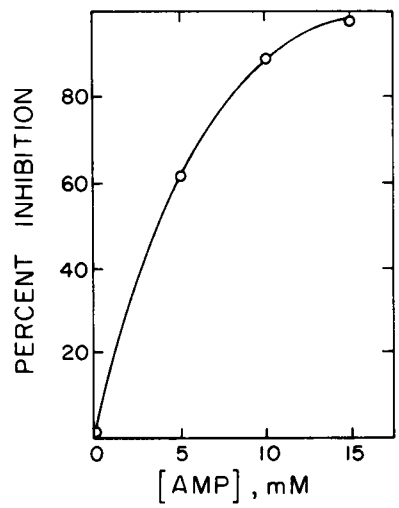

FIG. 5. Inhibition in the presence of AMP. Reaction mixtures contained 6.7 mg plastid protein, 10 nmol Proto and 10 μmol ATP in 1 ml total volume. Incubations were for 2 h at 30 C. The control samples produced 895 ± 33 pmol Mg-Proto.

Table VI. *Absolute Specificity of Mg Chelatase for ATP*

Each flask contained 7.5 mg plastid protein in experiment 1 and 7.1 mg in experiment 2, and 10 nmol Proto in 1.0 ml reaction mixture. Incubation lasted 2 h. All trinucleotide concentrations were 10 mM, ADP concentration was 20 mM.

Additions to Standard System	Mg-Proto
	pmol
Experiment 1	
ATP	937 ± 63
GTP	2.3 ± 0.7
UTP	1.5 ± 0.0
CTP	1.7 ± 0.1
ITP	1.9 ± 0.3
Experiment 2	
ATP	982 ± 7.5
ADP	1.9 ± 0.5

stabilize, the newly formed Mg-Proto, or to position Proto in the membrane prior to the actual chelation step.

In contrast with our findings on Mg chelatase, ferrochelatase, which has been purified 1,000-fold from *Spirillum itersonii* membranes (4) does not require ATP.

Present research is aimed toward elucidating the role of ATP and the nature of the inhibition by AMP. The most direct approach would be to follow Mg chelatase activity as a function of adenine nucleotide ratios. Unfortunately, this avenue is precluded by the presence of active ATP hydrolyzing enzyme(s) in our plastid preparations.

Acknowledgment—The authors are obliged to Dr. C. H. Lin for the preparation of the material for Figure 3A.

LITERATURE CITED

1. CASTELFRANCO PA, SCHWARCZ S 1978 Mg-Protoporphyrin-IX and δ-aminolevulinic acid synthesis from glutamate in isolated greening chloroplasts. Arch Biochem Biophys 186: 365–375
2. CASTELFRANCO PA, JD WEINSTEIN, S SCHWARCZ, AD PARDO, BE WEZELMAN 1979 The Mg insertion step in chlorophyll biosynthesis. Arch Biochem Biophys 192: 592–598
3. CASTELFRANCO PA, AD PARDO, BM CHERESKIN, JD WEINSTEIN 1979 Mg-chelatase in developing chloroplasts. Plant Physiol 63: S-96
4. DAILEY HA JR 1977 Purification and characterization of the membrane-bound ferrochelatase from *Spirillum itersonii*. J Bacteriol 132: 302–307
5. DIXON GH, HL KORNBERG 1959 Assay methods for key enzymes of the glyoxylate cycle. Biochem J 72: 3p
6. FISKE CH, Y SUBBAROW 1925 The colorimetric determination of phosphorus. J Biol Chem 66: 375–400
7. GASSMAN ML, JX DUGGAN, LC STILLMAN, LM VLCEK, PA CASTELFRANCO, B WEZELMAN 1978 Oxidation of chlorophyll precursors and its relation to the control of greening. *In* G Akoyunoglou, JH Argyroudi-Akoyunoglou, eds, Chloroplast Development Elsevier/North Holland, Amsterdam, pp 167–181
8. HARDY SI, PA CASTELFRANCO, CA REBEIZ 1970 The effect of the hypocotyl hook on greening in etiolated cucumber cotyledons. Plant Physiol 46: 705–707
9. HIATT AS 1961 Preparation and some properties of soluble succinic dehydrogenase from higher plants. Plant Physiol 36: 552–557
10. HOCK B, H BEEVERS 1966 Development and decline of the glyoxylate cycle enzymes in watermelon seedlings (*Citrullus vulgaris*. Schrad.) Effects of dactinomycin and cycloheximide. Z Pflanzenphysiol 55: 405–414
11. LEESE BM, RM LEECH, WW THOMPSON 1972 Isolation of plastids from different regions of developing maize leaves. *In* G Forti, M Avron, A Melandri, eds, Proc 2nd Int Congr Photosynthesis Res Stresa, June 1971. Dr W Junk, The Hague, pp 1485–1494
12. REYNOLDS ES 1963 The use of lead citrate at high pH as an electron-opaque stain in electron microscopy. J Cell Biol 17: 208–212
13. SMITH BB, CA REBEIZ 1979 Chloroplast biogenesis. XXIV. Intrachloroplastic localization of the biosynthesis and accumulation of protoporphyrin IX, magnesium-protoporphyrin monoester, and longer wavelength metalloporphyrins during greening. Plant Physiol 63: 227–231
14. SPURR AR 1969 A low-viscosity epoxyresin embedding medium for electron microscopy. J Ultrastruct Res 26: 31–43
15. STADTMAN ER 1957 Preparation and assay of acyl coenzyme A and other thiol esters; use of hydroxylamine. Methods Enzymol 3: 931–941
16. WATSON ML 1958 Staining of tissue sections for electron microscopy with heavy metals. J Biochem Biophys Cytol 4: 475–478
17. WEINSTEIN JD, PA CASTELFANCO 1977 Protoporphyrin IX biosynthesis from glutamate in isolated greening chloroplasts. Arch Biochem Biophys 178: 671–673