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Properties of Magnesium Chelatase in Greening Etioplasts

METAL ION SPECIFICITY AND EFFECT OF SUBSTRATE CONCENTRATIONS[1]

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ABSTRACT

Evidence was obtained by means of low temperature spectrofluorimetry and high pressure liquid chromatography for the exclusive chelation of Mg^{2+} into protoporphyrin IX by a plastid preparation extracted from greening cucumber cotyledons. Under the experimental conditions which were optimized for Mg^{2+} chelation, Zn^{2+} chelation was not detected. However, Zn^{2+} chelation was observed when ATP was omitted and $ZnCl_2$ was added to the incubation mixture. The observed Zn^{2+} chelation was partially nonenzymic. The requirements for the enzyme, Mg chelatase, are discussed in terms of the regulatory function of this enzyme.

Mg^{2+} chelation constitutes the first committed step in the synthesis of Chls. Metal ion insertion is the reaction which is responsible for the separation of two great classes of naturally occurring tetrapyrroles: the Cyts, which are derived from Fe-Proto,[4] and the Chls, which are derived from Mg-Proto.

The chelation of Mg^{2+} by Proto in the presence of greening etioplasts was reported in two prior communications from this laboratory (3, 8).

The identification of the reaction product as Mg-Proto rested on the comparison of its room temperature fluorescence spectrum in ether with that of authentic Mg- and Zn-Proto dimethyl esters (3). Preliminary results obtained with HPLC also supported the identification of the chelated metal as Mg.

Mg-Proto and Zn-Proto dimethyl esters differ by approximately 6 or 7 nm in their room temperature emission spectra (Table I; Fig. 2). However, etheral solutions of these two compounds could not be resolved by room-temperature spectrofluorimetry. Zn-Proto has been reported in many biological materials, although, so far, no biological function has been assigned to this metalloporphyrin. It has been shown in erythrocytes of patients affected with lead poisoning and Fe deficiency anemia (4) and in yeast cells cultured in the presence of Zn^{2+} salts (7). Synthesis of Zn-Proto has been demonstrated with cell-free systems obtained from *Rhodopseudomonas spheroides* (6), etiolated barley leaves (5), and greening cucumber cotyledons (9). We felt it was necessary to continue our studies on the nature of the metal to ascertain whether any Zn-Proto is synthesized under our experimental conditions.

MATERIALS AND METHODS

Reagents. EDTA (free acid), ATP (Na_2 salt), GSH, and NAD were purchased from Sigma. Hepes, Tes, Cys, and BSA (fatty-acid-poor) were obtained from Calbiochem. Diazald (*N*-methyl-*N*-nitroso-*p*-toluenesulfonamide) and 2-methylbutane were obtained from Aldrich Chemical Co. THF (stabilized analytical reagent) was obtained from Mallinckrodt. Proto was obtained from Porphyrin Products, Logan, UT; Mg-Proto-Me_2 and Zn-Proto-Me_2 were generous gifts of Dr. Kevin Smith, Department of Chemistry, University of California, Davis. Ammonium EDTA (NH_4^+EDTA) was prepared as follows: 1 eq EDTA was stirred with 1.5 eq aqueous NH_4OH. The solution was brought to pH 7.7 with KOH and diluted to yield a 100 mM EDTA solution. Diazomethane was made by the decomposition of Diazald in concentrated alkali, was co-distilled with ether, and was collected in a receiver chilled to 0 C.

Protoporphyrin IX Purification. Commercial available Proto was found to be of insufficient purity. Proto (Porphyrin Products) was, therefore, purified as follows. Approximately 10 mg Proto was suspended in 4 ml 95% ethanol containing 40 μmol KOH and centrifuged briefly to clarify the suspension. The supernatant was decanted. This procedure was repeated 8 or 10 times until little or no more porphyrin dissolved. The supernatants were combined and the total volume was measured. To the alcoholic extract were added 0.33 volume H_2O and 0.27 volume 1 M K-citrate (pH 4.0). The solution was brought back to pH with HCl. The alcoholic solution was extracted with 1 volume diethyl ether, and the lower phase was washed with 0.17 volume ether. The two ether extracts were combined, washed three times with equal volumes of H_2O, and stored at −15 C overnight. The ether was decanted without disturbing the ice crystals and was extracted with an equal volume of 10 mM KOH. The dissolved ether was removed by blowing N_2 over the dilute KOH phase. A small aliquot of the latter was diluted into 25% w/w HCl and the Proto concentration was determined spectrophotometrically [λ_{max} = 411 nm; E_{mM} = 278 (2)]. Purified Proto was estimated to be 99% homogenous by HPLC, using a 405-nm absorbance detector.

Preparation of Pheophytin. A hexane extract of Chl prepared from bean leaves (0.2 to 0.3 ml) was treated with an approximately equal volume of 6 M HCl. The mixture was shaken vigorously for 30 s in the dark. A large excess of H_2O (30 ml) was added, and the mixture was extracted three times with 5 ml diethyl ether. The ether extract was washed several times with H_2O to remove the HCl and was stored overnight at −15 C. The ether extract was decanted from the ice pellet and analyzed spectrofluorimetrically (Table I).

Preparation of Methyl Pheophorbide. Peak 1 of the HPLC effluent (Fig. 1) was collected. To 2.0 ml of this material in 90%

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[4] Abbreviations: Proto, protoporphyrin IX; Proto-Me_2, protoporphyrin IX dimethyl ester; Mg-Proto, Mg-protoporphyrin IX; Mg-Proto-Me_2, Mg-protoporphyrin IX dimethyl ester; Zn-Proto, Zn-protoporphyrin IX; Zn-Proto-Me_2, Zn-protoporphyrin IX dimethyl ester; Me-Chlide, methyl chlorophyllide; THF, tetrahydrofuran; EPA, diethyl ether-2-methylbutane-absolute ethanol (2:2:1 by volume); HPLC, high pressure liquid chromatography.

methanol, 6 ml H_2O was added, and the pH was adjusted to 2.0 with HCl. After standing in the dark at room temperature for 30 min, a few crystals (5 to 10 mg) of trisodium citrate were added, and the pH was adjusted to 4.5 with 0.1 M KOH. The aqueous solution was extracted three times with diethyl ether, and the ether was washed once with H_2O. The ether extract was stored overnight at −15 C and decanted from the ice pellet. The product was methylated with CH_2N_2 (see below) prior to spectrofluorimetric analysis (Table I).

Plant Material. Cucumber seeds (*Cucumis sativus* L. var. Beit Alpha), a gift of the Niagara Chemical Division, FMC Corporation, Modesto, CA, were germinated in complete darkness at approximately 25 C for 5.5 days. After 20 h illumination (40 to 60 $\mu E\ m^{-2}\ s^{-1}$, at 22 to 26 C), the cotyledons were harvested.

Chloroplast Isolation. Developing chloroplasts were isolated by a previously described procedure (8) except that "Pellet 2" was layered on 35 ml 0.6 M sucrose buffer (8) and centrifuged for 15 min at 500*g* in an HB-4 Sorvall rotor.

Divalent cation deficient plastids were prepared as follows. Pellet 2 was resuspended in 10 ml "EDTA buffer" [0.5 M sucrose, 10 mM Hepes, 20 mM Tes, 10 mM NH_4^+EDTA (pH 7.7)]. After 5 min at 0 to 4 C, the suspension was layered on 35 ml of a buffer containing 0.7 M sucrose, 10 mM Hepes, 20 mM Tes, 5 mM Cys, and 0.2% BSA (pH 7.7) and centrifuged at 1000*g* for 15 min in an HB-4 Sorvall rotor. The EDTA treatment was repeated and the final pellet was resuspended in incubation buffer.

Incubation Conditions. Routine incubations contained, in 1 ml: 500 μmol sucrose, 10 μmol Hepes, 20 μmol Tes, 4.0 μmol GSH, 0.6 μmol NAD, 2 mg BSA, 10 nmol Proto, varying amounts of ATP and metal salts, and 1 to 8 mg plastid protein as determined by the biuret method. Incubations were for 1 h at 28 to 30 C under ordinary laboratory illumination (2.0–4.5 $\mu E\ m^{-2}\ s^{-1}$) and were terminated by freezing. Deviations from the routine incubation are given in figure and table legends.

Determination of Mg^{2+} and Zn^{2+}. Plastid pellets were dried at 110 C and the residue was digested in boiling concentrated NHO_3 to clarity and diluted to volume with H_2O which had been distilled, deionized, and finally redistilled from an all-glass apparatus. Zn^{2+} and Mg^{2+} then were determined using a Perkin-Elmer 460 atomic absorption spectrophotometer against Zn^{2+} and Mg^{2+} standards purchased from Anderson Laboratories Inc., Fort Worth, TX, and Fisher Scientific, respectively.

Extraction of Metalloporphyrins. Routine extraction of metalloporphyrins has been described elsewhere (3). The diethyl ether solutions which contain large amounts of acetone and H_2O were suitable for room-temperature determination of porphyrins and metalloporphyrins. For spectrofluorimetry at 77 K and for HPLC, further purification of the ether extracts was required.

The crude diethyl ether solutions (2–6 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 three times with 10 ml diethyl ether. The four ether extracts were combined, washed once with 10 ml H_2O, and concentrated to 6 ml in a stream of N_2. The samples were stored overnight at −15 C. The ether was decanted from the ice pellet and treated with an ethereal solution of CH_2N_2 to a persisting straw-yellow color. After 15 min, the excess CH_2N_2 was removed in a stream of N_2.

Low Temperature Spectrofluorimetry. An aliquot of the ether solution containing the porphyrin methyl esters was mixed with an equal volume of 2-methylbutane and 0.5 volume absolute ethyl alcohol. Uncorrected fluorescence spectra were determined in a Perkin-Elmer MPF-44A fluorescence spectrophotometer, equipped with a liquid N_2 accessory. A 430-nm filter was used in the emission beam.

HPLC. For HPLC, the ethereal solutions of the porphyrin and metalloporphyrin methyl esters were evaporated to dryness in a stream of N_2. The residue was taken into 50 to 100 μl THF; a 5- to 20-μl aliquot was injected into a Waters Associates liquid chromatograph model 6000A equipped with a Rheodyne sample injector model 7125, a Waters Associates μBondapak C_{18} column, a Whatman Co Pell ODS precolumn, and a 405-nm absorbance detector (Waters Associates, model 440). The column was developed with 90% methanol, at a 1.2 ml/min flow rate. Excellent separations were obtained with 1 to 5 nmol of the synthetic metalloporphyrin dimethyl ester standards. The peaks were collected and their identity was confirmed by low temperature spectrofluorimetry. One ml of the column eluate in 90% methanol was mixed with 1 ml each of 2-methylbutane and diethyl ether. Three hundred μl H_2O was added. The two-phase mixture was separated; 0.5 ml of the upper phase was treated with 0.25 ml each of diethyl ether, 2-methylbutane, and absolute ethyl alcohol. Fluorescence was measured at 77 K as described above.

RESULTS

Greening etioplasts were incubated with Proto and with $ZnCl_2$ in the presence of ATP. The porphyrins and metalloporphyrins in the incubation mixture were extracted, methylated, injected into the chromatography column and developed with 90% methanol as described. Figure 1 shows the chromatogram tracing which was obtained. The four peaks were identified by spectrofluorimetry with reference to standards. Peak 1 was found even when Proto was omitted from the incubation mixture and in nonincubated zero-time controls. It was identified as Me-Chlide on the basis of the agreement of its fluorescence properties with those of Chl (Table I). Upon treatment with HCl to form methyl pheophorbide, the emission peak was shifted to a slightly longer wavelength at 295 K, and to a slightly shorter wavelength at 77 K. The same shifts were observed when Chl was converted to pheophytin

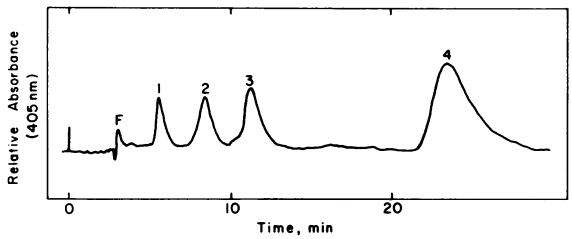

FIG. 1. High pressure liquid chromatogram: absorbance profile at 405 nm. This prophyrin sample was obtained from an incubation in the presence of 10 μM Proto, 10 mM $ZnCl_2$, 10 mM ATP, and endogenous Mg^{2+}. The chromatography was done on a μBondapak C-18 column. Elution solvent, 90% methanol; flow rate, 1.2 ml/min. F, solvent front; peak 1, Me-Chlide; peak 2, Mg-Proto-Me_2; peak 3, Zn-Proto-Me_2; peak 4, Proto-Me_2.

Table I. *Uncorrected Fluorescence Emission Peaks of Proto Derivatives under Different Conditions*

All emission measurements were taken with a 2-nm slitwidth and were reproducible to ±1 nm. Excitation was at 410 ± 10 nm.

Tetrapyrrole	Ether		EPA		90% Methanol, 295 K
	295 K	77 K	295 K	77 K	
Proto-Me_2	633	627	633	628	629
Mg-Proto-Me_2	593	599	593	599	593
Zn-Proto-Me_2	586	583	587	584	586
Me-Chlide	667		669	668	672
Me-pheophorbide			671	666	
Chl	665		667	669	671
Pheophytin			670	667	

(Table I). For these reasons, Peak 1 was ascribed to Me-Chlide, formed by the methylation of Chlide with CH_2N_2. Chlide was probably formed from Chl during the extraction by the action of chlorophyllase. Chlorophyllase has been shown to be very active in aqueous acetone solutions (1).

Peaks 2, 3, and 4 agreed with authentic Mg-Proto-Me_2, Zn-Proto-Me_2, and Proto-Me_2, respectively, in terms of HPLC retention times and fluorescence properties (Table I). The emission spectra of Mg-Proto-Me_2 and Zn-Proto-Me_2 were affected in opposite ways by temperature changes (Table I; Fig. 2). Figure 2 shows the liquid N_2 spectra of authentic Zn-Proto-Me_2 (A) and Mg-Proto-Me_2 (B) in EPA. A mixture of these two compounds was easily resolved at 77 K (C) but could not be resolved at room temperature (D). The excitation spectra of the two metalloporphyrins were also very different: Mg-Proto-Me_2 had an uncorrected excitation maximum at 433 nm in EPA at 77 K; under these conditions, Zn-Proto-Me_2 had a maximum at 421 nm. At 77 K, porphyrin dimethylester spectra were much more reproducible in EPA than in ether solution. At this temperature, ether often solidified into an opaque microcrystalline mass which interfered with the fluorescence measurements, whereas EPA always formed a clear glass.

At room temperature, the emission spectra of the nonmethylated compounds Mg-Proto and Zn-Proto were identical to those of their dimethyl esters. However, at 77 K, the free metalloporphyrins often gave nonreproducible artifactual peaks, particularly in the region between 589 and 591 nm. For these reasons, liquid N_2 spectra were always measured in EPA and on the dimethyl esters.

In our previous work on Mg chelatase (8), we were unable to demonstrate the dependence of the reaction upon added Mg^{2+} ion. Since plastids are known to be rich in Mg^{2+}, it seemed possible that the endogenous Mg^{2+} was sufficient to saturate this enzyme. Therefore, we endeavored to remove the endogenous Mg^{2+} by washing with NH_4^+ETDA. The Mg^{2+} and Zn^{2+} content of crude and EDTA-washed plastids is shown in Table II. It can be seen that roughly 97% of both ions were removed by this procedure.

Dependence of Mg chelatase upon added Mg^{2+} was demonstrated with EDTA-treated plastids (Fig. 3). Saturation with respect to Mg^{2+} was reached at about 10 mM Mg^{2+}. The chelation of Mg^{2+} shows an almost absolute dependence upon added Proto (Fig. 4). Saturation was reached at about 10 μM Proto.

We have previously shown (8) that Mg chelation requires ATP and is inhibited by AMP. At that time, we were not able to demonstrate the saturation of Mg chelatase with respect to ATP. In these experiments, using purified Proto along with increased greening time, we were able to show that the chelatase activity is saturated at about 10 mM ATP (Fig. 5).

The requirement for plastid protein in the chelation of Zn^{2+} ion is summarized in Table III. A substantial component of the total

Table II. *Metal Ion Content of Plastid Preparations*

The entries in Tables II and III give the means and the experimental ranges.

Sample	Mg^{2+}	Zn^{2+}
	μg/mg plastid protein	
Plastids before EDTA washing	6.06 ± 1.13	0.18 ± 0.02
Plastids after EDTA washing	0.18 ± 0.04	0.006 ± 0.002

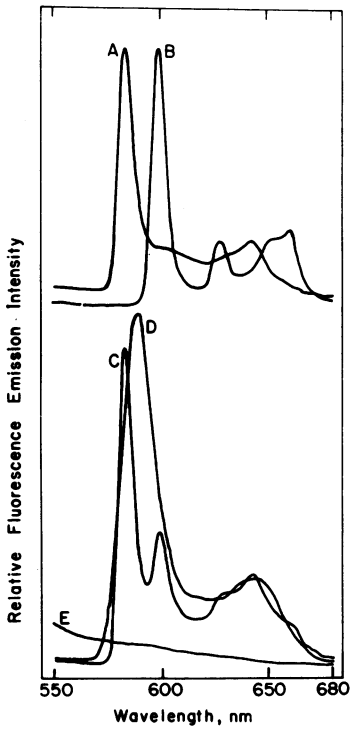

FIG. 2. Resolution of Mg-Proto-Me_2 and Zn-Proto-Me_2 by emission spectrofluorimetry in EPA at 77 K. Spectra are uncorrected. A, Zn-Proto-Me_2 at 77K; B, Mg-Proto-Me_2 at 77 K; C, mixture of Zn-Proto-Me_2 and Mg-Proto-Me_2 at 77 K; D, same as C, at 295 K; E, EPA solvent base line at 77 K.

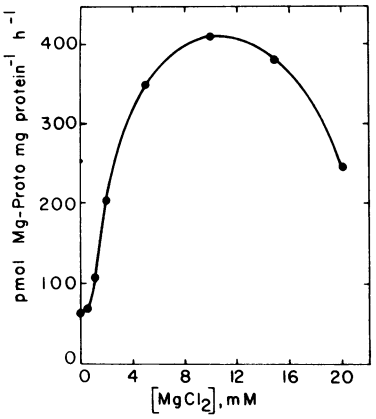

FIG. 3. Dependence of Mg-Proto synthesis on added $MgCl_2$ in EDTA-washed plastids. All the incubations contained10 mM ATP and 10 μM Proto.

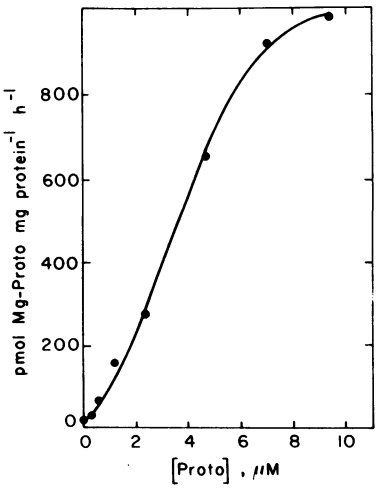

FIG. 4. Dependence of Mg-Proto synthesis on added Proto. The plastids were not EDTA-washed, and no Mg^{2+} was added. All incubations contained 10 mM ATP.

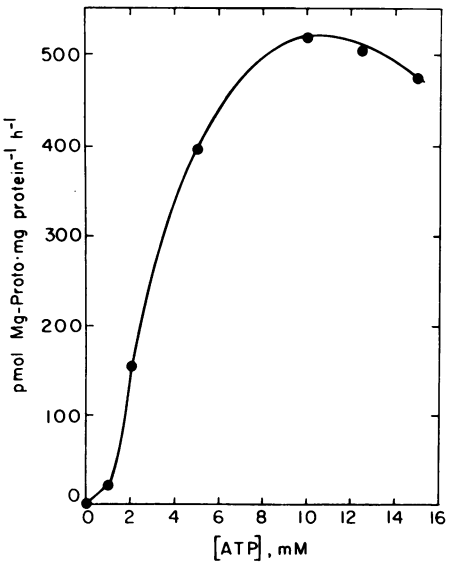

FIG. 5. Dependence of Mg-Proto synthesis on ATP. The plastids were not EDTA-washed, and no Mg^{2+} was added. All incubations contained 10 μM Proto.

Table III. *Plastid Protein Requirement for Zn^{2+} Chelation*

All samples contained 5 mM $ZnCl_2$. The "complete" sample contained 4.14 mg plastid protein.

Sample	Zn-Proto
	pmol
"Complete"	7014 ± 283
Boiled plastids	3650 ± 394
No plastids	4212 ± 61
Zero-time	782 ± 36

chelation must be nonenzymic, as seen from the boiled enzyme and minus protein controls. The zero time control value of 782 ± 36 pmol Zn-Proto does not mean that there is some Zn-Proto in the initial plastid preparation but, rather, that, in the presence of added $ZnCl_2$, this much Zn-Proto is formed (probably nonenzymically), even without the 1-h incubation.

DISCUSSION

It has been shown that Mg-Proto and Zn-Proto are easily resolved as the dimethyl esters by spectrofluorimetry in EPA at 77 K (Table I; Fig. 2). At this temperature, the emission maxima of these compounds are shifted in opposite directions (Table I). The emission of Zn-Proto-Me_2 moves toward higher frequencies and that of Mg-Proto-Me_2 moves toward lower frequencies. Therefore, although at room temperature the maxima differ by approximately 6 nm, at 77 K they are 16 nm apart. These compounds are also easily separated by HPLC (Fig. 1).

No detectable Zn-Proto was found in reaction mixtures incubated under our optimal conditions for Mg-Proto synthesis, which include 10 mM ATP. Analysis of the metals present in our plastid preparations has shown that the Zn^{2+} content is about 3% of the Mg^{2+} content on a weight basis (Table II). If $ZnCl_2$ is added to the incubation mixture, large amounts of Zn-Proto are synthesized; however, more than 50% of this synthesis is nonenzymic. We have endeavored to determine whether the enzymic component of Zn^{2+} chelation is dependent upon ATP, as reported by Little and Kelsey (5), but no conclusive results were obtained.

When the endogenous Mg^{2+} was removed from the greening etioplasts by EDTA washing, the synthesis of Mg-Proto became clearly dependent on the addition of $MgCl_2$ to the incubation mixture (Fig. 3). The curve is slightly sigmoidal with a marked inhibition of product formation above 10 mM Mg^{2+}. Half maximal product formation occurred at about 2 mM added $MgCl_2$.

The assay conditions used here have permitted us to observe the ATP saturation of Mg-Proto synthesis (Fig. 5). Again, the curve exhibits sigmoidal behavior, with an inhibition of product formation at ATP concentrations above 10 mM. Half maximal product formation occurred at about 3.5 mM ATP.

The dependence of Mg-Proto synthesis on added Proto was observed previously (3, 8). We have now determined a Proto concentration curve (Fig. 4). This curve is clearly sigmoidal. Half saturation occurred at about 3.5 μM Proto.

The sigmoidal behavior of the concentration curves for all three reactants is consistent with an allosteric nature of this enzyme, which is not unreasonable in view of its position in the pathway of tetrapyrrole synthesis. In fact, Mg chelatase is the first enzyme of the "Mg-branch" which leads to the Chls.

At this stage in our investigations, it is still too early to theorize about the mechanism of Mg^{2+} insertion and about the role of ATP in this process. However, it seems reasonable that the actual Mg^{2+} donor in the chelatase reaction is the Mg-ATP complex which is also the real substrate for most kinases.

The identification of the reaction product is not complete because we are still not certain of the extent of the methylation of the propionic acid side chain at position 6 of the macrocycle. Preliminary results by Castelfranco *et al.* (3) indicated that the product consists of a mixture of free acid and monomethyl ester. This important question is currently under investigation and will form the object of a future communication.

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