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Regeneration of Magnesium-2,4-Divinylpheoporphyrin a_5 (Divinyl Protochlorophyllide) in Isolated Developing Chloroplasts[1]

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ABSTRACT

A preparation of developing chloroplasts isolated from greening cucumber (*Cucumis sativus* L. var Beit Alpha) cotyledons was found capable of synthesizing divinyl protochlorophyllide (magnesium-2,4-divinylpheoporphyrin a_5) in the presence of glutamate, adenosine triphosphate, reducing power, *S*-adenosyl-L-methionine, and molecular oxygen. Both adenosine triphosphate and molecular oxygen were absolutely required while each of the other three was strongly promotive. Organelle intactness was essential. The divinyl protochlorophyllide (Pchlide) formed *in vitro* could be completely phototransformed. Regeneration of Pchlide was not inhibited by 0.3 millimolar chloramphenicol. The initial *in vitro* rate of Pchlide regeneration was considerably higher than the rate of Pchlide synthesis observed when greened cucumber seedlings were returned to darkness. However, Pchlide synthesis *in vitro* fell off exponentially with a half-life of approximately 21 minutes, whereas Pchlide synthesis *in vivo* was linear for at least 100 minutes. It is likely that the leveling off of the *in vitro* rate is due to the loss of chloroplast integrity during the incubation, because neither adding more cofactors, nor phototransforming the accumulated Pchlide in the middle of the incubation period, restored the high initial rate of Pchlide synthesis.

When a greening angiosperm tissue is placed in the dark, Chl accumulation ceases (6) while Pchlide regeneration takes place. The Pchlide reaches a concentration that is usually similar to the Pchlide concentration found in dark-grown tissue. Thereafter, the tetrapyrrole pathway is shut off, presumably at the initial step, the formation of ALA[2] (2). Although this is the general behavior of greening angiosperm tissue, some interesting exceptions have recently been described (1).

There is no doubt that the Pchlide accumulated during this dark interval is located in the plastid. However, there is no way of estimating the contribution of other cell compartments—cytosol, mitochondria, nucleus, ER, *etc.*—to this Pchlide resynthesis, so long as this phenomenon is followed in intact tissues. For this reason, we have studied Pchlide regeneration in isolated developing chloroplasts. Our results show that Pchlide regeneration *in organello* exhibits requirements for exogenous ATP, O_2, SAM and reducing power. Presumably, these requirements of our *in vitro* experimental system represent dependencies of the developing chloroplasts upon the cytosol and other compartments in the intact cell.

MATERIALS AND METHODS

Materials. Cucumber seed (*Cucumis sativus* L. var Beit Alpha) was a gift of Moran Seeds, Inc., P. O. Box 3091, Modesto, CA 95359. Chromatography and fluorimetry standards, Mg-2-vinyl, 4-ethyl pheoporphyrin a_5 (monovinyl Pchlide), and Mg-2,4-divinylpheoporphyrin a_5 (divinyl Pchlide) were obtained from dark-grown barley shoots and from a *Rhodopseudomonas spheroides* mutant V-3 culture, respectively. Both materials were extracted by large scale modifications of the routine extraction of porphyrins and metalloporphyrins in use in this laboratory (8). The Pchlide obtained from etiolated barley contains a small amount of the divinyl component (3). However, this was not enough to cause interference in our determinations.

Chemicals. ATP, CAP, Cys, EDTA, Glc-6-P, Glu, Hepes, Percoll, Tes were purchased from Sigma. BSA, was purchased from Calbiochem. Acetone, hexanes, and diethyl ether (for anesthesia) were obtained from Mallinckrodt. Isopentane (2-methylbutane) was purchased from Aldrich. Polyethylene (chromatography grade) was from Poly Sciences Inc., Warrington, PA.

Methods. *Plant Material and Chloroplast Isolation.* Cucumber seeds were germinated in complete darkness at room temperature for 5 to 6 d. After 20 h illumination with white fluorescent light (60 to 80 $\mu E \cdot m^{-2} \cdot s^{-1}$ PAR, at 28 to 30°C), the cotyledons were harvested and developing chloroplasts were isolated (12). The MgDVP initially present in the isolated plastids (11) was routinely removed by a 5 min exposure to white light (40–50 $\mu E \cdot m^{-2} \cdot s^{-1}$ PAR) prior to the *in vitro* incubation. Percent intactness of the isolated chloroplasts was determined by the $K_3Fe(CN)_6$ reduction method (18).

Incubation Conditions. Routine incubations contained in 1 ml:500 μmol sorbitol, 10 μmol Hepes, 20 μmol Tes, 1 μmol EDTA, 1 μmol $MgCl_2$, 2 mg BSA, and 3 to 5 mg of plastid protein as determined by the biuret method. Except as noted in legends, incubations were for 1 h at 29 to 30°C (pH 7.7), in complete darkness, which was achieved by wrapping incubation vessels in aluminum foil. Anaerobic conditions were attained by gently blowing N_2 from a N_2 gas tank through a hypodermic needle inserted in a rubber septum. This gassing was begun 5 min before the incubation, and continued throughout the incubation. The reaction was initiated by the addition of 50 μl of 100 mM ATP through the septum. Reactions were terminated by freezing.

Pigment Extraction and Assay Procedure. Routine extraction of nonphytylated pigments was carried out as described earlier

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[2] Abbreviations: ALA, 5-aminolevulinic acid; CAP, chloramphenicol; EPA, diethyl ether/2-methylbutane/absolute ethanol 2/2/1 by volume; Glc-6-P, glucose-6-phosphate; MgDVP, magnesium-2,4-divinylpheoporphyrin a_5 (divinyl protochlorophyllide) Pchlide; SAM, *S*-adenosyl-L-methionine.

(8). The ether fraction containing Mg-Porphyrin diacids and mono esters was assayed spectrofluorimetrically using a Perkin-Elmer MPF 44-A fluorescence spectrophotometer (9). Samples used for TLC and low temperature spectrofluorimetry were washed with 50 mM K-phosphate buffer (pH 6.8) and H_2O to remove acetone and other polar molecules. The ether extract was then stored overnight at −15°C and decanted carefully from the ice pellet (10).

Thin-Layer Chromatography. The *in vitro* reaction product was also identified by segregation on polyethylene thin layers, at room temperature in the dark using 90% acetone as the solvent (4, 11, 16). Authentic monovinyl and divinyl Pchlide were used as standards of comparison.

Low Temperature Spectrofluorimetry. The *in vitro* reaction product was also identified by excitation spectrofluorimetry at 77°K by a previously described method (3, 4, 11, 13).

Pchlide Accumulation in Vivo. Seedlings that had been illuminated for 20 h were placed in the dark at 30°C. At intervals, 25 cotyledon pairs (2.2 g) were harvested and ground in 8 ml of cold 95% acetone containing 0.1 M NH_4OH, under a green safelight. The homogenate was centrifuged for 8 min at 13,000*g*, and the pellet was extracted once more under the same conditions. The two supernatants were combined; H_2O was added to yield an acetone content of 75%, and the H_2O-acetone mixture (15–20 ml) was extracted five times with 10 ml of hexanes to remove the Chl. One-fifth volume of saturated NaCl was added to the remaining lower phase, and the pigments were extracted into ether (8) and assayed by spectrofluorimetry (9).

RESULTS

Accumulation of MgDVP required ATP, SAM, and reducing power (Table I, experiment 1). For the latter an equimolar mixture of NADPH plus Glc-6-P was used, which was slightly more effective than NADPH alone (data not shown).

O_2 was absolutely required (Table I, experiment 2). Organelle intactness was necessary. The ability to form MgDVP was totally destroyed by freeze-thawing, by sonication, or by osmotic shock (data not shown). The percent intactness in the Percoll-isolated chloroplast pellet determined by the $K_3Fe(CN)_6$ reduction method (18) was found to be between 89 and 92% in different preparations. This value was confirmed by direct examination of the chloroplast suspension under phase contrast microscopy (23).

Table I. *Cofactor Requirements of MgDVP Formation*

In experiment 1, intact developing chloroplasts, 4.4 mg protein, were incubated aerobically with 5 mM ATP, 1 mM SAM, 5 mM NADPH, 5 mM Glc-6-P, and 6 mM Glu. In experiment 2, 3.3 mg of plastid protein was added per sample. Anaerobiosis was achieved by gassing with N_2 as described in "Materials and Methods."

Incubation Conditions	Mg-2,4-Divinylpheoporphyrin a_5	
	pmol·h^{-1}·mg protein^{-1}	Percent of control
Experiment 1		
Complete	359	100
Minus Glu	123	34
Minus ATP	17	5
Minus SAM	115	32
Minus (NADPH + Glc-6-P)	125	35
Experiment 2		
Complete	225	100
Minus Glu	150	59
Minus O_2	<5	<2
Minus Glu, minus O_2	<5	<2

The reaction product was identified as MgDVP—*i.e.* as *divinyl* Pchlide—by TLC on polyethylene layers with 90% acetone as the solvent (4, 11, 16) and by excitation fluorescence spectroscopy at room and liquid N_2 temperatures (3, 4, 11, 13) (Table II). As standards of comparison, *monovinyl* Pchlide isolated from etiolated barley tissue and *divinyl* Pchlide isolated from a culture of *Rhodopseudomonas spheroides* V-3 mutant were used. The *in vitro* accumulated MgDVP was phototransformable (Table III).

The stimulation due to exogenous Glu was found to be variable (Table I), but was consistently saturated by 5 mM exogenous Glu (data not shown), and the timecourse of the reaction was similar with and without added Glu (Fig. 1). Attempts to enhance the dependence of MgDVP synthesis on exogenous Glu by adding the inhibitors of plastidic Glu synthesis, L-albizziine and azaserine (19), resulted in marked inhibition of Pchlide re-synthesis that was not reversed by Glu addition (data not shown).

Pchlide regeneration was not inhibited when 0.3 mM CAP was added directly to the incubation (data not shown).

The rate of MgDVP accumulation *in vitro* was maximal at the beginning, but decreased exponentially with time (Fig. 1). Very little product was accumulated after 60 min. With or without added Glu, the rate of MgDVP accumulation could be expressed by an equation of the form:

$$\frac{dP(t)}{dt} = V_0 \cdot e^{-\frac{t \cdot \ln 2}{t_{½}}}$$

where V_0 is the initial rate of MgDVP accumulation and $t_{½}$ is the half-life for the decay of the Pchlide synthetic capacity. The experimental points were fitted by the nonlinear least square method and the values for V_0 and $t_{½}$ were determined.

With added Glu, V_0 was 5.78 pmol·mg protein^{-1}·min^{-1} and $t_{½}$, 22.14 min. Without added Glu, V_0 was 4.04 pmol·mg protein^{-1}·min^{-1} and $t_{½}$, 20.36 min. The two $t_{½}$ values were not deemed to be significantly different.

In contrast, Pchlide regeneration in intact seedlings that were returned to darkness proceeded linearly for at least 100 min at a rate of 14.36 pmol g fresh wt^{-1}·min^{-1} (Fig. 2).

In the *in vitro* system, when a second addition of Glu, ATP, NADPH plus Glc-6-P, or SAM was made after 50 min of incubation, and the reaction product was determined after an additional 50 min, the final yield of MgDVP was the same as

Table II. *Identification of the in Vitro Incubation Product*

Pchlide Species	TLC on Polyethylene with 90% Acetone R_F	Fluorescence Excitation Maximum	
		Ether, 298°K	EPA, 77°K
Monovinyl	0.67	433	447
Divinyl	0.50	437	453
Incubation product	0.48–0.51	437	453

Table III. *Phototransformability of MgDVP Synthesized in Vitro*

Three mg of plastid protein per sample were used. Light intensity was 40 to 50 μE·m^{-2}·s^{-1}; the dark samples were covered with aluminum foil. Other incubation conditions were the same as in Table I.

Illumination Conditions	Mg-2,4-Divinylpheoporphyrin a_5	
	pmol·h^{-1}·mg protein^{-1}	Percent of control
Dark, 60 min	147	100
Light, 60 min	4	3
Dark, 60 min; light, 5 min	5	3

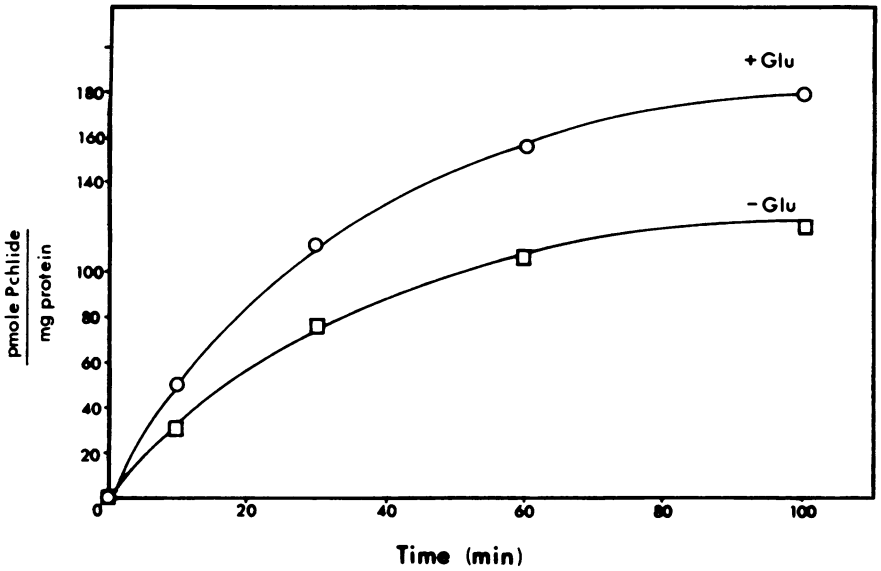

FIG. 1. *In vitro* time course of MgDVP formation in the presence and absence of added Glu. The composition of the incubation mixture was as indicated in Table I; 4.6 mg of plastid protein was added per sample. The reaction was terminated by freezing in a dry ice-acetone bath. The experimental data points fitted curves of the form:

$$\frac{dP(t)}{dt} = V_0 \cdot e^{-\frac{t \cdot \ln 2}{t_{1/2}}}$$

$$\left[\text{integrated form: } P(t) = \frac{V_0 \cdot t_{1/2}}{\ln 2}\left(1 - e^{-\frac{t \cdot \ln 2}{t_{1/2}}}\right)\right]$$

where V_0 is the initial velocity and $t_{1/2}$ is the half-life for the decay of $dP(t)/dt$. A nonlinear least squares method was used. With added Glu: $V_0 = 5.78$ pmol·mg protein^{-1}·min^{-1}; $t_{1/2} = 22.14$ min; $R^2 = 0.996$. Without added Glu: $V_0 = 4.04$ mg protein^{-1}·min^{-1}; $t_{1/2} = 20.36$ min; $R^2 = 0.980$.

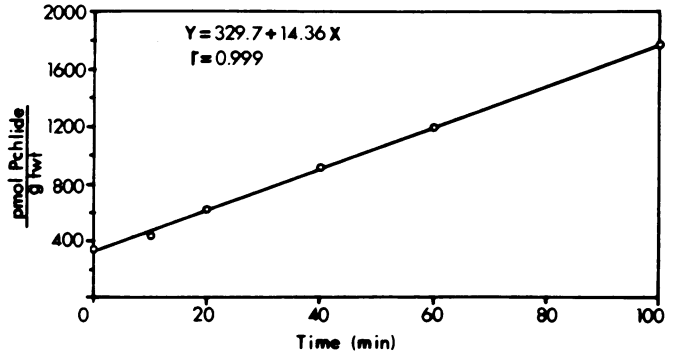

FIG. 2. *In vivo* time course of Pchlide regeneration when intact seedlings, that had been exposed to continuous light for 20 h, were returned to darkness.

without the second addition of substrate and cofactors (data not shown).

After 30 min incubation, the accumulated Pchlide was removed by a 5 min exposure to white fluorescent light; the samples were then incubated again in the dark for another 30 min. Pchlide removal did not restore the capacity to accumulate additional Pchlide at anywhere near the original rate (data not shown).

DISCUSSION

The synthesis of MgDVP in isolated developing chloroplasts required ATP, reducing power, SAM, O_2, and organelle intactness. These requirements were altogether consistent with what is known about the Chl biosynthetic pathway as far as Pchlide (7). ATP is required in ALA synthesis (14, 17) and in Mg^{2+} chelation (20). NADPH is required in ALA synthesis (14, 17) and in the formation of the isocyclic ring (25). O_2 is required in the oxidation of coproporphyrinogen to protoporphyrin (15) and in the formation of the isocyclic ring (9). SAM is required for the methylation of Mg-protoporphyrin IX (21) and is not made in the chloroplasts (24). Organelle intactness is required for Mg^{2+} chelation (12).

The stimulation by exogenous Glu was qualitatively consistent but quantitatively variable. We were unable to find incubation conditions under which MgDVP regeneration was absolutely dependent on added Glu. Chloroplasts are known to be the main site of Glu synthesis in green plant tissues (19, 22), and therefore a supply of endogenous substrate for Pchlide synthesis would be expected to be present in the organelles.

The lack of CAP inhibition indicates that Pchlide regeneration at this stage of greening proceeds without concomitant organellar protein synthesis. This finding is consistent with earlier results from our own laboratory about Chl synthesis from ALA in isolated developing chloroplasts (5). In a sense, it is not surprising that at this rather late stage of greening (after 20 h of continuous illumination), Chl biosynthesis is insensitive to inhibitors of organellar protein synthesis. Presumably, all the protein factors needed to sustain short-term synthesis of Pchlide and Chl (5) are already present in these plastids.

The rate of MgDVP regeneration *in vitro* under our optimal conditions extrapolated to 4.04 pmol·mg protein^{-1}·min^{-1} at zero time in the absence of added Glu, and to 5.78 pmol·mg protein^{-1}·min^{-1} in the presence of added Glu. In our chloroplast isolation, 0.863 mg of protein were recovered in the intact chloroplast pellet from each g of fresh cotyledon tissue. Furthermore, 7% of the Chl in the tissue was recovered in the isolated chloroplast pellet (data not shown) indicating a 7% recovery of the chloroplast population. Finally, it was observed that only 90% of the plastids in the 'intact chloroplast pellet' were actually intact by the $K_3Fe(CN)_6$ reduction assay (N Arroyave, PA Castelfranco, unpublished data). By combining this information, we are able to express the initial *in vitro* rates in terms of fresh cotyledonary tissue.

Without added Glu:

$$4.04 \times 0.863 \times \frac{100}{7} \times \frac{100}{90} = 55.34 \text{ pmol·g fresh wt}^{-1}\text{·min}^{-1}$$

With added Glu:

$$5.78 \times 0.863 \times \frac{100}{7} \times \frac{100}{90} = 79.18 \text{ pmol·g fresh wt}^{-1}\text{·min}^{-1}.$$

Comparing these with the *in vivo* rate of Pchlide accumulation (Fig. 2) which is 14.36 pmol·g^{-1}·min^{-1}, it is clear that the initial *in vitro* rates are higher than the *in vivo* rate. However, the *in vitro* rates decreased exponentially (Fig. 1), whereas the *in vivo* rate remained constant for at least 100 min (Fig. 2). Adding Glu increased the initial rate, V_0, from 55.34 to 79.18. But the half-life of the Pchlide-synthetic capacity was virtually unaffected (22.14 min with and 20.36 min without added Glu).

Since a second addition of substrate and cofactors in the middle of the incubation period had no effect on the final yield of MgDVP, we conclude that the process does not level off

because the system runs out of any of these materials.

The possibility that the observed leveling off of Pchlide synthesis *in vitro* reflects a feedback inhibition by the accumulated Pchlide, was ruled out by phototransforming the Pchlide that had accumulated during 30 min and placing the vessels back into the dark. It was observed that phototransformation in no way restored the original rates of Pchlide synthesis.

Possibly, our incubation medium is not an appropriate environment for the prolonged survival of isolated chloroplasts and therefore the latter disintegrate during the incubation. We believe that this is the likely explanation for the observed decay of the *in vitro* Pchlide synthetic rates.

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