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Effect of N-Methylmesoporphyrin IX on the Branch Point of the Tetrapyrrole Pathway in Pea (*Pisum sativum* L.) Chloroplasts

Gyung-Hee Yu*

Department of Biochemistry, College of Sciences, Yonsei University, Seoul 120-749, Korea
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Abstract: Administering δ-aminolevulinic acid (ALA) to isolated pea (*Pisum sativum* L.) chloroplasts resulted in an increase of heme synthesis in the heme branch of the tetrapyrrole pathway. At 0.1 mM ALA, in the presence of 1 mM $FeSO_4$ heme synthesis was stimulated up to 7 fold of that in the absence of $FeSO_4$. N-Methylmesoporphyrin IX (NMMP), a powerful inhibitor of ferrochelatase, inhibited heme synthesis by 95% at one micromolar concentration. The addition of ATP to the chloroplasts caused not only heme synthesis, but Mg-protoporphyrin IX synthesis in the chlorophyll branch of the tetrapyrrole pathway. In the presence of NMMP, however, inhibition of Mg-protoporphyrin IX synthesis was not observed whereas heme synthesis was inhibited completely.
Key words: chloroplasts, N-methylmesoporphyrin IX, tetrapyrrole pathway.

Chloroplasts have all the enzymatic machinery required for the tetrapyrrole pathway leading to heme and chlorophyll synthesis (reviewed in Beal and Weinstein, 1990). The hemes are not only a functional prosthetic group of hemoprotein, such as electron transport cytochromes, peroxidase, and catalase, but a precursor of the chromophore of phytochrome in higher plants and phycocyanobilins in algae. Both heme and chlorophyll synthesis share a common tetrapyrrole pathway up to protoporphyrin IX and diverge at the branch point of ferrochelatase (protoheme ferrolyase; EC 4.99.1.1) and Mg-chelatase (Castelfranco and Beal, 1981). Ferrochelatase catalyzes the insertion of Fe^{2+} into protoporphyrin IX to achieve heme synthesis, whereas Mg-chelatase catalyzes the insertion of Mg^{2+} into protoporphyrin IX to give Mg-protoporphyrin IX synthesis which is the first unique step of chlorophyll synthesis. Recently, the method of measuring the flux at the branch point was developed in pea chloroplasts and it was found that the addition of ATP gave rise to a decrease in heme synthesis by 68% and a simultaneous increase in Mg-protoporphyrin IX synthesis. Moreover, the preferential turnover of heme compared to Mg-protoporphyrin IX was elucidated in these studies (Yu and Weinstein, 1995; Walker *et al.*, 1995).

An N-alkylated porphyrin, N-methylmesoporphyrin IX (NMMP) can act as neither a Mg-chelatase nor a ferrochelatase substrate. However, NMMP is a powerful inhibitor of ferrochelatase (Dailley and Fleming, 1983) and causes the accumulations of porphyrin intermediates, which may change the flux of porphyrins through the heme and chlorophyll branches of the tetrapyrrole pathway. In the present study, we have attempted to examine the flux of heme and Mg-protoporphyrin IX synthesis at the branch point of the tetrapyrrole pathway by using NMMP. The administering of NMMP may give some information on the regulation of the branch point.

*To whom correspondence should be addressed.
Tel: 82-2-361-2566, Fax: 82-2-362-9897

Materials and Methods

Plant material

Pea (*Pisum sativum* L.) seeds purchased from Asgrow Seed Co. were rinsed of excess fungicide, soaked in water for 90 min, and grown for 7 days under a 12 h light/12 h dark cycle.

Chloroplast isolation

Intact chloroplasts were isolated from 7-days-old pea leaves through a Percoll pad with procedures as previously described (Walker and Weinstein, 1991). Residual Percoll, BSA and DTT were removed by washing the intact chloroplasts with wash buffer (grinding buffer lacking BSA and DTT). Chloroplast intactness was examined by the latency of 6-phosphogluconate dehyd-

rogenase activity (Joumet and Douce, 1985).

Chloroplast incubations

For the synthesis of heme and Mg-protoporphyrin IX, intact chloroplasts were incubated at 30℃ in darkness for 30 min. The reactions were initiated by the addition of chloroplasts. For heme synthesis the 1 ml of incubation mixtures contained iso-osmotic incubation medium [0.5 M sorbitol, 50 mM Tricine, 1 mM EDTA (ethylenediaminetetraacetic acid), 1 mM $MgCl_2$, 5 mM DTT (dithiothreitol), pH 7.8] which was supplemented with 1 μCi δ-[4-^{14}C] aminolevulinic acid (ALA) (40~60 mCi/mmol, New England Nuclear) at final concentrations of 100 μM. For the simultaneous measurements of heme and Mg-protoporphyrin IX synthesis, the incubations were performed under the same conditions except when nonradioactive ALA was used (Walker *et al.*, 1995).

Quantitating heme and Mg-protoporphyrin IX

Heme synthesis was quantitated by the incorporation of radioactivity into noncovalently bound heme (Yu and Weinstein, 1995). The entire noncovalently bound heme was extracted with acid-acetone (Weinstein and Beale, 1983), and purified by adsorption of heme from the extraction solvent on a reversed-phase C-18 column (Waters Sep-Pak C-18 cartridge, 100 mg). The heme was further purified by reversed-phase high pressure liquid chromatography (HPLC) equipped with Nova-Pak ODS column (3.7×75 mm, 4 μm particles). The absorbance was monitored at 398 nm. The heme was quantified by comparison of the area of heme peaks to a standard curve. The amount of newly synthesized heme was obtained by estimation of the ^{14}C counts present in the heme peak.

The Mg-protoporphyrin IX synthesis was measured by the previously described stopped assay procedure (Walker and Weinstein, 1991). After incubation, the chloroplasts were extracted with ammoniacal acetone and Mg-protoporphyrin IX was measured fluorometrically.

Other procedure

Plastid protein was measured with the method developed by Bradford (1976), using bovine serum albumin as a standard.

Results and Discussion

The first committed intermediate of the tetrapyrrole pathway is ALA (Beal, 1990). Two molecules of ALA, then, are converted to porphobilinogen (PBG) by ALA dehydratase (Dresel and Falk, 1953).

We have attempted to drive *de novo* heme synthesis

Table 1. Heme accumulation in the chloroplasts administered ALA and PBG

Additions	Heme accumulation (pmol·mg^{-1} plastid protein)
Control (no incubation)	530± 10
2 mM ALA	638± 77
1 mM PBG	553± 7

All incubations were done in the presence of 1 mM $FeSO_4$. The data in all tables are represented as the mean± deviation.

Table 2. Effect of NMMP on the accumulation of the newly synthesized heme in the chloroplasts administered ALA

Additions	Heme (pmol·mg^{-1} plastid protein)
ALA	9± 0
ALA+$FeSO_4$	65± 2
ALA+NMMP	0.7± 0.1
ALA+$FeSO_4$+NMMP	4± 1

The administering ALA to the chloroplasts was performed in the presence and absence of $FeSO_4$. Radioactive [^{14}C-]ALA was supplemented to a final concentration of 0.1 mM. Concentrations of $FeSO_4$ and NMMP were 1 mM and 1 μM, respectively.

sation of two molecules of ALA yields one molecule in isolated intact chloroplasts by administering two intermediates of the tetrapyrrole pathway, ALA and PBG, in the presence of $FeSO_4$ (Table 1). Since the condenof PBG, we administered 2 mM ALA and 1 mM PBG, respectively. We found that the heme was already present in significant amounts in the chloroplasts when the entire non-covalently bound heme was directly extracted with acid-acetone. This pre-existing heme was 530± 10 pmol·mg^{-1} protein. The chloroplasts incubated with 2 mM ALA accumulated heme of 638± 77 pmol·mg^{-1} protein. It was a 40% increase of heme compared to that which pre-existed in the chloroplasts. Administering 1 mM PBG to the chloroplasts gave a 5% increase of the heme accumulation. For the heme synthesis in intact chloroplasts, therefore, ALA was more effective compared to PBG as a substrate.

Since significant amounts of heme were present in the chloroplasts, quantitating the new heme synthesis required using radioactive labeled ALA as a substrate. The newly synthesized heme was measured by incubating the chloroplasts with [^{14}C-]ALA and quantitating the [^{14}C-]heme.

The heme synthesis is catalyzed by ferrochelatase which inserts iron into protoporphyrin IX (Jones, 1968; Matringe *et al.*, 1994). In the presence of $FeSO_4$, the heme synthesis increased up to 7 fold of that when

Table 3. Effect of NMMP on the accumulation of the newly synthesized heme and Mg-protoporphyrin IX in the chloroplasts administered ALA in the presence of $FeSO_4$ and ATP

Additions	Heme	Mg-protoporphyrin IX
	(pmol·mg^{-1} plastid protein)	
ALA+$FeSO_4$+ATP	35±4	906±50
ALA+$FeSO_4$+ATP+NMMP	N.D.	906±10

Concentrations of ALA and $FeSO_4$ were 0.1 and 1 mM, respectively. ATP was present at 4 mM with a regenerating system. (20 mM phosphoenolpyruvate, 5.4 U/ml pyruvate kinase).
N.D. : not detected.

$FeSO_4$ was absent (Table 2). It was noticeable that heme synthesis was seen in the absence of $FeSO_4$. Under these conditions, heme synthesis was much smaller than that in the presence of $FeSO_4$. In fact, the endogenous iron pool in chloroplasts is well documented. An iron-storing protein, ferritin, is located in chloroplasts and considered an endogenous iron pool (Theil, 1987).

N-Methylmesoporphyrin IX is an N-alkylated porphyrin which has a powerful inhibitory effect on ferrochelatase (Dailey and Fleming, 1983). In pea chloroplasts, 1 μM NMMP strongly inhibited the heme synthesis by 95% and 75% at 50 μM and 2 mM ALA, respectively (Yu and Weinstein, 1995). Heme synthesis was also inhibited by 50% at 2.5 μM NMMP in *Euglena gracilis* (Beal and Foley, 1982). One micromolar of NMMP, in the present study, showed to be a powerful inhibitor of the *de novo* heme synthesis by almost 95% in the presence and absence of $FeSO_4$ (Table 2).

Both the Mg-protoporphyrin IX and heme synthesis utilize the protoporphyrin IX pool formed from ALA with the co-substrate, Mg^{2+} and Fe^{2+}, respectively (Beal and Weinstein, 1990). We found that NMMP shut down the heme synthesis. Thus we have attempted to examine the ability of *de novo* Mg-protoporphyrin IX synthesis in the presence of NMMP. Both the Mg-protoporphyrin IX and heme synthesis were measured simultaneously in the presence and absence of 1 μM NMMP (Table 3). All incubations included 4 mM ATP with a regenerating system (20 mM phosphoenolpyruvate, 5.4 U/ml pyruvate kinase) and 1 mM $FeSO_4$ since ATP is required for Mg-chelatase and $FeSO_4$ is required for ferrochelatase.

N-Methylmesoporphyrin IX had no effect on the Mg-protoporphyrin IX synthesis whereas it had a strong inhibitory effect on the heme synthesis. The heme synthesis was undetectable in the presence of NMMP. In the absence of NMMP, Mg-protoporpyrin IX exceeded that of heme by 25 fold. On the other hand, protoporphyrin IX was not detectable under these conditions (data not shown). This result is consistent with the suggestion that Mg-chelatase activity is present in sufficient amounts to utilize all of the protoporphyrin IX produced from ALA in pea chloroplasts (Walker *et al.*, 1995).

Until the recent studies, there have been two controversial models for the regulation of this branch point of the tetrapyrrole pathway (Beal and Weinstein, 1990). One is the "single pathway" model which proposes that ferrochelatase and Mg-chelatase utilize the same porphyrin pool originating from a common tetrapyrrole pathway. The other is the "dual pathway" model which argues that there are two separated porphyrin pools produced from different pathways.

In our data, the Mg-protoporphyrin IX synthesis was not affected by inhibition of the heme synthesis in the presence of NMMP. Since Mg-chelatase and ferrochelatase utilize the same substrate, the accumulations of porphyrins caused by a shut-down of heme synthesis would have resulted in an increase of the Mg-protoporphyrin IX synthesis. The ineffectiveness of NMMP in changing the rate of the Mg-protoporphyrin IX synthesis would tend to favor the "dual pathway" model. Nonetheless we can not rule out the possibility of the "single pathway" model. It is possible that the overall rate of the porphyrin flux may be indirectly affected by the blocking of the heme synthesis, resulting in the constant amounts of the Mg-protoporphyrin IX synthesis.

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