Chloroplast biogenesis 76. Regulation of 4-vinyl reduction during conversion of divinyl Mg-protoporphyrin IX to monovinyl protochlorophyllide *a* is controlled by plastid membrane and stromal factors*

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Abstract

Most of the chlorophyll (Chl) *a* of green plants is formed *via* two biosynthetic routes, namely the carboxylic divinyl and monovinyl chlorophyll biosynthetic routes. These two routes are linked by (4-vinyl) reductases that convert divinyl tetrapyrroles to monovinyl tetrapyrroles by reduction of the vinyl group at position four of the macrocycle to ethyl. The activities of these two routes are very sensitive to cell disruption. For example in barley leaves, cell disruption, a mandatory step during plastid isolation, results in partial inactivation of the carboxylic divinyl route. Investigations with subplastidic fractions revealed that the carboxylic divinyl and monovinyl biosynthetic routes were regulated by a delicate interaction that involved plastid membranes, stroma, and reduced pyridine nucleotides. While the monovinyl biosynthetic route was very active in isolated plastid membranes, activation of the divinyl biosynthetic route required the joint presence of plastid membranes and stroma. Contrary to expectation, activity of the carboxylic divinyl biosynthetic route was greatly enhanced by addition of NADPH to the lysing buffer used during plastid membranes and stroma preparation. NADPH in cooperation with the plastid stroma may play an important regulatory role during the biosynthesis of divinyl and monovinyl protochlorophyllide *a*.

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Abbreviations: ALA, 5-aminolevulinic acid; Chl, chlorophyll; Chlide, chlorophyllide; DV, divinyl (vinyl groups at position 2 and 4 of the macrocycle); MV, monovinyl (vinyl group at position 2 and ethyl group at position 4 of the macrocycle); Pchlide, Protochlorophyllide; Proto, Protoporphyrin IX; Mpe, Mg Proto monomethyl ester. Unless preceded by MV or DV, tetrapyrroles are used generically to designate metabolic pools that may consist of MV and DV components.

Additional key words: *Hordeum vulgare*; NADPH.

Introduction

Biosynthetic heterogeneity refers to the biosynthesis of a particular metabolite by an organelle, tissue or organism *via* multiple biosynthetic routes (Arigoni 1994). It has been documented in 5-aminolevulinic acid (ALA), chlorophyll (Chl) *a*, and vitamin B_{12} biosynthesis (Arigoni 1994, Rebeiz *et al.* 1994, Scott 1994). In green plants Chl *a* is formed *via* parallel biosynthetic routes, namely divinyl (DV) and monovinyl (MV) Chl *a* carboxylic routes (Leeper 1991, Richards 1993, Rebeiz *et al.* 1994) (Fig. 1). Intermediates of the DV carboxylic route consist of dicarboxylic and monocarboxylic tetrapyrroles with vinyl groups at positions 2 and 4 of the macrocycle such as DV protoporphyrin IX (Proto), DV Mg-Proto, DV Mg-Proto monomethyl ester (Mpe), DV protochlorophyllide (Pchlide) *a*, and DV chlorophyllide (Chlide) *a* (Figs. 1 and 2). The MV carboxylic routes involve dicarboxylic and monocarboxylic tetrapyrroles including MV Mg-Proto, MV Mpe, MV Pchlide *a*, and MV Chlide *a*, which have one vinyl and one ethyl group at positions 2 and 4 of the macrocycle, respectively (Figs. 1 and 2). The ratio of MV to DV Pchlide *a* biosynthesis and accumulation is dependent on the plant species and plant age (Shioi and Takamiya 1992, Ioannides *et al.* 1994). In higher plants, the end product of the Chl *a* biosynthetic heterogeneity are invariably MV Chl *a* and *b*, with the only known exception of a lethal maize mutant (Bazzaz 1981) which forms only DV Chl *a* and *b*. However, in the prochlorophyte picoplankton of the subtropical waters of the North Atlantic as well as in the picoplankton of the euphotic zone of the world tropical and temperate oceans, and the Mediterranean sea, DV Chl *a* and *b* are the predominant Chl species (Chisholm *et al.* 1990, 1992, Veldhuis and Kraay 1990, Goerike and Repeta 1992). In green plants, the multiplicity of Chl *a* biosynthetic routes produces different pools of MV Chl *a*, at different sites of the photosynthetic membranes (Rebeiz *et al.* 1983, 1994).

The DV and MV Chl *a* routes are linked at the level of DV Mg-protoporphyrin IX (Proto), DV protochlorophyllide (Pchlide) *a*, and DV chlorophyllide (Chlide) *a* by (4-vinyl)reductase(s) that convert the 4-vinyl group at position 4 to ethyl, thus converting a DV tetrapyrrole to a MV tetrapyrrole (Tripathy and Rebeiz 1988, Parham and Rebeiz 1992, 1995, Kim and Rebeiz 1996). Ellsworth and Hsing (1974) also reported that Mpe was converted to MV Mpe by an NADH-dependent enzyme. However, so far no one including ourselves has been able to duplicate these results (Kim and Rebeiz 1996). The number of active (4-vinyl)reductases is uncertain at this stage. Whyte and Griffiths (1993) have interpreted the accumulation of DV and MV Pchlide *a* in term of a dual pathway with a single vinyl reductase of broad specificity. In their scheme the major route converts DV Pchlide *a* to MV Chl *a* *via* DV Chlide *a* which is reduced to MV Chlide *a*. The minor route consists of the formation of MV Chlide *a*, and by inference MV Chl *a* *via* DV Pchlide *a*, which is converted to MV Pchlide *a* by the non-specific vinyl reductase. This hypothesis is not compatible with the following observations: (*a*) During DV and MV Pchlide *a* biosynthesis, only a fraction of the MV Pchlide *a* pool can be formed by reduction of DV Pchlide *a*

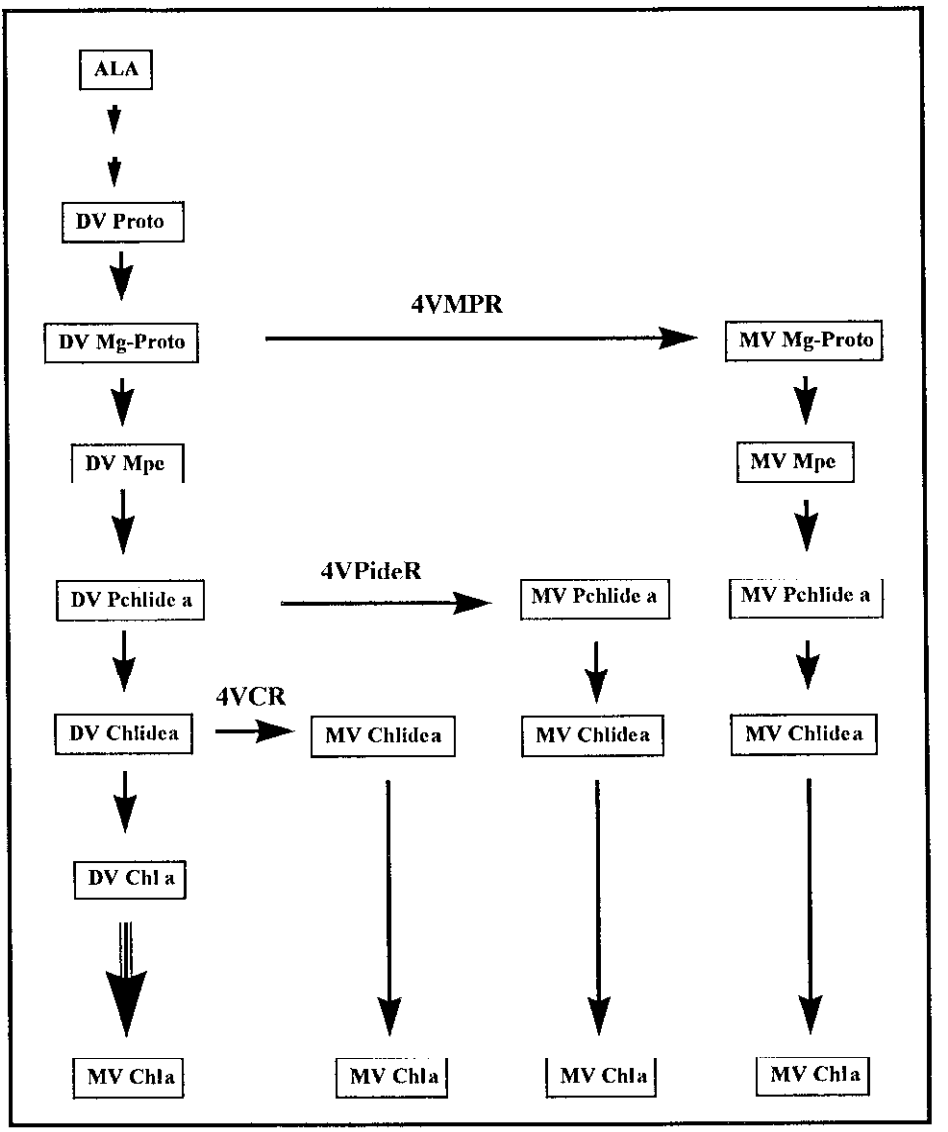
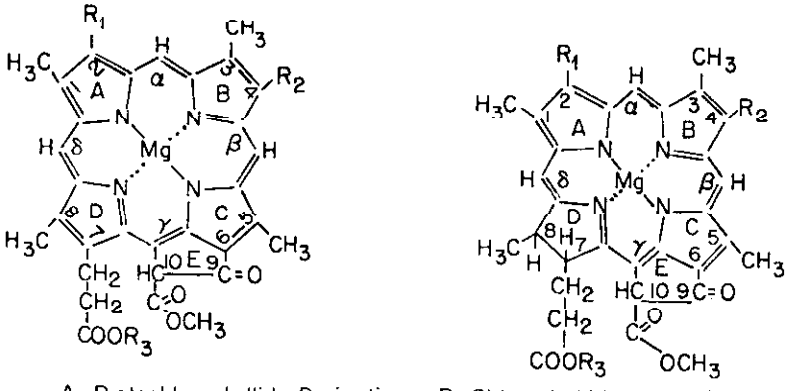


Fig. 1. The MV and DV monocarboxylic routes of chlorophyll *a* biosynthesis in green plants. DV = divinyl, MV = monovinyl, ALA = 5-aminolevulinic acid, Proto = protoporphyrin IX, Mpe = Mg-Proto monomethyl ester, Pchlide = protochlorophyllide, Chlide = chlorophyllide, Chl = chlorophyll, 4VPideR = [4-vinyl] protochlorophyllide *a* reductase, 4VCR = [4-vinyl] chlorophyllide *a* reductase. Arrows joining the DV and MV branches refer to reactions catalyzed by [4-vinyl] reductases. Triple-lined arrows point to putative reactions. Adapted from Rebeiz *et al.* (1983, 1994).

(Tripathy and Rebeiz 1988). (*b*) In *Rhodobacter capsulatus* in which the *bchJ* gene that codes for the DV Pchlide *a* reductase enzyme has been deleted, in addition to the accumulation of DV Pchlide *a*, the accumulation of MV Mg Proto and its monoester (precursors of Pchlide *a*) has been observed (Suzuki and Bauer 1995). This in turn

indicates that separate [4-vinyl] reductases exist which act prior to DV Pchlide *a* and DV Chlide *a* vinyl reduction, and which are responsible for the accumulation of MV Mg protoporphyrins in plants (Belanger and Rebeiz 1982). This in turn is compatible with the recent detection of [4-vinyl] Mg-Proto reductase in barley (Kim and Rebeiz 1996). This work shows that the DV and MV Chl *a* carboxylic biosynthetic routes are regulated by an interaction of plastid membranes, stromal factors, and reduced pyridine nucleotides.



A. Protochlorophyllide Derivatives B. Chlorophyllide Derivatives

	R_1	R_2	R_3	Compound
Aa.	$CH=CH_2$	CH_2-CH_3	H	MV Pchlide
Ab.	$CH=CH_2$	$CH=CH_2$	H	DV Pchlide
Ac.	$CH=CH_2$	CH_2-CH_3	longchain alcohol	MV Pchlide ester
Ad.	$CH=CH_2$	$CH=CH_2$	longchain alcohol	DV Pchlide ester
Ba.	$CH=CH_2$	CH_2-CH_3	$C_{20}H_{39}$	MV Chl a
Bb.	$CH=CH_2$	CH_2-CH_3	H	MV Chlide a
Bc.	$CH=CH_2$	$CH=CH_2$	H	DV Chlide a
Bd.	CH_2-CH_3	CH_2-CH_3	H	Meso Chlide a

Fig. 2. Chemical structures of protochlorophyllide *a* and chlorophyllide *a* derivatives.

Materials and methods

Plants: Barley (*Hordeum vulgare* L. cv. Robust) seeds were planted in moist vermiculite in plastic trays. The latter were wrapped in aluminum foil and incubated in darkness at 28 °C. Etiolated leaves were harvested after six days of germination.

Chemicals: DV Mg-Proto (disodium salt) was purchased from *Porphyrin Products* (Logan, USA). All other chemicals were purchased either from *Sigma* (St. Louis, USA) or from *Aldrich* (Milwaukee, USA).

Preparation of etiochloroplasts: All procedures including etiochloroplast preparation were carried out under subdued laboratory light (0.5 W m^{-2}). 28 g of etiolated barley

leaves were hand-ground in a cold ceramic mortar containing 90 cm^3 of homogenization buffer. The latter consisted of 500 mM sucrose, 15 mM Hepes, 30 mM Tes, 1 mM $MgCl_2$, 1 mM EDTA, 5 mM cysteine, and 0.2 % (m/v) bovine serum albumin (BSA), at a room temperature, pH 7.7. The homogenate was filtered through two layers of *Miracloth* (*Calbiochem*) and 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 10 min at 1 °C. The pelleted etiochloroplasts were gently resuspended in incubation buffer A or lysing buffer using a small paintbrush. For further plastid purification, the pelleted etiochloroplasts were resuspended in 5 cm^3 of homogenization buffer and were purifed by *Percoll* density centrifugation (Lee *et al.* 1991). The pelleted, *Percoll*-purifed etiochloroplasts were resuspended in incubation medium A. Incubation medium A consisted of 500 mM sucrose, 200 mM Tris, 20 mM $MgCl_2$, 2.5 mM Na_2EDTA, 20 mM ATP, 40 mM NAD^+, 1.25 mM methanol, 0.15 % (m/v) BSA, and 1.5 mM NADPH, adjusted to pH 7.7 at room temperature. The pH was adjusted with KOH and HCl. The NAD^+, ATP, and tetrapyrrole substrates readily penetrate etiochloroplast membranes (Rebeiz *et al.* 1982, Fuesler *et al.* 1984, Tripathy and Rebeiz 1986). Unless otherwise indicated, the lysing buffer consisted of 25 mM Tris, 2 mM $MgCl_2$, 2.5 mM EDTA, and 0.1 % (m/v) BSA, at a room temperature, pH 7.7.

Preparation of plastid membranes: Etiochloroplasts were suspended in 4 cm^3 of lysing buffer. Where indicated, NADPH, $MgCl_2$ or Mg-Proto were added to the lysing medium. The lysed plastid suspension was centrifuged at 235 000×*g* for 1 h in a *Beckman 80 Ti* fixed angle rotor at 1 °C. The supernatant (stromal fraction) which was free of tetrapyrroles (Smith and Rebeiz 1979) was collected, and the pelleted membranes were resuspended in incubation medium B. Incubation medium B consisted of 200 mM Tris, 3 mM $MgCl_2$, 2.5 mM EDTA, 20 mM ATP, 0.15 % (m/v) BSA, and 3 mM NADPH, at a room temperature, pH 7.7.

Cyclopentanone ring synthetase assay: When using whole etiochloroplasts, the cyclopentanone ring synthetase reaction was carried out in flat-bottomed tubes (2.2×6.5 cm) containing 0.33 cm^3 of etiochloroplasts (0.4-0.7 mg proteins) suspended in incubation medium A, an additional 0.33 cm^3 of incubation medium A, 0.32 cm^3 distilled water, and 30 mm^3 of 50 μM DV Mg-Proto solution in Tris buffer (pH 7.6). When using plastid membranes, the reaction was carried out in flat-bottomed tubes (2.2×6.5 cm) containing 0.33 cm^3 of plastid membranes (0.53-0.69 mg proteins) suspended in incubation medium B, 0.33 cm^3 of stromal fraction (0.15-0.19 mg proteins) or 0.33 cm^3 of lysing medium, 30 mm^3 of 50 μM DV Mg-Proto in Tris buffer (pH 7.6), and 0.32 cm^3 distilled water. The reaction mixtures were preincubated in the light (3.2 W m^{-2}) for 3 min in order to deplete the endogenous Pchlide *a* pool by conversion to Chlide *a*. After adding substrate, the reaction mixture was incubated in an oscillating water bath [0.83(oscillation) s^{-1}] in darkness, at 30 °C, for the indicated times. The reaction was terminated by addition of 10 cm^3 acetone:0.1 M NH_4OH (9:1, v/v).

Protochlorophyllide *a* extraction and determination: Pigments were extracted by adding 10 cm^3 acetone:0.1 M NH_4OH (9:1, v/v) to the reaction mixture. Following centrifugation at 39 000×*g* for 12 min at 1 °C, the acetone extract was decanted and stored at -80 °C until use. Chlorophylls and other fully esterifed tetrapyrroles were transferred from acetone to hexane by extraction with an equal volume of hexane, followed by a second extraction with one-third volume of hexane. The remaining hexane-extracted acetone residue which was devoid of fully esterifed pigments and contained all monocarboxylic tetrapyrroles such as Pchlides and Mpe, and all dicarboxylic tetrapyrroles such as Proto and Mg-Proto, was used for quantitative Pchlide *a* determination by spectrofluorometry at room temperature (Rebeiz *et al.* 1975). Pchlide *a* was determined with a precision of 7.5 %. Minimum detection levels amounted to about 0.2 pmol cm^{-3}. The net amount of Pchlide *a* formed after incubation was calculated by subtracting the amount of Pchlide *a* detected at the beginning of incubation (0 h control) from the amount of Pchlide *a* detected after incubation.

Quantitative determination of MV and DV Pchlide *a*: The amount of MV and DV Pchlide *a* was determined from the total amount of Pchlide *a* determined at room temperature (see above), and from the MV to DV Pchlide *a* ratio which was determined by spectrofluorometry in diethyl ether at 77 K. Transfer of monocarboxylic tetrapyrroles from the hexane-extracted acetone fraction to diethyl ether was achieved by solvent extraction. Monocarboxylic tetrapyrroles were extracted into diethyl ether by adding to the hexane-extracted acetone fraction 1/5 volume of diethyl ether, 1/17 volume of saturated NaCl, and 1/70 volume of 0.37 M KH_2PO_4 (pH 7.0). The mixture was thoroughly mixed, and the phases were separated by a brief centrifugation for 30 s at room temperature. The ether phase was collected with a Pasteur pipette, and the ether-extracted acetone residue was re-extracted 1 5 times with small volumes of diethyl ether until all the Mpe, Pchlide and Chlide had been transferred to diethyl ether. The remaining ether-extracted acetonic phase contained mostly dicarboxylic tetrapyrroles such as Proto and/or Mg Proto. The ether extract containing monocarboxylic tetrapyrroles was used for the determination of the MV to DV Pchlide *a* ratio from fluorescence excitation spectra recorded at 77 K in ether as described in Tripathy and Rebeiz (1985).

Spectrofluorometry: Fluorescence spectra were recorded on a fully corrected photon-counting, high-resolution *SLM* spectrofluorometer model *8000C*, interfaced with an *IBM* model *486* microcomputer. Room temperature spectra were recorded on an aliquot of the hexane-extracted acetone fraction in a cylindrical microcell 3 mm in diameter at emission and excitation bandwidths of 4 nm. Low temperature fluorescence spectra (77 K) were recorded at emission and excitation bandwidths that varied from 0.5 to 4 nm depending on signal intensity (Tripathy and Rebeiz 1985, Wu *et al.* 1989). The photon count was integrated for 0.5 s at each 1 nm increment.

Protein determination: Protein was determined by the BCA method on an aliquot of the plastid preparation after delipidation (Smith *et al.* 1985). Absorbance at 562 nm was determined on a *Sequoia-Turner* spectrophotometer model *340*.

Statistical analysis: Statistical analyses were performed using ANOVA analysis of variance (*SAS Institute*, Carey, USA). As indicated in every table, either a randomized complete block or a split plot design was used. The least significance difference (LSD) was used to partition all multiple pairwise differences between means at the indicated levels of significance.

Results

The DV Chl *a* carboxylic biosynthetic route predominates during conversion of DV Mg-Proto to Pchlide *a* by isolated barley etiochloroplasts in darkness: Barley is a dark MV/light DV plant species. In darkness, barley leaves biosynthesize and accumulate mainly MV Pchlide *a* *via* the MV Chl *a* carboxylic biosynthetic route, while in the light, they form and accumulate mainly DV Pchlide *a* (Carey and Rebeiz 1985).

Table 1. Preferential conversion of DV Mg-Proto to DV Pchlide *a* by barley etiochloroplasts in darkness. Values are means of two (Exp. A, C) or three (Exp. B) replicates. The values were treated as a randomized complete block. Within columns, values followed by different letters are significantly different at the indicated level of significance, while values followed by the same letter are not significantly different. Exp. = experiment. Exp. A: incubation in medium A; Exp. B: incubation in medium B; Exp. C: *Percoll*-purified etiochloroplasts incubated in medium A. Since prior to incubation the Pchlide *a* content in Exp. C was close to zero, the statistic of this experiment was not done. For medium composition see Materials and Methods. Significance level is given in [%].

Exp.	Incubation [min]	Total Pchlide *a* [μmol kg^{-1}(protein)]	DV Pchlide *a*	MV Pchlide *a*	MV/DV Pchlide *a*
A	0	49.6a	15.8a	33.8a	2.1
	30	205.4b	154.1b	51.3b	0.3
	60	255.8c	178.3c	77.5c	0.4
	120	230.0c	151.4b	78.6c	0.5
	Significance level	1.2	1.7	0.5	
	LSD	20.0	17.9	3.4	
B	0	44.9a	12.5a	32.5a	2.6
	60	172.8b	106.7b	66.2b	0.6
	Significance level	4.6	2.4	13.3	
	LSD	23.1	12.1	11.2	
C	0	0.3	0.2	0.1	0.5
	60	44.2	40.5	3.7	0.6

The fully esterifed Pchlide pool, *i.e.*, the Pchlide ester pool, is not affected by the light and dark treatments and consists mainly of MV Pchlide ester. Contrary to expectation, incubation of isolated barley etiochloroplasts with DV Mg-Proto in incubation medium A in darkness, resulted in its conversion mainly to DV Pchlide *a* (Table 1, Exp. A). Similar results were observed in incubation medium B (Table 1, Exp. B), or by incubating Percoll-purifed plastids in medium A (Table 1, Exp. C).

The spectrofluorometric profile of the Pchlide *a* pool formed during conversion of DV Mg-Proto to DV Pchlide *a* and lesser amounts of MV Pchlide *a* is depicted in Fig. 3, curve *a*. These results were in sharp contrast to previous results, where photoperiodically grown barley seedlings converted added ALA mainly to MV Pchlide *a* in darkness (Carey and Rebeiz 1985) and where isolated etiochloroplasts prepared from kinetin + giberellic acid-treated etiolated barley leaves converted DV Mg-Proto mainly to MV Pchlide *a* (Tripathy and Rebeiz 1986). The endogenous Pchlide *a* content of kinetin + giberellic acid-treated etiochloroplasts prepared from etiolated barley leaves is similar to that reported in Table 1 (Daniell and Rebeiz 1984, Rebeiz *et al.* 1984). It amounts to about 10-15 % of the total Pchlide *a* pool of etioplasts that have not been exposed to any light. In an effort to understand the reason for this discrepancy, various factors that may affect the onset of the DV and MV carboxylic biosynthetic routes during Pchlide *a* biosynthesis were investigated (see below).

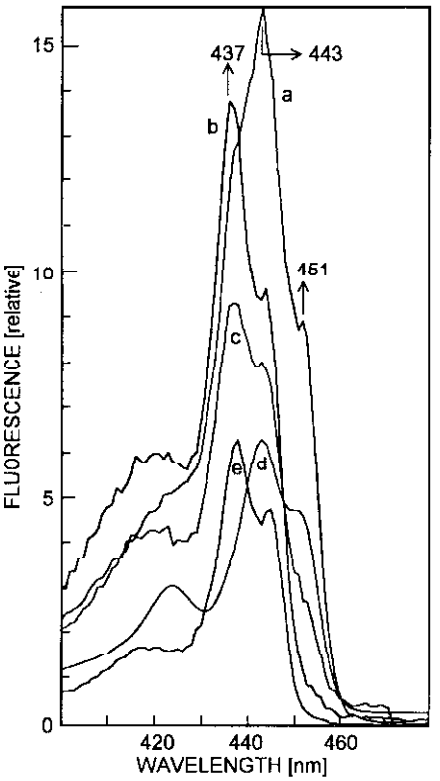


Fig. 3. Fluorescence excitation profiles in ether at 77 K of the protochlorophyllide (Pchlide) *a* pool formed during conversion of DV Mg Proto mainly to DV and MV Pchlide *a* by plastidic and subplastidic preparations. The spectra were recorded at an emission wavelength of 625 nm (the emission maximum of MV and DV Pchlide *a*). *a* = barley etiochloroplasts incubated with DV Mg-Proto in medium A; *b* = barley etiochloroplast membranes incubated with DV Mg-Proto in medium B; *c* = barley etiochloroplast membranes prepared in lysing buffer containing NAPH and incubated in the presence of DV Mg-Proto, stroma, and NADPH in medium B; *d* = authentic DV Pchlide *a*; *e* = authentic MV Pchlide *a*. Arrows point to emission maxima: at 443 nm [B_y (0'-0) transition of pure DV Pchlide *a*, and B_x(0'-0) transition of pure MV Pchlide *a*]; at 437 nm [B_y(0'-0) transition of pure MV Pchlide *a*], and at 451 nm [B_x(0'-0) transition of pure DV Pchlide *a*] (Tripathy and Rebeiz 1985).

The MV Chl *a* carboxylic biosynthetic route predominates during conversion of DV Mg-Proto to Pchlide *a* by barley etiochloroplast membranes in darkness: Incubation of etiochloroplast membranes with DV Mg-Proto for 1 h in darkness, in incubation medium B in the absence of added stroma resulted in a 3-fold decrease in the rate of conversion of the DV Mg-Proto substrate to Pchlide *a* (Tables 1 and 2, A1). Addition

of DV Mg-Proto to the lysing buffer, a strategy that worked well in preserving the activity of Mg-Proto chelatase, the enzyme that inserts Mg into Proto (Lee *et al.* 1992) during plastid lysis, had no effect on the reaction rate (Table 2, A1, B1). Neither did the addition of $MgCl_2$ which preliminary experiments suggested it might have an effect on the reaction (Table 2, A1, C1). However, contrary to expectations,

Table 2. Conversion of DV Mg-Proto to DV or MV Pchlide *a* in darkness depends on the interaction of plastid stroma, plastid membranes, and NADPH. Values are means of two replicates. The values were treated as a split-plot. Within columns, values followed by different letters are significantly different at the indicated level of significance, while values followed by the same letter are not significantly different. M = plastid membranes, S = plastid stroma. Incubation was in medium B (see Materials and methods).

Exp.	Additives to lysing buffer	Enzyme source	Change in Pchlide *a* after 1 h incubation			
			Total Pchlide *a*	DV Pchlide *a*	MV Pchlide *a*	MV/DV Pchlide *a*
			[μmol kg^{-1}(protein) s^{-1}]			
A1	None	M	21.0a	0.5ab	20.5ab	40.9
A2	None	M+S	21.3a	2.1abc	19.2a	9.1
B1	0.5 mM Mg-Proto	M	21.6a	0.3a	21.3ab	76.8
B2	0.5 mM Mg-Proto	M+S	23.1a	3.6c	19.4a	5.4
C1	10 mM $MgCl_2$	M	25.1a	2.4bc	22.7b	9.5
C2	10 mM $MgCl_2$	M+S	24.7a	3.8c	20.9ab	5.4
D1	1 mM NADPH	M	21.9a	-0.03a	21.9a	infinite
D2	1 mM NADPH	M+S	41.0de	11.7d	29.3e	2.5
E1	1 mM NADPH + 0.5 mM Mg-Proto	M	23.4a	2.5b	20.9ab	8.5
E2	1 mM NADPH + 0.5 mM Mg-Proto	M+S	38.0c	14.9e	23.1bc	1.6
F1	1 mM NADPH + 10 mM Mg-Proto	M	31.2b	1.2ab	30.0e	24.5
F2	1 mM NADPH + 10 mM Mg-Proto	M+S	43.6d	17.7f	25.9cd	1.5
Significance level [%]			0.05	0.03	0.08	
LSD			5.1	2.3	3.1	

90 to 99 % of the synthesized Pchlide *a* consisted of MV instead of DV Pchlide *a* (Table 2, A1, B1, C1). The spectrofluorometric profile of the Pchlide *a* pool formed during conversion of DV Mg-Proto mainly to MV Pchlide *a* is depicted in Fig. 3, curve *b*. These results indicated that isolated plastid membranes were more similar to green barley leaves in their MV Pchlide *a* biosynthetic capabilities than isolated plastids. It also suggested that the plastid stroma may somehow be involved in the regulation of the DV and MV Pchlide *a* ratio during Pchlide *a* formation from DV Mg-Proto. Reconstitution of plastids by addition of stroma to plastid membranes resulted in a significant albeit small increase in the amount of synthesized DV Pchlide *a*, when Mg-Proto and $MgCl_2$ were added to the lysing buffer (Table 2, A1, B2, C2).

Lysis of barley plastids in the presence of added NADPH, followed by reconstitution of plastids by addition of stroma to plastid membranes, enhances the activity of the DV Chl *a* carboxylic biosynthetic route: Addition of 1 mM NADPH to the lysing buffer during plastid membrane preparation had no apparent effects on the DV/MV Pchlide *a* ratio, or the conversion rate of DV Mg-Proto to Pchlide by plastid membranes (Table 2, A1, D1). Most of the synthesized Pchlide *a* consisted, however, of MV Pchlide *a*. Addition of DV Mg-Proto to the lysing buffer in addition to added NADPH was also without a significant effect on the reaction (Table 2, A1, D1, E1), while addition of $MgCl_2$ resulted in increased formation of total Pchlide *a* and MV Pchlide *a* (Table 2, A1, D1, F1). However, when plastid lysis was carried out in the presence of added NADPH, and when stroma, plastid membranes, and NADPH were present in the reaction mixture, a significant increase in the rate of DV Mg-Proto conversion to Pchlide *a* was observed (Table 2, D1, D2). Similar results were noted with addition of NADH (values not shown). In addition, it was observed that the DV Chl *a* carboxylic biosynthetic route was strongly activated, as evidenced by a considerable increase in the amount of synthesized DV Pchlide *a* (Table 2, D1, D2). Addition of Mg-Proto or $MgCl_2$ in addition to added NADPH during plastid lysis resulted in essentially similar results (Table 2, E1, E2, and F1, F2). Addition to the reconstituted system of S-adenosylmethionine (SAM) which is involved in the conversion of DV Mg-Proto to DV Mg-Proto monomethyl ester during Pchlide *a* formation, was without effect (Table 3). This in turn indicates that the reconstituted system contains an endogenous methyl source which is large enough to support the conversion of Mg-Proto to Mg-Proto monomethyl ester at the rates of Pchlide *a* formation reported in Table 2.

Table 3. S-adenosyl methionine (SAM) has no effect on DV Pchlide *a* formation during incubation of plastid membranes + stroma and NADPH with DV Mg-Proto. Plastid lysis was performed in the presence of 1 mM NADPH. Values are means of two replicates. The values were treated as a randomized complete block. Within columns, values followed by the same letter are not significantly different at the 5 % level, while values followed by different letters are significantly different. Incubation was in medium B.

Cofactor	Total Pchlide *a* [μmol kg^{-1}(protein) s^{-1}]	DV Pchlide *a*	MV Pchlide *a*
No SAM	40.6a	12.3a	28.4a
1 mM SAM	44.6a	12.6a	31.9a

Discussion

Walker *et al.* (1991) reported that both stroma and plastid membranes of cucumber etiochloroplasts were essential for the conversion of DV Mg-Proto or Mpe to Pchlide *a*. In this work we showed that barley etiochloroplast membranes were perfectly capable of catalyzing the conversion of exogenous DV Mg Proto to Pchlide *a*, in the absence of added stroma (Table 2). These results emphasize the ease with which

some of the components of the enzyme system responsible for the conversion of Mg-Proto and its monomethyl ester to Pchlide *a*, can be solubilized during plastid membrane preparation.

It is also shown that plastid isolation results in the disruption of the regulatory mechanism(s) that control the proportion of DV and MV Pchlide *a* formation. For example, although barley seedlings form mainly MV Pchlide *a* during dark incubation (Carey and Rebeiz 1985), isolated barley plastids formed mainly DV Pchlide *a* (Table 1). Such a situation could arise if NADPH became unavailable to the isolated plastids. It has been our experience, however, that isolated plastids contain enough endogenous NADPH to meet the needs of [4-vinyl] reductase(s). For example, the rate of endogenous NADPH-dependent DV Chlide *a* reduction in isolated etiochloroplasts amounts to about 57 nmol kg^{-1}(plastid protein) s^{-1} (Parham and Rebeiz 1992), which is several fold higher than the rates of MV Pchlide *a* formation reported in Table 2. We also consider the possibility that isolated etiochloroplasts may be contaminated with mitochondria whose diaphorase may oxidize extraplastidic NADPH and renders the latter unavailable to the isolated plastids. This hypothesis did not survive experimentation since mitochondria-free plastids prepared by *Percoll* purification formed more DV Pchlide *a* than crude plastids, and exhibited a higher DV/MV ratio (Table 1, Exp. C).

Further investigations of the *in vivo-in organello* discrepancy revealed that an interaction of plastid membranes, stroma, and reduced pyridine nucleotides was involved in the regulation of DV and MV Pchlide *a* biosynthesis (Table 2). This regulation appeared to be very sensitive to organelle disruption, as different DV and MV Pchlide *a* biosynthetic profiles were observed depending on the presence or absence of NADPH during plastid lysis (Table 2). However, contrary to expectation, addition of reduced nucleotides and stroma to plastid membranes activated the DV instead of the MV Chl *a* carboxylic biosynthetic route (Table 2). Kay and Griffiths (1983) reported that the presence of NADPH during etiochloroplast lysis protected NADPH:protochlorophyllide oxidoreductase (PCR) from degradation by endogenous proteases. It was conjectured that since PCR forms a complex with DV Pchlide *a* and might protect it from the action of [4-vinyl] Pchlide *a* reductase, degradation of PCR caused by the absence of added NADPH during lysis may result in the reduction of most of the unprotected DV Pchlide *a* to MV Pchlide *a*. This phenomenon would not occur in whole etiochloroplasts, which are not subjected to lysis-induced PCR degradation, and may in turn account for the high DV Pchlide *a* formation in isolated etiochloroplasts (Table 1). This hypothesis was not compatible, however, with the observations (*a*) of MV Pchlide *a* accumulation in darkness in barley leaves (Carey and Rebeiz 1985), and (*b*) that PCR was as active in etiochloroplasts which were lysed in the absence of added NADPH as in whole plastids (Table I in Parham and Rebeiz 1992). In that work, the DV Chlide *a* substrate, which was reduced to MV Chlide *a*, was generated by photoconversion of endogenous DV Pchlide *a* by PCR.

Altogether, these results indicate that, in cooperation with the plastid stroma, NADPH plays an important regulatory role during the biosynthesis of DV and MV Pchlide *a*.

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