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Site of Prenylation Reaction in Synthesis of Phylloquinone (Vitamin K_1) by Spinach Chloroplasts

Gernot SCHULTZ, Bernd H. ELLERBROCK, and Jürgen SOLL

Institut für Tierernährung, Arbeitsgruppe für Phytochemie und Futtermittelkunde, and Botanisches Institut, Tierärztliche Hochschule, Hannover

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In spinach chloroplasts, 1,4-dihydroxy-2-naphthoate is prenylated by phytyldiphosphate and subsequently methylated by *S*-adenosylmethionine to form phylloquinol. The site of the prenylation reaction is the chloroplast envelope membrane.

1,4-Dihydroxy-2-naphthoate [1,2] as the crucial intermediate in the biosynthesis of menaquinones in bacteria is formed from shikimate [3] via chorismate [2] and *o*-succinylbenzoate [1]. Prenylation [4] and methylation [1] result in formation of menaquinones of different length of side chain. The principal steps of the synthesis of the naphthoquinone moiety are similar for both plants and bacteria [5–7]. In the present paper we wish to report on the prenylation of 1,4-dihydroxy-2-naphthoate by phytyldiphosphate and subsequent methylation by *S*-adenosylmethionine to form phylloquinol in spinach chloroplasts. The site of the prenylation reaction is the chloroplast envelope membrane.

MATERIALS AND METHODS

Radiochemicals and Chemical Synthesis

S-[*methyl*-^{14}C]Adenosylmethionine (56 Ci/mol) was purchased from Amersham Buchler (Braunschweig, FRG), [U-^{14}C]phytol (80 Ci/mol) from NEN (Dreieich, FRG). [U-^{14}C]Phytyl diphosphate was prepared according to [8] modified as follows: 0.75 mg (≈ 200 µCi) of the above labeled phytol dissolved in 25 µl acetonitrile was added dropwise within 2 h by a micropipette to a 250-µl reaction vessel containing 100 µl di(triethylammonium) phosphate (3.3% in dry acetonitrile) and 10 µl trichloroacetonitrile. After leaving overnight at 0 °C in the dark, phytyl phosphates were precipitated by adding 100 µl acetone plus 5 µl concentrated NH_3. After 2 h at −20 °C the precipitate was washed twice with 100 µl 0.28 M NH_3 in methanol to remove phytyl monophosphate. The yield of the diphosphate was 30 µCi. 1,4-Dihydroxy-2-naphthoic acid was prepared from 1-hydroxy-2-naphthoic acid according to [2], 1,4-Naphthoquinones and 2-methyl-1,4-naphthoquinones with phytyl, geranylgeranyl, farnesyl and geranyl side chain, respectively, (2-prenyl-1,4-naphthoquinones and 2-methyl-3-prenyl-1,4-naphthoquinones) were synthesized as described under 'synthesis of menaquinones, general procedure' in [9] modified as follows. Tetrahydrofuran was used as solvent with BF_3 etherate. The etheral solution of the prenylnaphthoquinol was shaken with aqueous solution of sodium dithionite and was alkalinized by NAOH. After oxidizing the quinol by Ag_2O in the dark in dry diethylether, the product was chromatographed on a column of silicagel (1.5 × 40 cm) with light petroleum/diethylether (20–10:1) as solvent. If necessary, the product was rechromatographed with the same system on thin-layer. For chloroplast experiments, prenylated and non-prenylated naphthoquinols were formed by reduction from the corresponding quinones just prior to use: some crystals of $NaBH_4$ were added to a mixture containing 50 µl of the 1 mM quinone, 2.5 ml methanol, 2.5 ml diethyl ether and 0.5 ml water. After 1 min the quinol was extracted with 10 ml diethyl ether, washed three times with 5 ml water each and quantified photometrically (approximated $\varepsilon_{243\,nm}$ 27500 $mol^{-1}\,l\,cm^{-1}$, see [9,10]). The required amount was evaporated under nitrogen in the incubation vessel of the chloroplast experiments.

Isolation of Broken Chloroplasts

Broken chloroplasts were prepared from intact chloroplasts isolated according to [11,12]; however, the concentration of $MgCl_2$ in all the media [11] was 2 mM, $MnCl_2$ 1 mM and EDTA 1 mM. The chloroplast pellet which resulted from centrifugation of suspension in the modified medium A [11] was suspended in the modified medium C [11] (about 2 mg chlorophyll/ml medium C). The chloroplasts were further purified on a linear Percoll gradient (Pharmacia, Uppsala, Sweden) (3500 × *g* at 0 °C for 45 min) [12]. The lower of the two bands contained the purified intact chloroplasts. To remove adhering Percoll, the intact chloroplasts were washed twice with modified medium C [11]. To obtain 'broken chloroplasts', the above intact ones were osmotically shocked (1 ml water per > 1 mg chlorophyll for 20 min).

Isolation of Chloroplast Subfractions

Chloroplast subfractions were isolated from the pellet of the above intact chloroplasts after purification by a Percoll gradient. After an osmotic shock, thylakoid and envelope membranes as well as stroma phase were separated on a sucrose gradient according to [14] (see also [13]). For criteria of purity of subfraction see [14].

Dedicated to Prof. Dr Helmut Holzer, Freiburg/Br., on the occasion of his 60th birthday.

Reaction Mixtures in Chloroplast and Subfraction Experiments

Reaction mixtures in experiments in Tables 1–3 and Fig. 1–2 contained the substrates and broken chloroplasts (or chloroplast subfractions) suspended in 1 ml of medium C according to [11] which was modified as mentioned above (2 mM $MgCl_2$; 1 mM EDTA). The concentrations of the substrates added were, if not otherwise defined: 1,4-dihydroxy-2-naphthoate 5 μM (1 μl of the 5 mM acid in ethanol was added to the reaction mixture); 2-methyl-1,4-naphthoquinol 5 μM; ATP 0.1 mM; [U-^{14}C]phytol 12 μM; *S*-[*methyl*-^{14}C]-adenosylmethionine 16 μM (for further details see tables and figures). Reaction mixtures were incubated at 20 °C for 80 min. The light intensity of the illumination was 0.1 J × cm^{-2} × s^{-1}. Aliquots (200–300 μl) were taken at different times. The reaction was stopped and the lipids extracted as described in [13]. Protein contents were determined by the method of Lowry and chlorophyll by the method of Arnon.

Purification and Identification of Labeled Products

Unlabeled carrier substances (about 25 μg per aliquot) identical to the expected labeled products were added to the extraction solution. All the naphthoquinols were oxidized for better purification before chromatography. The resulting 2-prenyl-1,4-naphthoquinones and 2-methyl-3-prenyl-1,4-naphthoquinones were chromatographed on silica gel layers with light petroleum (b.p. 60–80 °C)/diethyl ether (10:1) as solvent and rechromatographed on cellulose layers (Schleicher & Schüll, Einbeck-Dassel, FRG), impregnated with 7% paraffin oil in petrol (b.p. 100–140 °C) with aceton/methanol/H_2O as solvent. The solvent system in the case of phytyl derivatives were 13:7:0.5, with geranylgeranyl derivatives 13:7:1, with farnesyl derivatives 13:7:2 and with geranyl derivatives 13:7:3. Non-prenylated naphthoquinones were chromatographed on silica gel layer with light petroleum/diethyl ether (5:1) and rechromatographed on silica gel layers with $CHCl_3$/CCl_4 (1:1) as solvent. All the substances were cochromatographed with authentic samples and detected by ultraviolet quench at 254 nm. Labeled products were counted as previously described [13].

RESULTS

In spinach chloroplasts the last two steps of phylloquinol formation were identified:

Table 1. *Incorporation of* ^{14}C *from [U-*^{14}*C]phytol plus ATP into 1,4-dihydroxy-2-naphthoate by spinach chloroplasts*
1.2 mg chlorophyll per experiment (1 ml); for further details see Materials and Methods

Incubation with	2-[*phytyl*-^{14}C]Phytyl 1,4-naphthoquinol formed in the	
	light	dark
	pmol × mg chlorophyll^{-1} × h^{-1}	
1,4-Dihydroxy-2-naphthoate + [U-^{14}C]phytol + ATP	28.0	3.0
1,4-Dihydroxy-2-naphthoate + [U-^{14}C]phytol	43.5	1.5
[U-^{14}C]Phytol + ATP	1.0	0.5

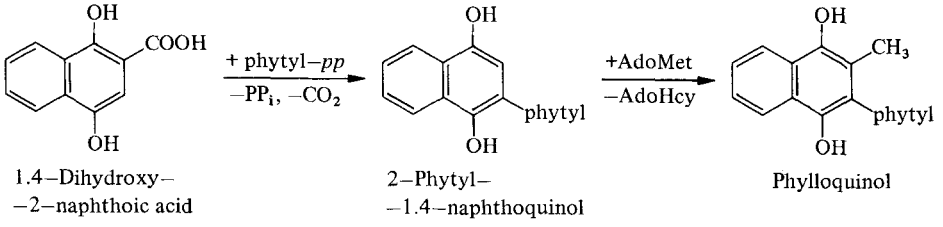
Formation of 2-Phytyl-1,4-naphthoquinol from 1,4-Dihydroxy-2-naphthoate in Spinach Chloroplasts

Adding phytol in the light, 2-phytyl-1,4-naphthoquinol was formed from 1,4-dihydroxy-2-naphthoate by purified chloroplasts, which were slightly osmotically shocked in concentrated suspension (> 1 mg chlorophyll/ml). Maximal rate obtained was 63 pmol × mg chlorophyll^{-1} × h^{-1}. The rate was not enhanced by adding ATP to the suspension. An active kinase which pyrophosphorylates phytol is located in the stroma of the chloroplast [13]. Thus, it may be concluded that only phytol but not ATP was transferred across the slightly shocked envelope membrane: addition of ATP in the dark had minimal effects (Table 1). ATP required for pyrophosphorylation of phytol was probably formed by photophosphorylation of chloroplast thylakoids in the light.

The mechanism of prenylation of the naphthalenic moiety resembles that of bacteria, but differs from animals, which are capable of forming menaquinones from 2-methyl-1,4-naphthoquinone (or the quinol) plus prenyl diphosphate [16, 17]. In chloroplasts 2-methyl-1,4-naphthoquinol in not prenylated by phytol plus ATP in the light nor is 1,4-dihydroxy-2-naphthoate methylated by *S*-adenosylmethionine (Table 2).

Phylloquinol Formation from 2-Phytyl-1,4-naphthoquinol in Spinach Chloroplasts

When *S*-adenosylmethionine was additionally applied in experiments with 1,4-dihydroxy-2-naphthoate plus phytol,



Scheme. *The last two steps of the formation of phylloquinol.* Phytyl-*PP*, phytyl diphosphate; AdoMet, *S*-adenosylmethionine; Ado-Hcy, *S*-adenosylhomocysteine

The mechanism is analogous to bacteria; however, only the phytyl moiety (from phytyl diphosphate) is used in the prenylation reaction. According to the tocopherol synthesis [15] only the quinol and not the quinone was prenylated.

phylloquinol was formed along with 2-phytyl-1,4-naphthoquinol (Table 3). Thus, the intermediately formed 2-phytyl-1,4-naphthoquinol reacted with *S*-adenosylmethionine to form phylloquinol. Furthermore, when 2-phytyl-1,4-naphtho-

Table 2. *Incorporation of ^{14}C from [U-^{14}C]phytol and S-[methyl-^{14}C]-adenosylmethionine, respectively, into 1,4-dihydroxy-2-naphthoate, and the same for [U-^{14}C]phytol into 2-methyl-1,4-naphthoquinol by spinach chloroplasts*
1.17 mg chlorophyll per experiment (1 ml); for further details see Materials and Methods

Incubation conditions	Product formed	
	type	amount
		pmol × mg chlorophyll^{-1} × h^{-1}
1,4-Dihydroxy-2-naphthoate + [U-^{14}C]phytol + ATP; light	2-[*phytyl*-^{14}C]phytyl-1,4-naphthoquinol	63
1,4-Dihydroxy-2-naphthoate + *S*-[*methyl*-^{14}C]adenosyl-methionine; dark	2-[*methyl*-^{14}C]methyl-1,4-naphthoquinol	3.5
2-Methyl-1,4-naphthoquinol + [U-^{14}C]phytol + ATP; light	[*phytyl*-^{14}C]phyllo-quinol	2.5

Table 3. *Incorporation of ^{14}C from [U-^{14}C]phytol and S-adenosylmethionine into 1,4-dihydroxy-2-naphthoate by spinach chloroplasts*
1.3 mg chlorophyll per experiment (1 ml); for further details see Materials and Methods

Incubation with	*S*-Adenosyl-methionine	Formation of	
		2-[*phytyl*-^{14}C]-phytyl-1,4-naph-thoquinol	[*phytyl*-^{14}C]-phyllo-quinol
	μM	pmol × mg chloro-phyll^{-1} × h^{-1}	
1,4-Dihydroxy-2-naph-thoate + [U-^{14}C]-phytol + ATP	–	22.5	0.15
	10	27.0	0.45
	100	24.9	1.05
[U-^{14}C]Phytol + ATP	–	0.3	0.10

quinol (5 μM) was used as substrate, it was methylated by *S*-adenosylmethionine to yield phylloquinol. The maximal rate obtained was 6 pmol × mg chlorophyll^{-1} × h^{-1}. Increasing the applied concentration of 2-phytyl-1,4-naphthoquinol from 1–5 mM, enhanced the phylloquinol formation in chloroplasts only slightly. Consequently, the reaction might be controlled by diffusion of substrate across the envelope membrane. In testing the substrate specificity, the 2-phytyl-1,4-naphthoquinol was preferred to the geranylgeranyl derivative (ratio of phylloquinol : menaquinol-formation was (1 : 0.3). Triton X-100 (0.2%) (see [4]) exhibited no stimulatory effect on the methylation reaction. Elimination of an endogenous byproduct, which is methylated at high rates at the thylakoids was a technical problem in determining the methylation rate. For this reason, experiments using farnesyl and geranyl derivatives were not conclusive.

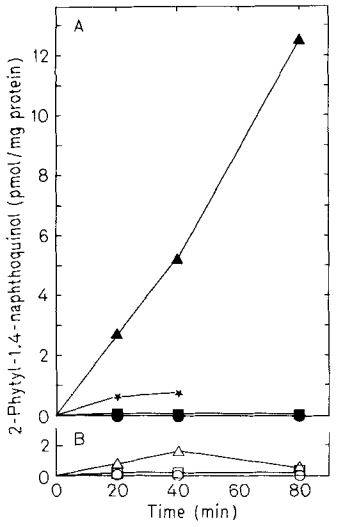


Fig. 1. *Formation of 2-[phytyl-^{14}C]phytyl-1,4-naphthoquinol from 1,4-dihydroxy-2-naphthoate (10 μM) plus [U-^{14}C]phytol plus ATP by chloroplast subfractions in the dark (A), and the same without adding 1,4-dihydroxy-2-naphthoate (B).* (★) Envelope membranes (5.0 mg protein/ml). (▲, △) Envelope membranes (1.54 mg protein) plus stromal phase (2.3 mg protein) per ml; results are calculated on the basis of envelope protein. (■, □) Stromal phase (4.63 mg protein/ml). (●, ○) Thylakoid membranes (14.4 mg protein; 1.5 mg chlorophyll) plus stromal phase (2.3 mg protein) per ml; results are calculated on the basis of thylakoid protein. For further details see Materials and Methods

Synthesis of 2-Phytyl-1,4-naphthoquinol by Chloroplasts Subfractions

In applying phytol plus ATP in the dark, only envelope membranes are active in synthesizing 2-phytyl-1,4-naphthoquinol from 1,4-dihydroxy-2-naphthoate. The required phytyl diphosphate is formed by the kinase in the stromal phase [13], which was added to the envelope membranes in the test. Neither thylakoid membranes nor stromal protein are capable of forming the quinol (Fig. 1).

In the experiment in Fig. 2, phytyl diphosphate instead of phytol plus ATP was applied. In this system the optimal substrate concentrations for prenyltransferase reaction in envelope membranes are: 1 μM 1,4-dihydroxy-2-naphthoate and about 3 μM phytyl diphosphate. Equal rates are obtained with 20 μM phytyl diphosphate [56 pmol × (mg envelope protein)$^{-1}$ × h^{-1}]. 1mM $MgCl_2$ instead of 2 mM $MgCl_2$ reduces the rates by about the half. (Mg^{2+} concentration optimum of the reaction is at 2 mM; data not shown.) Thylakoid membranes are also inactive in the system using phytyl diphosphate.

DISCUSSION

Phylloquinone and generalized prenylquinone synthesis in chloroplasts (e.g. the synthesis of tocopherols, plastoquinone and phylloquinone) [13] exhibits a sequence of coordinated reactions starting with the synthesis of the quinone moiety [18] and of isopentenyl diphosphate in the stromal phase to the synthesis of the prenylated quinones at the membranes, preferentially the envelope membrane [13, 19]. The group of Douce demonstrated [20] that the prenylpyrophosphates are formed from isopentenyl diphosphate by a soluble transferase of the stroma which is effectively activated by chloroplast membranes, both envelope and thylakoid membranes. Fur-

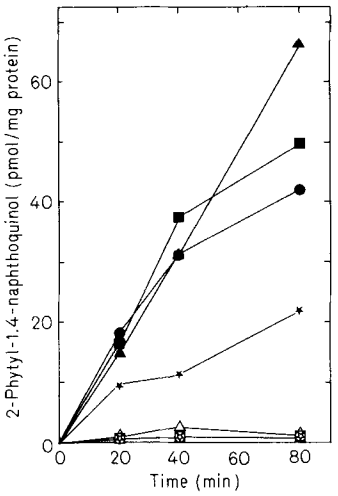


Fig. 2. *Formation of 2-[*phytyl-^{14}*C]phytyl-1,4-naphthoquinol from 1,4-dihydroxy-2-naphthoate plus [U-*^{14}*C]phytyl diphosphate (3 μM).* Filled symbols: envelope membranes (1.25 mg protein/ml) (▲) 1 μM 1,4-dihydroxy-2-naphthoate, 2 mM $MgCl_2$; (■) 10 μM 1,4-dihydroxy-2-naphthoate, 2 mM $MgCl_2$; (★) 10 μM 1,4-dihydroxy-2-naphthoate, 1 mM $MgCl_2$; (●) 0.1 mM 1,4-dihydroxy-2-naphthoate, 2 mM $MgCl_2$. Open symbols: thylakoid membranes (9.15 mg protein/ml; 1.05 mg chlorophyll; (△) 1 μM 1,4-dihydroxy-2-naphthoate, 2 mM $MgCl_2$; (□) 10 μM 1,4-dihydroxy-2-naphthoate, 2 mM $MgCl_2$; (☆) 10 μM 1,4-dihydroxy-2-naphthoate, 1 mM $MgCl_2$; (○) 0.1 mM 1,4-dihydroxy-2-naphthoate, 2 mM $MgCl_2$. For further details see Materials and Methods

thermore, most of the prenyl diphosphate is dispersed in membranes [20]. Thus, during biosynthesis the isoprenidic tails of the diphosphates may be positioned in membranes. In chloroplasts, however, phytyl diphosphate is used in the synthesis of the prenylquinones α-tocopherol [13] and phylloquinone, whereas geranylgeranyl diphosphate is preferred in the synthesis of chlorophylls [21]. The hydrogenation of geranylgeranyl diphosphate to yield phytyl diphosphate [22] may occur in membranes. As with plastoquinone and tocopherol synthesis [13], the present work reveals that also in phylloquinone synthesis the prenylation step occurs in the envelope membrane: 2-phytyl-1,4-naphthoquinol is formed from 1,4-dihydroxy-2-naphthoate and phytyl diphosphate at an optimal concentration range of 1 μM each. Phylloquinones are located in thylakoid and envelope membranes both [23], but at present its function in plants is unknown and there is no indication if it acts in γ-carboxyglutamate synthesis, as widely demonstrated in animals by Suttie et al. [24] and recently described in bacteria [25].

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G. Schultz and J. Soll, Institut für Tierernährung, Tierärztliche Hochschule, Bischofsholer Damm 15, 3000 Hannover 1, Federal Republic of Germany

B. H. Ellerbrock, Botanisches Institut, Tierärztliche Hochschule, Bünteweg 17d, 3000 Hannover-Kirchrode, Federal Republic of Germany