Plant Physiol. (1983) 71, 849–854
0032-0889/83/71/0849/06/$00.50/0

Hydrogenation of Geranylgeraniol[1]

TWO PATHWAYS EXIST IN SPINACH CHLOROPLASTS

Received for publication September 20, 1982 and in revised form December 15, 1982

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ABSTRACT

The reduction of geranylgeranylpyrophosphate to phytylpyrophosphate in spinach chloroplasts is described for the first time. The reductase is localized in the chloroplast envelope. By contrast, the reduction of the geranylgeranyl moiety in Chl synthesis is catalyzed in the thylakoids (via Chl synthetase). NADPH functions as electron donor in both reactions. Chl synthetase is firmly bound to the thylakoid membranes, and very little activity is found in the stroma fraction. Chl synthetase in chloroplasts can use the pyrophosphate ester of either phytol, geranylgeraniol, or farnesol, phytylpyrophosphate being the preferred substrate. Exogenous Chlide exhibits no influence on Chl synthesis by chloroplast subfractions.

Ph,[2] a monounsaturated terpenoid alcohol ($C_{20}H_{39}OH$), forms the side chains of Chl, tocopherol (vitamin E), and phylloquinone (vitamin K) in green tissue. GG is the most likely precursor of Ph, but until recently (20) there was no direct evidence of a conversion of GG to Ph.

Ph had been shown to be labeled by a number of different precursors: the hydrogen donor NADPH, basic carbon precursors, such as acetate, mevalonate, and IPP, but not by GG (see Refs. 8, 23, 22, 5, and 24, respectively). GG is known to occur as the side chain in bacteriochlorophyll of purple bacteria (7), and it was later found in trace amounts esterified to Chl *a* in greening etiolated tissue (12, 25). Rüdiger *et al.* (17) showed that an enzyme different from chlorophyllase, *i.e.*, Chl synthetase, was responsible for the esterification of Chlide in etiolated tissue. Subsequently, this enzyme was demonstrated in spinach chloroplasts (6, 20) and Chl-free chromoplasts from daffodil (11). Further examination of the last steps of Chl biosynthesis in greening etiolated oat seedlings showed a stepwise reduction of the initial product, Chl_{GG}, via Chl dihydrogeranylgeraniol and Chl tetrahydrogeranylgeraniol to Chl_{Ph} (2, 18). In a preliminary report (20), we showed that this sequence also exists in the natural green system. Very little is known about the regulation of this important final step in Chl synthesis in chloroplasts.

In studies of tocopherol and phylloquinone synthesis, we found that GGPP could not substitute for PPP in the enzymic prenylation of the aromatic precursor of either vitamin (19, 21). This is in contrast to Chl synthetase which uses GGPP and PPP and to a lesser extent even FPP in the etioplast system (17). In a recent short communication (20), we showed for the first time that GGPP was directly incorporated into Ph. No data were available for the localization and regulation of this reductase activity. The data presented below further characterize the reduction of GGPP and show that two distinct sites and two distinct pathways exist for the conversion of the geranylgeranyl moiety to the phytyl moiety in spinach chloroplasts: the chloroplast envelope for the hydrogenation of GGPP to PPP, and the thylakoids for the esterification of chlide with GGPP and stepwise reduction of Chl_{GG} to Chl_{Ph}.

MATERIALS AND METHODS

Reagents. The reagents were from commercial sources and were of the highest purity available.

Radiochemical and Chemical Syntheses. [1,5,9-^{3}H]FPP (6.1 Ci/mmol) was purchased from New England Nuclear (Draieich, F. R. G.), and was diluted with unlabeled FPP to 70 μCi/μmol. [*ethyl*-1-^{14}C]Bromoacetate (19.6 mCi/mmol) was purchased from Amersham Buchler (Braunschweig). [1-^{14}C]GG was synthesized from [*ethyl*-1-^{14}C]bromoacetate and all *trans*-farnesylacetone (6,-10,14-trimethyl-5,9,13-pentadecatrien-2-one) by a modified Horner-Wittig reaction as described previously (20). [1-^{14}C]Ph was synthesized by an identical procedure as described for [1-^{14}C]GG, but 6,10,14-trimethylpentadecan-2-one was used instead of farnesylacetone. 6,10,14-Trimethylpentadecan-2-one was made from natural phytol by ozonolysis (15). FPP, GGPP, and PPP, either labeled or unlabeled, were synthesized from the corresponding alcohol as described in Ref. 26. Chlide *a* was prepared from Chl *a* with chlorophyllase as described in Ref. 3.

Preparation of Broken Chloroplasts. Chloroplasts were isolated from freshly picked field-grown spinach by standard methods (16). They were further purified by Percoll gradient centrifugation (10). The purified chloroplasts were then subjected to osmotic shock in hypotonic buffer solution containing 10 mM Hepes, pH 7.6, and 4 mM $MgCl_2$ (buffer A), yielding broken chloroplasts to ensure free passage of ATP and isoprenoid pyrophosphates through the chloroplast envelope.

Isolation of Chloroplast Subfractions. Chloroplasts were purified and submitted to osmotic breakage as described above. The separation of the chloroplast compartments was achieved by a discontinuous sucrose density gradient (1.2, 0.96, and 0.6 M sucrose) (9). The top phase of the gradient was used as the chloroplast extract. Thylakoids were washed twice in buffer A prior to use. Chl was determined according to (1), protein according to (14).

Assay Conditions for the Reduction and Incorporation of GGPP. The complete reaction mixture contained, if not otherwise defined, 50 mM Hepes, pH 7.6, 4 mM $MgCl_2$, 2 mM $MnCl_2$, 0.1 mM ATP, 0.1 mM NaF, 3 mM NADPH. Suspensions of broken chloroplasts contained an additional 10 mM $NaHCO_3$. Reaction mixtures were illuminated with white light in a water bath with 0.1 J/

[1] This work was aided by grants from the Deutsche Forschungsgemeinschaft.

[2] Abbreviations: Ph, phytol; GG, geranylgeraniol; IPP, isopentenylpyrophosphate; $Chl_{GG,DHGG,THGG,Ph}$, Chl esterified with GG, dihydro-GG, tetrahydro-GG, and Ph; GGPP and PPP, GG- and Ph-pyrophosphate; Phae, phaeophytin; FPP, farnesol-PP; Chl a_F, Chl $a_{farnesol}$.

$cm^2 \cdot s$ light intensity. Reactions were carried out at 20 ± 2°C. The assay volume was 1 ml in experiments with broken chloroplasts; with chloroplast subfractions, it was 0.5 ml.

Analytical Methods. PPP was very labile to hydrolysis in aqueous acidic solutions. For that reason, PPP was purified in organic solvents in the presence of ammonia. In contrast, Chl was susceptible to oxidation and hydrolysis at alkaline pH and was therefore converted through acid treatment to the more stable Phae.

Purification of PPP. Aliquots were taken at different times from the incubation mixture. The reaction was stopped by the addition of three volumes of $CHCl_3$:methanol:NH_3 (1:2:0.2, v/v/v), and the resultant monophasic solution was evaporated under vacuum to 100 to 200 μl. The flasks were rinsed five times with 200 μl $CHCl_3$, and the combined extracts were subjected to TLC on Kieselguhr-Silica Gel 60 precoated plates (Merck). A solvent system of propan-1-ol:butan-1-ol:2 N NH_3 (5:2:3, v/v/v) was used (hereafter called TLC system I). Fifty μg PPP were added as carrier to every TLC assay. The PPP band on the TLC plate (still contaminated with [^{14}C]GGPP) was visualized by spraying one-tenth of the TLC plate with 20% phosphomolybdic acid in methanol (w/v) and heating at 120°C. The residual PPP zone was scraped off the plate, suspended in 2 ml H_2O, acidified with 0.1 N H_2SO_4 to a final pH of 1.7 to 2.0, and hydrolyzed for 30 min at 100°C. The acid hydrolysis yielded Ph and GG, along with the isoalcohols (isophytol and geranyllinalool) and an elimination product (phytadiene). At this stage, either HCl or methanol should be avoided inasmuch as both give rise to further degradation and modified products. The hydrolysate was extracted with diethyl ether, and the extract was washed until free of acid and then rechromatographed on silica gel plates containing 10% $AgNO_3$ (w/w) with *n*-hexane:diisopropyl ether:ethyl acetate (2:1:1, v/v/v) as developing solvent (hereafter called TLC system II). TLC system II resolved Ph from GG, but not isophytol from Ph or geranyllinalool from GG; if these separations are desired, the same solvents in a ratio of 6:1:1 (v/v/v) can be used. The TLC plates were checked with a TLC radioscanner for accurate chromatography. The relevant radioactive areas were then scraped off the plates and dissolved in a mixture of 2 ml methanol and 20 μl saturated NaCl solution. Five ml of scintillation fluid (Hydroluma, Baker, Groβ-Gerau) were added and the radioactivity was determined using a Packard liquid scintillation counter (Tricarb 3255). The recovery rate of Ph after the purification was completed was about 30% according to independent experiments.

Purification of Chlorophylls. Aliquots were taken from the incubation mixture at the same time intervals as for the PPP synthesis. The reaction was stopped with 2 ml acetone:water (3:1, v/v) and the solution was then extracted three times with 3 ml petroleum ether (b.p. 40–60°C). The Chl was converted to Phae by acidification with 1 ml 1 N HCl. The petroleum ether was washed until free of acid and evaporated to dryness, and the Phae was subjected to TLC on precoated silica gel plates (Schleicher & Schüll, G-1500). A solvent system of CCl_4:acetone (91:9, v/v) was used. The Phae *a* and Phae *b* regions were scraped off the plates separately and eluted from the gel with acetone (2 × 2 ml). The Phae was further purified and the $Phae_{Ph}$, $Phae_{THGG}$, $Phae_{DHGG}$, and $Phae_{GG}$ components were separated by HPLC on a column (4 × 250 mm) of Li-Chrosorb RP-8 (5–10 μm) with methanol:water (95:5, v/v) (2). Radioactivity was determined in a scintillation counter (Beckman LS-100C) with Rotiszint 22 (Roth, Karlsruhe).

RESULTS

Recently obtained data not only showed that chloroplasts contained a Chl synthetase (6, 20) but also indicated that they possessed the ability to reduce the GG moiety by two different routes (20). To analyze these processes further, broken chloroplasts and their subfractions were incubated with [1-^{14}C]GGPP, and the responses of different effectors on Chl and PPP synthesis were determined.

Phytylpyrophosphate Synthesis. The phytyl side chain of Chl is labile to hydrolysis by chlorophyllase, which had been shown to be active in chloroplasts in the presence of high levels of organic solvent, *e.g.*, 40% acetone, and to transesterification with alcohols, especially methanol. The product of this hydrolysis and transesterification is Ph. To establish the reduction of free GGPP to PPP, the synthesis of PPP and not Ph had to be documented by special methods (see "Materials and Methods"). Assays with broken chloroplasts contained a very high Chl concentration (about 3 mg/ml, thus avoiding dilution of necessary cofactors), and these broken chloroplasts reduced GGPP to PPP (Table I). The rate of reduction was not altered when NADPH was omitted in the light, indicating that broken chloroplasts formed sufficient NADPH to support the GGPP reduction. GGPP reductase activity was found in broken chloroplasts also in the dark without NADPH (data not shown). Thus, neither light nor exogenous NADPH influenced the formation of PPP from GGPP (Table I). Using GG instead of GGPP as substrate resulted in a 25% decrease of the reduction to PPP, indicating that the PPi form was the substrate for the reductase and not the alcohol form.

A pH optimum of 7.6 for the synthesis of PPP with a decrease in the acidic range was observed. When broken chloroplasts (1.6 mg Chl/assay) were incubated for 60 min as described in "Materials and Methods;" the rate of reduction was 1.2, 0.9, and 0.2 nmol/mg Chl·h for pH 7.6, 7.1, and 6.6, respectively.

To localize GGPP reductase activity, isolated chloroplast subfractions were tested for their ability to reduce GGPP. As

Table I. *Reduction of GGPP in the Synthesis of Chl a_{Ph} and PPP*

One-ml samples of broken chloroplasts containing 0.1 mM [^{14}C]GGPP or 0.4 mM GG were incubated with or without light and with or without NADPH. Samples were analyzed as described under "Materials and Methods."

Incubation Conditions	PPP	Chl a_{Ph}
	pmol/mg Chl·h	
GGPP + light + NADPH	50	780
GGPP + light − NADPH	49	810
GGPP − light + NADPH	45	190
GG + light + NADPH	36	50

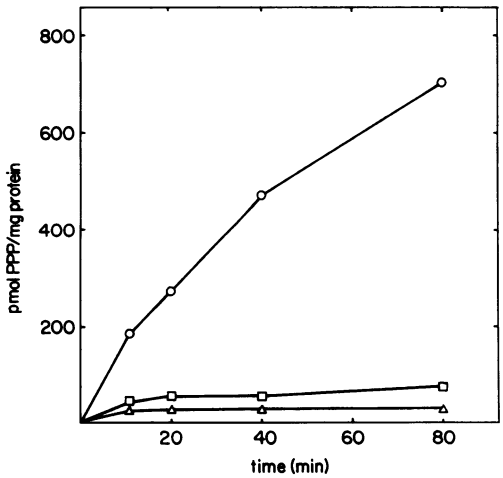

FIG. 1. Localization of GGPP reduction in spinach chloroplasts. Envelope (○), thylakoids (□), and stroma (△) were isolated from purified chloroplasts and tested for their ability to reduce GGPP in a time course experiment.

Figure 1 shows, GGPP reductase was located solely in the chloroplast envelope. No increase of activity was achieved by recombining chloroplast extract with envelope or thylakoid membranes (data not shown). Omitting NADPH resulted in a sharp decrease of PPP formation in the envelope. Envelope membranes (equivalent to 1.6 mg protein) were incubated with [1-^{14}C]GGPP (0.25 mM) with or without NADPH as described in "Materials and Methods." PPP was formed at 390 pmol/h·mg protein in the presence of NADPH, while without NADPH the rate dropped to 80 pmol/h·mg protein. GG could not replace GGPP as precursor of PPP when incubated only with the envelope fraction. The synthesis of PPP from GG could be partly restored if envelope membranes were incubated together with stroma and ATP (0.1 mM). The rate of PPP formation from GG plus ATP in this recombined system was up to 50% of comparabale conversion rates from GGPP. The reduction of GG and GGPP at the chloroplast envelope yielded only PPP; no Ph was detectable.

Chlorophyll Synthesis. Chl synthesis was studied in time course experiments (1 h) and remained linear over the course of the experiment. The existence of Chl a_{DHGG} and Chl a_{THGG} in broken chloroplasts and thylakoids was shown by HPLC techniques. The reduction of GGPP in Chl synthesis showed significant differences from the same process involved in PPP synthesis. As can be deduced from the results in Table I, Chl a_{Ph} synthesis from GGPP was light-dependent, probably due to the light-dependent conversion of Pchlide to Chlide. Furthermore, GG could not replace GGPP in Chl synthesis by Chl synthetase at a significant rate (Table I). Additional distinctions between the reduction of GGPP and the GG side chain of Chl were made by measuring the apparent K_m for GGPP. In contrast to PPP the rate of Chl a_{Ph} synthesis from GGPP increased to 0.67 mM GGPP (Table II). Chl b_{Ph} synthesis is saturated at 0.17 mM GGPP in broken chloroplasts (Table III). The Chl synthetase in broken chloroplasts showed little response to changes in pH. The incorporation rates of GGPP at pH 7.6, 7.1, and 6.6 were 2.1, 2.3, and 1.4 nmol/mg Chl·h, respectively. The reduction of Chl a_{GG} in chloroplasts is very fast, as we concluded from the time course studies. After 10 min, the main product of Chlide esterification with labeled GGPP was Chl a_{Ph} (see also Ref. 20). When thylakoids were used, the amount of Chl $a_{GG,DHGG,THGG}$ increased but Chl a_{Ph} still represented at least 30% of the products formed (Tables IV and VI).

Specificity of the Chlorophyll Synthetase. Chloroplasts and thylakoids were supplied with different isoprenoid pyrophosphates to determine the specificity of the esterification step. Chl synthetase had been shown to have a relative specificity of 6:3:1 for GGPP/PPP/FPP in etioplast membranes of oats (17). In contrast, the Chl synthetase in the overall synthesis of Chl *a* showed a 4-fold preference to PPP over GGPP in broken chloroplasts and at the thylakoid membranes. Broken chloroplasts and thylakoids were incubated with 1.2 mM [^{14}C]GGPP or PPP, respectively. Broken chloroplasts incorporated GGPP into Chl *a* at a rate of 1.1 nmol/mg Chl *a*·h, while PPP yielded 5.6 nmol Chl *a*/mg Chl *a*·h. With the thylakoids, the rates were lower, 0.72 (GGPP) and 2.6 nmol/mg Chl *a*·h (PPP). Under these conditions, GGPP and PPP were incorporated at the same rate into Chl *b* by broken chloroplasts and thylakoids (data not shown). For the first time, Chl b_{GG} was proven to exist in green tissue (see also Tables III and V).

Chl synthetase can also use FPP (0.1 mM [^{3}H]FPP) to esterify Chlide. Chl a_F does not exist *in vivo* but is formed under *in vitro* conditions by broken chloroplasts in high yields (0.87 nmol/mg Chl *a*·h) when supplied with exogenous FPP. The absence of Chl a_F *in vivo* is probably due to a lack of free FPP in the chloroplasts.

A very active prenyltransferase, which used the exogenously supplied FPP and endogenous IPP to form GGPP, was present in the broken chloroplast preparation. When FPP (0.1 mM [^{3}H]FPP) was added to the chloroplasts, a good yield of Chl a_{GG} was obtained (0.15 nmol/mg Chl *a*·h, in comparison to 0.71 nmol/mg Chl *a*·h from 0.06 mM [^{14}C]GGPP). Chl a_{Ph} was also formed at a rate of 0.021 nmol/mg Chl·h from 0.1 mM [^{3}H]FPP. FPP was not incorporated in Chl $b_{F,GG,Ph}$. The synthesis of Chl a_{Ph} and Chl a_F increased over time during the experiment (up to 40 min), while the synthesis of Chl a_{GG} from FPP reached a plateau after 20 min. This probably reflected limited IPP availability, therefore decreasing the rate of Chl a_{GG} formation with time and finally the rate of Chl a_{GG} formation equaled that of Chl a_{GG} conversion to Chl a_{Ph}.

The effect of exogenous Chlide on Chl synthesis was studied with chloroplast subfractions and a recombined system of thylakoids and soluble protein. Added Chlide *a* did not stimulate the rate of Chl a_{Ph} formation either by thylakoids or in combination with stroma (Table IV). Stroma always contained a low amount of residual Chl synthetase activity which had become solubilized during hypotonic lysis of the chloroplasts (Tables IV–VI). We were unable to detect any effect of Chlide on the solubilized Chl synthetase activity (Tables IV and V). Chl b_{Ph} and Chl b_{GG} syntheses by the membrane-bound enzyme were stimulated by Chlide *a* when assayed in combination with thylakoids, while no effect was detected in the recombined system of thylakoids plus stroma and stroma alone.

Under photosynthetic conditions, the broken chloroplasts produced enough reducing equivalents (NADPH) to reduce the unsaturated form of the pigment (Table I). However, Chl a_{Ph} and Chl a_{GG} synthesis was increased by NADPH when thylakoids or thylakoids together with stroma were used as Chl-synthesizing systems (Table VI). When FAD was used in experiments together with NADPH, no additional effect was detected (data not shown). We assume that NADPH is the sole electron donor for this reaction.

DISCUSSION

We were able to demonstrate that two pathways exist in spinach chloroplasts for the reduction of the GG moiety to the Ph moiety, the reduction of GGPP to PPP in the chloroplast envelope, and the reduction of GG as side chain of Chl via Chl a_{DHGG} and Chl a_{THGG} to Chl a_{Ph} in the thylakoid. These processes are shown in chloroplasts for the first time.

Table II. *Effect of GGPP Concentration on the Synthesis of PPP and Chl a*

Broken chloroplasts equivalent to 5.2 mg ml^{-1} were incubated in the light for 60 min with different amounts of [1-^{14}C]GGPP in a final volume of 1.9 ml. Aliquots were taken at different times and assayed for PPP and Chl as described in "Materials and Methods."

[1-^{14}C]GGPP	PPP	Chl a_{Ph}		Chl $a_{GG,DHGG,THGG}$		
mM	*nmol/mg Chl*	*nmol/mg Chl a*	*nCi/nmol Chl* a_{Ph}	*nmol/mg Chl a*	*nCi/nmol Chl* a_{GG}	*nmol [^{14}C]Chl* a_{Ph}*/nmol [^{14}C]Chl* a_{GG}
0.67	1.2	8.6	0.061	0.75	0.61	11.5
0.17	1.44	5.9	0.042	0.40	0.47	14.75
0.04	0.35	2.0	0.015	0.20	0.23	10

Table III. *Effect of GGPP Concentration on the Synthesis of Chl b*

Broken chloroplasts (5.2 mg Chl ml^{-1}) were assayed in the light for 60 min with different amounts of [1-^{14}] GGPP in a final volume of 1.9 ml. Aliquots were taken at different times and assayed for Chl as in "Materials and Methods."

[1-^{14}C]GGPP	Chl b_{Ph}		Chl $b_{GG,DHGG,THGG}$		
mM	nmol/mg Chl b	nCi/nmol Chl b_{Ph}	nmol/mg Chl b	nCi/nmol Chl b_{GG}	nmol[^{14}C]Chl b_{Ph}/ nmol[^{14}C]Chl b_{GG}
0.67	1.4	0.011	1.1	0.23	1.27
0.17	1.5	0.011	1.24	0.35	1.21
0.04	0.43	0.003	0.38	0.1	1.1

Table IV. *The Effect of Exogenously Added Chlorophyllide on the Chlorophyll Synthesis in Chloroplast Subfractions*

Thylakoids (T) (1.1 mg Chl ± 100 nmol Chlide), thylakoids plus stroma (S) (0.55 mg Chl + 1.4 mg stroma protein ± 50 nmol Chlide), and stroma (2.8 mg protein ± 20 nmol Chlide) were incubated in the light for 40 min with 0.2 mM [^{14}C]GGPP.

	Chl a_{Ph}		Chl $a_{GG,DHGG,THGG}$		
	nmol/mg Chl a	nCi/nmol Chl a_{Ph}	nmol/mg Chl a	nCi/nmol Chl a_{GG}	nmol[^{14}C]Chl a_{Ph}/ nmol[^{14}C]Chl a_{GG}
T + Chlide	0.30	0.0022	0.62	0.48	0.48
T − Chlide	0.31	0.0022	1.32	1.6	0.24
T + S + Chlide	0.34	0.0025	0.75	0.66	0.45
T + S − Chlide	0.44	0.0032	1.25	1.6	0.35
S + Chlide[a]	0.012		0.003		4
S − Chlide[a]	0.013		0.003		4.3

[a] Values are expressed as nmol/mg protein.

Table V. *The Effect of Exogenous Chlorophyllide on Chlorophyll b Synthesis*

Thylakoids (T) (1.1 mg Chl ± 100 nmol Chlide), thylakoids + stroma (S) (0.55 mg Chl + 1.4 mg stroma protein ± 50 nmol Chlide), and stroma (2.8 mg protein ± 20 nmol Chlide) were incubated in the light for 40 min with 0.2 mM [^{14}C]GGPP.

	Chl b_{Ph}		Chl $b_{GG,DHGG,THGG}$		
	nmol/mg Chl b	nCi/nmol Chl b_{Ph}	nmol/mg Chl b	nCi/nmol Chl b_{GG}	nmol[^{14}C]Chl b_{Ph}/ nmol[^{14}C]Chl b_{GG}
T + Chlide	1.4	0.01	0.80	4.7	1.75
T − Chlide	0.81	0.006	0.19	0.9	4.3
T + S + Chlide	1.4	0.01	0.16	0.9	8.75
T + S − Chlide	1.5	0.01	0.3	1.8	5
S + Chlide[a]	0.11		0.002		5.5
S − Chlide[a]	0.11		0.002		5.5

[a] Values are expressed as nmol/mg protein.

Table VI. *NADPH Dependence of GGPP Incorporation and Reduction in Chlorophyll Synthesis by Spinach Chloroplasts and their Subfractions*

[^{14}C]GGPP (0.08 mM) was incubated with or without NADPH in the presence of broken chloroplasts (equivalent to 3.5 mg Chl), thylakoids (T) (equivalent to 0.83 mg Chl), thylakoids + stroma (S) (equivalent to 0.45 mg Chl + 0.8 mg stroma protein), or stroma (equivalent to 1.7 mg protein) for 40 min in the light.

	Chl a_{Ph}		Chl $a_{GG,DHGG,THGG}$		
	nmol/mg Chl a	nCi/nmol Chl a_{Ph}	nmol/mg Chl a	nCi/nmol Chl a_{GG}	nmol[^{14}C]Chl a_{Ph}/ nmol[^{14}C]Chl a_{GG}
Chloro + NADPH	0.42	0.003	0.29	1.0	1.5
T + NADPH	0.41	0.003	0.75	1.7	0.55
T − NADPH	0.30	0.002	0.53	0.48	0.57
T + S + NADPH	0.92	0.007	0.9	3.0	1.02
T + S − NADPH	0.31	0.002	0.7	1.1	0.44
S + NADPH[a]	0.008		0.003		2.7

[a] Values are expressed per mg protein.

The formation of GGPP from IPP in chloroplasts is due to soluble prenyltransferases. To become fully active, however, the presence of chloroplast membranes, either the envelope or thylakoid membrane, is necessary (6). As shown by Block *et al.* (6) and as depicted in Figure 2, the isoprenoid pyrophosphates are bound to the envelope during the elongation of IPP to GGPP. The

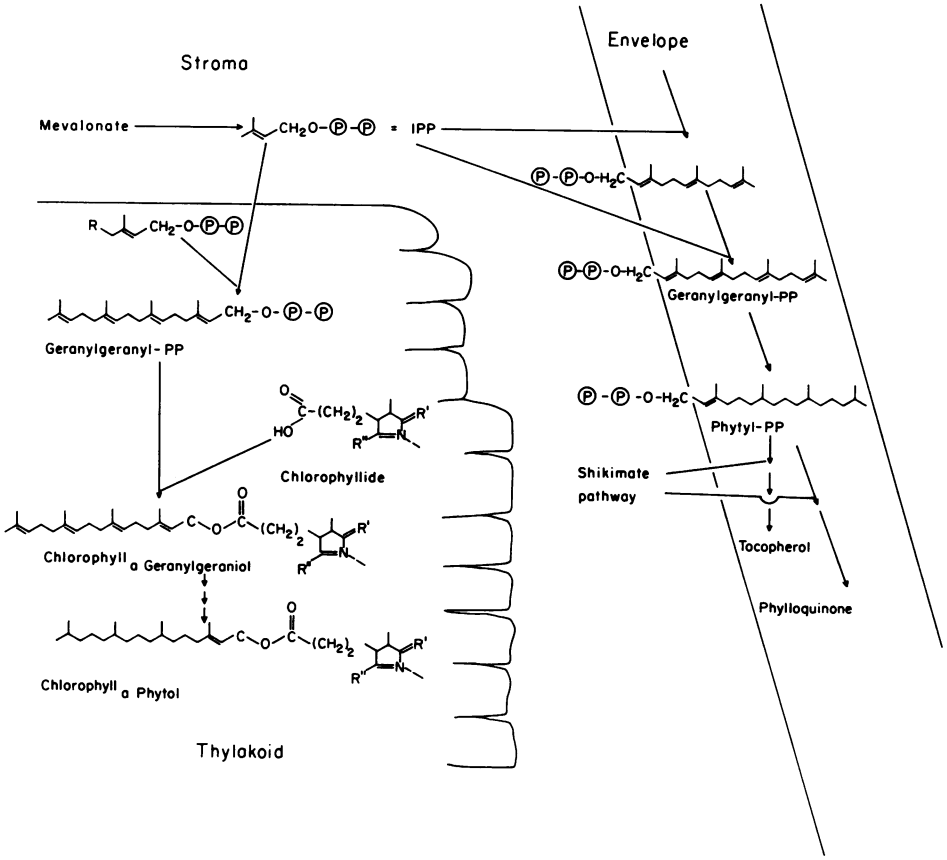

FIG. 2. Reduction of GGPP in prenylquinone and Chl synthesis and its compartmentalization in chloroplasts.

further steps of GGPP reduction to PPP and PPP utilization in the formation of α-tocopherol and phylloquinone are achieved by enzymes which are bound exclusively in the chloroplast envelope (19, 21). The subsequent enzymes, necessary to complete α-tocopherol synthesis, are also in the envelope (21). The GGPP reductase in the envelope produces more PPP than necessary for tocopherol and phylloquinone synthesis, especially if the losses during PPP purification are considered (see "Materials and Methods").

GGPP and not GG serves as substrate for GGPP reductase, as shown with isolated envelopes. The reductase activity with GG, however, is restored when ATP was supplied together with chloroplast extract. This is due to a kinase which forms GGPP from GG and ATP. A kinase which yields PPP from Ph and ATP was recently demonstrated in chloroplast extract (21). It was not examined here whether the Ph-kinase also pyrophosphorylates GG to GGPP, which is likely for the following reasons. Under *in vivo* conditions, GG probably does not occur in spinach chloroplasts; it does, however, exist under *in vitro* conditions with high chlorophyllase activity (6). Furthermore, the existence of free Ph (13) and a Ph-pool (4) has been shown in seedlings while the same is not valid for GG, which was only found esterified as Chl_{GG}. Accordingly, the conversion of GG to GGPP synthesis is likely due to the Ph-kinase.

Chl synthetase was first described in greening etiolated seedlings as catalyzing the esterification of Chlide with isoprenoid pyrophosphates (17). It was subsequently found in chromoplasts from daffodil (11) and spinach chloroplasts (6, 20). No details were available to compare the well described Chl synthetase from etioplasts with that from chloroplasts. The stepwise 2-electron transfer mechanism of Chl a_{GG} reduction via Chl a_{DHGG} and Chl a_{THGG} to Chl a_{Ph} was also found in chloroplasts. Chl synthetase from chloroplasts exhibits a different substrate specificity, with PPP being preferred 4:1 to GGPP, while the enzyme from etioplasts shows a 2:1 preference for GGPP. As pointed out earlier, PPP is formed in the envelope with higher yields than necessary for prenylquinone synthesis. This PPP, together with free Ph or a Ph pool which exists in plants (see Refs. 13 and 4, respectively), can in turn compete with or substitute for GGPP in Chl formation. GGPP is also needed for carotenoid synthesis and thus might not be available in sufficient amounts. The chloroplast therefore possesses a very flexible system for the last steps of Chl formation.

The Chl synthetase in chloroplasts is more stable than that from etioplasts. The main product of GGPP incorporation in Chlide by Chl synthetase is Chl a_{Ph} while Chl a_{Ph} is a minor compound in the etiolated system. The increase of Chl $a_{GG,DHGG,THGG}$ in experiments with thylakoids indicated a lability similar to that of the membranes from the etiolated system. This is also expressed in the ratios of the specific activity of newly formed Chl a_{Ph} to Chl a_{GG} [(nmol[^{14}C]Chl a_{Ph})/(nmol[^{14}C]Chl a_{GG})] which changed by 1 order of magnitude (compare tables). NADPH exhibits a pronounced positive effect on the reduction and increases the yield of Chl a_{Ph} in the thylakoids.

Chl *b* is also labeled from [^{14}C]GGPP and for the first time Chl b_{GG} is found in chloroplasts. Whether Chl b_{Ph} and Chl b_{GG} are synthesized from Chlide *a* or Chlide *b* is not clear. Our data suggest that GGPP is esterified to Chlide *a* yielding Chl a_{GG} and after reduction, Chl a_{Ph}, both of which can be transformed to Chl *b*. This is indicated also by the fact that GGPP and PPP are incorporated into Chl *b* to the same extent and not with a 4-fold preference for PPP as in Chl *a* synthesis. Finally, no Chl b_F is found while FPP is a very good substrate for Chl a_F formation.

In brief, the chloroplasts possess a very flexible system for the formation of the phytyl moiety in the synthesis of prenylquinones and Chl.

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