Chloroplast Biogenesis: [4-Vinyl] Chlorophyllide *a* Reductase Is a Divinyl Chlorophyllide *a*-Specific, NADPH-Dependent Enzyme[†]

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ABSTRACT: Some properties of [4-vinyl] chlorophyllide *a* reductase are described. This enzyme converts divinyl chlorophyllide *a* to monovinyl chlorophyllide *a*. The latter is the immediate precursor of monovinyl chlorophyll *a*, the main chlorophyll in green plants. [4-Vinyl] chlorophyllide *a* reductase plays an important role in daylight during the conversion of divinyl protochlorophyllide *a* to monovinyl chlorophyll *a*. [4-Vinyl] chlorophyllide *a* reductase was detected in isolated plastid membranes. Its activity is strictly dependent on the availability of NADPH. Other reductants such as NADH and GSH were ineffective. The enzyme appears to be specific for divinyl chlorophyllide *a*, and it does not reduce divinyl protochlorophyllide *a* to monovinyl protochlorophyllide *a*. The conversion of divinyl protochlorophyllide *a* to monovinyl protochlorophyllide *a* has been demonstrated in barley and cucumber etiochloroplasts and appears to be catalyzed by a [4-vinyl] protochlorophyllide *a* reductase [Tripathy, B. C., & Rebeiz, C. A. (1988) *Plant Physiol.* 87, 89–94]. On the basis of reductant requirements and substrate specificity, it is possible that two different 4-vinyl reductases may be involved in the reduction of divinyl protochlorophyllide *a* and divinyl chlorophyllide *a* to their respective 4-ethyl analogues.

The demonstration of metabolic pathways is a multistep process. It involves at least three stages: (a) the detection and characterization of metabolic intermediates, (b) the demonstration of precursor–product relationships between putative intermediates, and (c) purification and characterization of the enzymes involved in metabolic interconversions.

The notion of multiple biosynthetic routes for chlorophyll (Chl)[1] *a* formation in green plants was proposed in the early 1980s (Rebeiz et al., 1981, 1983; Rebeiz & Lascelles, 1982). It was initially based on the detection of various putative metabolic intermediates and end products which have been characterized by chemical derivatization and spectroscopic techniques (Belanger & Rebeiz, 1980a–c, 1982; Belanger et al., 1982a; Wu & Rebeiz, 1984, 1985, 1988; Shedbalkar et al., 1991). This was followed by the demonstration of precursor–product relationships among various intermediates, in vivo and in vitro (Belanger & Rebeiz, 1980c; Duggan & Rebeiz, 1982b; Tripathy & Rebeiz, 1986, 1988).

Most of the Chl *a* in green plants appears to be formed via two biosynthetic routes, namely, divinyl (DV) and monovinyl (MV) monocarboxylic routes (Rebeiz et al., 1983; Leeper, 1991). The DV monocarboxylic route is populated by monocarboxylic tetrapyrroles having vinyl groups at positions 2 and 4 of the macrocycle (Tripathy & Rebeiz, 1986, 1988). The MV monocarboxylic route is populated by monocarboxylic tetrapyrroles having vinyl and ethyl groups at positions 2 and 4 of the macrocycle, respectively (Tripathy & Rebeiz, 1986, 1988). The two monocarboxylic routes appear to be linked by 4-vinyl reductase(s) at the levels of DV-Chlide *a* (Duggan & Rebeiz, 1982b), DV-Pchlide *a* (Tripathy & Rebeiz, 1988), DV-Mg-Proto monomethyl ester (Ellsworth & Hsing, 1974), and somewhere between DV-Mg-Proto monomethyl ester and DV-Pchlide *a* (Tripathy & Rebeiz, 1986). What is not clear at this stage is (a) whether one 4-vinyl reductase or several 4-vinyl reductases are involved in the conversion of DV tetrapyrroles to MV tetrapyrroles and (b) whether one set of enzymes of broad specificity or two sets of enzymes of narrow specificity catalyze the reactions between DV-Proto and DV-Pchlide *a* and between MV-Proto and MV-Pchlide *a* (Tripathy & Rebeiz, 1986).

In this work, we report about the properties of [4-vinyl]-Chlide *a* reductase (4VCR). This enzyme has been detected in cucumber etiochloroplasts, a DDV/LDV plant species, but its cofactor requirement and its substrate specificity have not yet been etermined. [4-Vinyl] chlorophyllide *a* reductase was detected in the plastid membranes. It does not appear to be capable of reducing DV-Pchlide *a* to monovinyl-Pchlide *a*. This in turn suggests that two different 4-vinyl reductases may be involved in the reduction of DV-Pchlide *a* and DV-Chlide *a* to their respective 4-ethyl analogues.

MATERIALS AND METHODS

Plant Material. Cucumber (*Cucumis sativus* L. Beit Alpha MR) seeds (J. Mollema and Sons, Inc., Grand Rapids, MI) were planted in moist vermiculite. Germination was carried out in a growth room illuminated by six 1000-W metal halide lamps (21.1 mW cm²) under a 14-h light/10-h dark photoperiod. The temperature ranged from 27 °C in the light to 21 °C in darkness. Seedlings were watered with Hoagland's nutrient solution.

Induction of DV-Pchlide a Accumulation. Induction of DV-Pchlide *a* accumulation in etiolated cucumber cotyledons was adapted from Duggan and Rebeiz (1982a). Four-day-

1 Abbreviations: 4VCR, 4-[vinyl] chlorophyllide *a* reductase; ALA, δ-aminolevulinic acid; Chl, chlorophyll; Chlide, chlorophyllide; D, dark; DV, divinyl (vinyl groups at positions 2 and 4 of the macrocycle); G6PDH, gluconate-6-phosphate dehydrogenase; L, light; MV, monovinyl (vinyl group at position 2 and ethyl group at position 4 of the macrocycle); Pchlide, protochlorophyllide; Proto, protoporphyrin IX.

old etiolated cucumber cotyledons were harvested in darkness under a safe green light, and the hypocotyl hooks were removed. The cotyledons were placed in a large Petri dish, 15 cm in diameter, containing 9 mL of distilled water. The Petri dish was placed 11 cm below a photographic Sunpack flash, Model Auto 611 (Berkey Marketing Co., Woodside, NY), that generated a 2.5-ms actinic white flash. A reflecting mirror was placed 4 cm below the Petri dish and aluminum foil was placed all around the flash and the Petri dish, in order to increase the amount of incident light reflected back onto the tissue. Each 2.5-ms light flash was followed by 45 min of darkness. After three light–dark treatments, the tissue regenerated only DV-Pchlide *a* (Duggan & Rebeiz, 1982a).

Isolation of DV-Pchlide a-Enriched Plastids. Plastid isolation was performed under a safe green light that transmitted light between 510 and 520 nm and which did not photoconvert DV-Pchlide *a* to DV-Chlide *a*. Five-gram batches of DV-Pchlide *a*-enriched cotyledons were hand homogenized in a cold mortar. The tissue was ground in 12.5 mL of homogenization buffer. The latter consisted of 500 mM sucrose, 15 mM Hepes, 1 mM $MgCl_2$, 1 mM EDTA, 9 mM Tes, 5 mM cysteine, and 0.2% BSA (w/v), adjusted with KOH to pH 8.0 at room temperature. The resulting homogenate was filtered through two layers of Miracloth (Calbiochem, La Jolla, CA) and centrifuged at 200*g* for 5 min at 1 °C in a Beckman JA-20 angle rotor. The supernatant was decanted and centrifuged at 1500*g* for 10 min at 1 °C. The pelleted etiochloroplasts (about 5 mg of protein) were gently resuspended with a paint brush, in 5.0 mL of incubation buffer. Unless otherwise indicated, the latter consisted of 500 mM sucrose, 1.0 mM $MgCl_2$, 2.5 mM EDTA, 20.0 mM ATP, 1.0 mM NAD^+, 1.25 mM methanol, 200.0 mM Tris, and 0.2% BSA (w/v) adjusted to pH 7.7 at room temperature.

Measurement of Plastid Intactness. Measurement of etiochloroplast intactness was adapted from Lee et al. (1991) and consisted in the evaluation of gluconate-6-phosphate dehydrogenase (G6PDH) activity, a stromal marker. The reaction was carried out in a total volume of 1.0 mL containing 300 mM sucrose, 20 mM Tes–NaOH (pH 7.5), 10 mM $MgCl_2$, 0.17 mM $NADP^+$, and 0.2 mM gluconate 6-phosphate. The reaction was initiated by addition of etiochloroplasts (0.1 mL) resuspended in incubation medium, and the change in absorbance was recorded at 340 nm. Etiochloroplasts were lysed by addition of 0.1% Triton X-100 or by osmotic shock (see below). Plastid breakage was evaluated from the relative rates of $NADP^+$ reduction by etiochloroplasts lysed by 0.1% Triton X-100 and by osmotic shock (Lee et al., 1991).

Preparation of Plastid Stroma and Membranes. Preparation of plastid stroma and membranes was also performed under the safe green light which did not photoconvert DV-Pchlide *a* to DV-Chlide *a*. Separation of plastid stroma from membranes was adapted from Lee et al. (1991). Etiochloroplasts (about 5 mg of protein) were suspended in 5 mL of lysing buffer composed of 1.0 mM $MgCl_2$, 2.5 mM EDTA, 20.0 mM ATP, 1.0 mM NAD^+, 1.25 mM methanol, 0.2% BSA (w/v), and, unless otherwise indicated, 25 mM Tris-HCl. The pH was adjusted to 7.7 at room temperature with KOH and HCl. The lysed plastid suspension was centrifuged at 235000*g* for 1 h in a Beckman 80 Ti fixed angle rotor at 1 °C. This centrifugation separated the suspension into a colorless soluble-protein supernatant (stroma) and a yellowish pellet (membranes) (Lee et al., 1991). The stromal fraction was decanted, and the pelleted membranes were either resuspended in the lysing medium at a rate of 5 mL/5 g of homogenized tissue or were washed once before use. To this

effect, plastid membranes from 5 g of tissue (about 3 mg) were resuspended in 8 mL of 146 mM Tris-HCl adjusted to a room temperature pH of 7.7, and containing all of the additives present in the lysing buffer. The suspension was centrifuged at 235000*g* for 0.5 h at 1 °C. The supernatant was decanted, and the pelleted membranes were resuspended in 3 mL of lysing buffer containing or lacking cofactors.

Reduction of DV-Chlide a to MV-Chlide a. Plastids and subplastidic membranes enriched in DV-Pchlide *a* were prepared from cucumber cotyledons, as described above. DV-Chlide *a* reduction was initiated by conversion of most of the DV-Pchlide *a* to DV-Chlide *a* by a single, 2.5-ms flash of actinic white light (Duggan & Rebeiz, 1982a). The reduction of DV-Chlide *a* to MV-Chlide *a* was allowed to proceed in darkness for 20 min.

Pigment Extraction. Incubations were stopped by addition of 10 mL of cold acetone/0.1 N NH_4OH (9:1 v/v)/mL of incubate. The resulting mixture was centrifuged at 39000*g* for 12 min at 1 °C. Chlorophylls and other fully esterified 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 contained monocarboxylic and dicarboxylic tetrapyrroles, was used for quantitative pigment determination by spectrofluorometry at room temperature (Rebeiz et al., 1975). Spectrofluorometric determinations at 77 K were performed after transfer of the pigments to ether (Tripathy & Rebeiz, 1985; Wu et al., 1989).

Determination of MV- and DV-Chlorophyllide a. MV- and DV-Chlide *a* were determined spectrofluorometrically from the total amount of Chlide *a* which was determined at room temperature (Rebeiz et al., 1975) and from the proportion of MV- and DV-Chlide *a*, which was determined at 77 K (Wu et al., 1989).

Spectrofluorometry. Fluorescence spectra were recorded on a fully corrected photon-counting, high-resolution SLM spectrofluorometer Model 8000C, interfaced with an IBM Model XT microcomputer. Room temperature determinations were performed 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. Fluorescence spectra at 77 K were recorded at emission and excitation bandwidths that varied from 0.5 to 4 nm depending on signal intensity (Tripathy & Rebeiz, 1985; Wu et al., 1989). The photon count was integrated for 0.5 s at each 1-nm increment. Digital spectral data were automatically converted by the computer into quantitative values. Minimum detection levels were about 0.2 pmol/mL of hexane-extracted acetone extract.

Protein determination. Protein was determined according to Smith et al. (1985).

RESULTS

Plastid Integrity Is Not a Requirement for [4-Vinyl] Chlorophyllide a Reductase Activity. First, it was determined whether whole plastids were needed for 4VCR activity. Isolated plastids were osmotically lysed, and the 4VCR activity of the lysed plastids was compared to that of unlysed plastids. The extent of plastid lysis, as evidenced by G6PDH activity, a stromal marker, was about 92%. As shown in Table I, conversion of DV-Chlide *a* to MV-Chlide *a* proceeded at a higher rate in lysed than in unlysed preparations. This higher conversion rate may be due, however, to higher substrate concentration in the lysed preparation. Altogether, these results indicated that plastid integrity was not a mandatory

Table I: Comparison of 4VCR Activity of Etiochloroplasts before and after Lysis

etiochloroplasts	net change[a] in DV-Chlide a in 20 min (nmol/100 mg of protein)	net change[a] in MV-Chlide a in 20 min (nmol/100 mg of protein)
before lysis	−6.7 (−53.8%)[b]	6.8[c]
after lysis[d]	−18.0 (−65.1%)	19.1

[a] Mean of two replicates. [b] Values in parentheses represent the net change in DV-Chlide a, as a percent of the total DV-Chlide a present before incubation. [c] In all cases either no MV-Chlide a or trace amounts of MV-Chlide a were detected at the beginning of incubation. [d] Lysing buffer contained 25 mM Tris-HCl.

Table II: NADPH Requirement of Membrane-Bound 4VCR

etiochloroplast membranes[a]	net change[b] in DV-Chlide a in 20 min (nmol/100 mg of protein)	net change[b] in MV-Chlide a in 20 min (nmol/100 mg of protein)
without added reductants	−2.1 (−19.8%)[c]	2.9[d]
with added NADPH	−19.8 (−94.3%)	20.5
with added NADH	−2.9 (−24.6%)	3.8
with added GSH	−3.1 (−25.8%)	3.0

[a] Etiochloroplasts were lysed in lysing buffer containing 25 mM Tris-HCl. [b] Mean of two replicates. [c] Values in parentheses represent the net change in DV-Chlide a, as a percent of the total DV-Chlide a present before incubation. [d] In all cases, either no MV-Chlide a or small amounts of MV-Chlide a were detected at the beginning of dark incubation.

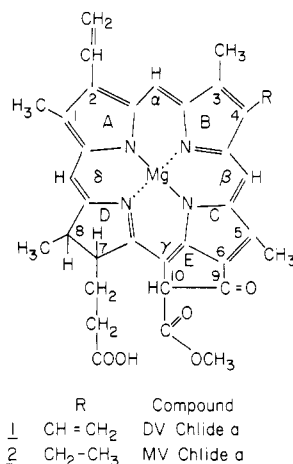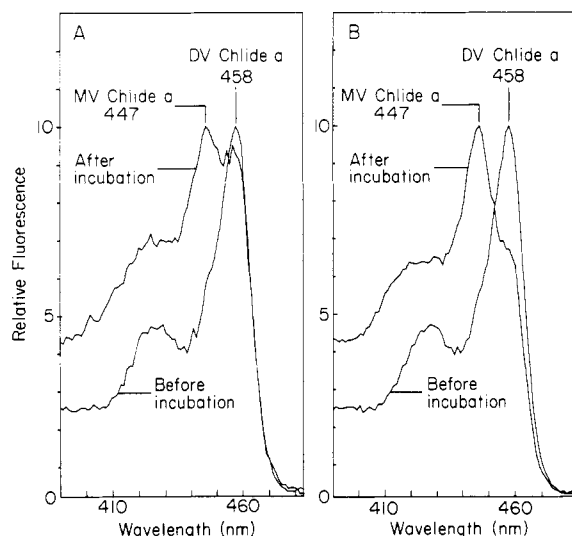| R	Compound
1 CH=CH₂	DV Chlide a
2 CH₂-CH₃	MV Chlide a

FIGURE 1: Chemical structures of DV-Chlide a and MV-Chlide a.

FIGURE 2: Fluorescence excitation spectra in ether at 77 K of (A) unlysed and (B) lysed plastids, before and after incubation for 20 min in darkness. The excitation spectra were recorded at the emission maximum of Chlide a at 675 nm, at a slit width of 4 nm. Arrows at 458 and 447 nm point to the Soret excitation maxima of DV- and MV-Chlide a, respectively. The spectra were normalized to the same amplitude at their Soret excitation maxima; 48% and 61% of the DV-Chlide a substrate were converted to MV-Chlide a in panels A and B, respectively.

requirement for 4VCR activity. Figure 2 describes the spectral changes that accompanied the reduction of DV-Chlide a to MV-Chlide a in lysed and unlysed plastids.

[4-Vinyl] Chlorophyllide a Reductase Is Detectable in Plastid Membranes and Requires NADPH for Activity. Ultracentrifugation of lysed plastids yielded two fractions: a clear stromal fraction that contained the bulk of the G6PDH activity (88%) and a yellowish membrane pellet that contained

nearly all the tetrapyrroles (98.4%). The membrane fraction exhibited erratic 4VCR activity that ranged from a few nanomoles of MV-Chlide a formed per 100 mg of plastid protein during 20 min of dark incubation to none. Addition of the stromal fraction to the membrane fraction did not improve activity (data not shown). Addition of exogenous NADPH to isolated plastid membranes improved dramatically the enzymatic conversion of DV-Chlide a to MV-Chlide a (Table II). Other reductants such as NADH⁺ and GSH had a minimal effect on the reduction (Table II). Altogether, these results indicated that NADPH was most likely the specific reductant involved in the enzymatic conversion of DV-Chlide a to MV-Chlide a.

Membrane-Bound 4VCR Is Not Active in the Reduction of Endogenous Divinyl Protochlorophyllide a to Monovinyl Protochlorophyllide a. To determine the possible involvement of 4VCR in the reduction of DV-Pchlide a as well as that of DV-Chlide a, the DV- and MV-Pchlide a and Chlide a pools were monitored simultaneously in the absence and presence of added NADPH, before and after dark incubation. After three 2.5-ms light/60 min dark cycles followed by a fourth 2.5-ms flash, the tetrapyrrole pool of the isolated plastid membranes consisted of DV-Pchlide a and DV-Chlide a (Table III). Although the latter was converted to MV-Chlide a during 20 min of dark incubation in the presence of NADPH, DV-Pchlide a was not affected by the treatment (Table III). Altogether, these results indicated that NADPH-dependent 4VCR was specific for DV-Chlide a and was not active toward endogenous DV-Pchlide a.

No Other Cofactor Besides NADPH Appears To Be Involved in the Conversion of Divinyl Chlorophyllide a to Monovinyl Chlorophyllide a. As pointed out in Materials and Methods, the incubation medium used in this work, in addition to Tris, sucrose, and BSA, contained several additives, namely ATP, Mg, EDTA, NAD⁺, and methanol. This incubation medium which supports high rates of Pchlide a and Chl a biosynthesis as well as thylakoid membrane assembly in isolated plastids (Rebeiz et al., 1984) has been routinely used for pigment biosynthesis studies in vitro. In order to determine whether any of the above-mentioned additives played a role in the conversion of DV-Chlide a to MV-Chlide a, their effect on 4VCR activity was investigated. Plastid membranes were isolated as described in Materials and Methods with the following modifications: (a) The lysing buffer contained 146 mM Tris-HCl instead of 25 mM, (b) the cofactor under investigation was omitted from the lysing buffer while all other additives were included, (c) isolated plastid membranes from 5 g of tissue (about 5 mg of protein) were washed once by resuspension in 8 mL of lysing buffer lacking the cofactor under investigation and were pelleted by centrifugation at 235000g for 0.5 h at 1 °C, (d) NADPH (0.56

Table III: Substrate Specificity of Membrane-Bound 4VCR

etiochloroplast membranes[a]	Pchlide *a* present after				Chlide *a* present after			
	3 LD + L[b]		3 LD + L + 20 min of dark		3 LD + L[b]		3 LD + L + 20 min of dark	
	DV (nmol/100 mg of protein)	MV (nmol/100 mg of protein)	DV (nmol/100 mg of protein)	MV (nmol/100 mg of protein)	DV (nmol/100 mg of protein)	MV (nmol/100 mg of protein)	DV (nmol/100 mg of protein)	MV (nmol/100 mg of protein)
without added NADPH[c]	19.5[d]	0.0	21.8	0.0 (0.0%)[e]	44.8	1.1	45.8 (2.2%)	4.1
with added NADPH[c]	24.3	0.0	23.8	0.0 (0.0%)	69.5	0.0	4.2 (−93.5%)	70.8

[a] Etiochloroplasts were lysed in lysing buffer containing 25 mM Tris-HCl. [b] Three, 2.5-ms light/45-min dark treatments followed by a fourth 2.5-ms flash. [c] Values represent the mean of two replicates. [d] DV-Pchlide *a* that was not converted to DV-Chlide a by the fourth 2.5-ms flash. [e] Values in parentheses represent the net change in MV-Pchlide *a* or DV-Chlide *a*, as a percent of the total MV-Pchlide *a* or DV-Chlide *a* present before incubation.

Table IV: Lack of Significant Effect of Various Additives on Membrane-Bound 4VCR

etiochloroplast membranes[a]	net change[b] in DV-Chlide *a* in 20 min (nmol/100 mg of protein)	net change[b] in MV-Chlide *a* in 20 min (nmol/100 mg of protein)
without added NADPH	−3.7 (−13.6%)[c]	4.9[d]
with added NADPH	−42.6 (−93.4%)	41.3
without added NAD	−87.3 (−95.0%)	80.0
with added NAD	−86.9 (−94.2%)	86.9
without added ATP	−30.2 (−89.6%)	34.4
with added ATP	−30.0 (−91.0%)	34.3
without added Mg	−114.5 (−95.0%)	115.5
with added Mg	−119.3 (−95.8%)	115.6
without added EDTA	−40.2 (−93.9%)	42.5
with added EDTA	−44.3 (−93.9%)	43.4
without added methanol	−64.9 (−96.6%)	61.8
with added methanol	−59.1 (−94.5%)	68.6
without pooled additives[e]	−32.4 (−86.4%)	34.0
with pooled additives	−35.8 (−93.7%)	37.9

[a] Etiochloroplasts were lysed in lysing buffer containing 146 mM Tris-HCl. The membranes were washed once by resuspension in lysing buffer lacking the cofactor under investigation and were pelleted by centrifugation at 235000g for 0.5 h at 1 °C. NADPH (0.56 mM) was added to the suspension of washed plastid membranes. The washed membranes were then incubated with the pooled additives, but in the absence of the particular additive under investigation. [b] Mean of two replicates. [c] Values in parentheses represent the net change in DV-Chlide *a*, as a percent of DV-Chlide *a* present before incubation. [d] Either no MV-Chlide *a* or trace amounts of MV-Chlide *a* were detected at the beginning of incubation, except for the ATP experiments. In this case, the MV-Chlide *a* content at the beginning of incubation amounted to 12.2 and 11.1 nmol of MV-Chlide *a*/100 mg of protein for −ATP and +ATP, respectively. [e] Pooled additives consisted of NAD+ + ATP + Mg + EDTA + methanol.

mM) was added to the suspension of washed plastid membranes which were incubated with the aforementioned additives, in the presence and absence of the additive under investigation. It was conjectured that if an additive was required for 4VCR activity, its omission from the incubation medium should be expressed by depressed 4VCR activity.

As shown in Table IV, none of the aforementioned additives appeared to be involved in the conversion of DV-Chlide *a* to MV-Chlide *a*. This was evidenced by similar 4VCR reaction rates in the absence and presence of the various additives. The same phenomenon was observed when ATP, Mg^{2+}, EDTA, NAD^+, and methanol were simultaneously omitted from the incubation medium, which in this case consisted of sucrose, Tris-HCl, BSA, and NADPH (Table IV). Altogether, these results indicated that NADPH was most probably the only cofactor required for 4VCR activity.

DISCUSSION

The conversion of DV-Chlide *a* to MV-Chlide *a* is a major reaction in green plants. Normal green plants do not contain detectable amounts of DV-Chl *a*. In DDV/LDV and DMV/LDV plants, in daylight, MV-Chl *a* is synthesized via DV-Pchlide *a* (Carey & Rebeiz, 1985; Carey et al., 1985). The latter is apparently converted to DV-Chlide *a*, which in turn is reduced to MV-Chlide *a* by 4VCR, as evidenced by the detection of a sizable MV-Chlide *a* pool in green plants (Nandihalli et al., 1990). MV-Chlide *a* is in turn converted to MV-Chl *a* by phytylation (Daniell & Rebeiz, 1984).

[4-Vinyl] chlorophyllide *a* reductase activity was monitored via the simultaneous disappearance of the DV-Chlide *a* substrate and the formation of MV-Chlide *a*. In most instances, the stoichiometry of DV-Chlide *a* disappearance and MV-Chlide *a* appearance was within 10–12%, which is within the range of experimental error. At low DV-Chlide *a* conversion rates of a few nanomoles per 100 mg of protein, as was observed with washed plastid membranes in the absence of added NADPH, this stoichiometry was not observed. This aberration may have been caused by the formation of small amounts of MV-Chlide *a* by hydrolysis of MV-Chl *a*, during dark incubation.

As reported in Tables II–IV, sizable 4VCR activity was detected in the plastid membranes. In this work, it was not possible to measure directly the 4VCR activity of the stroma. This is because the 4VCR assay relies on an endogenous DV-Chlide *a* substrate, which is membrane-bound and which is not present in the stroma. Very recently, a 4VCR assay that reduces exogenous DV-Chlide *a* has been developed and has permitted a comparison of the 4VCR activities of plastid membranes and stroma (R. Parham and C. A. Rebeiz, manuscript in preparation). The stroma was completely inactive.

Dramatic improvement in 4VCR activity was observed in the presence of NADPH, but not in the presence of other reductants such as NADH or GSH. This in turn indicates that 4VCR is an NADPH-dependent enzyme. In nature, most of the MV-Chl *a* is formed in daylight via DV-Chlide *a* which is reduced to MV-Chlide *a* by 4VCR. Under these conditions, the requirement for NADPH is likely to be readily met by the formation of NADPH during photosynthesis. Etioplasts prepared from etiolated cucumber cotyledons incapable of photosynthesis also exhibited 4VCR activity in the presence of added NADPH (R. Parham and C. A. Rebeiz, manuscript in preparation).

[4-Vinyl] chlorophyllide *a* reductase appears to be specific for DV-Chlide *a* and does not reduce endogenous DV-Pchlide *a* to MV-Pchlide *a* (Table III). Using a newly developed 4VCR assay for the reduction of exogenous DV-Chlide *a*, it has also been demonstrated that 4VCR was not active toward exogenous DV-Pchlide *a* (R. Parham and C. A. Rebeiz, manuscript in preparation). The reduction of DV-Pchlide *a* to MV-Pchlide *a* has been demonstrated in barley and cucumber etiochloroplasts. The activity in cucumber is about 4-fold lower, however, than in barley (Tripathy & Rebeiz, 1988). Nevertheless, the strong 4VCR activity toward DV-Chlide *a* and the complete lack of 4VCR activity toward DV-Pchlide *a* (Table III) suggests that 4VCR and [4-vinyl] protochlorophyllide *a* reductase are most probably two

different enzymes. On the basis of reductant requirements and substrate specificity, it is therefore proposed that two different 4-vinyl reductases are probably involved in the reduction of DV-Pchlide *a* and DV-Chlide *a* to their respective 4-ethyl analogues.

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