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SITE OF METHYLATION OF 2-PHYTYL-1,4-NAPHTHOQUINOL IN PHYLLOQUINONE (VITAMIN K_1) SYNTHESIS IN SPINACH CHLOROPLASTS

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Abstract—Phylloquinol (the quinol form of vitamin K_1) is synthesized from 2-phytyl-1,4-naphthoquinol and *S*-adenosylmethionine at the thylakoid membranes of spinach chloroplasts The addition of soluble stroma protein (chloroplast extract) is necessary *S*-Adenosylhomocysteine acts as strong competitive inhibitor

INTRODUCTION

The menaquinones (vitamin K_2 series) are formed in bacteria via the following sequence of reactions shikimate→chorismate→2-succinylbenzoate→CoA-ester of 2-succinylbenzoate [1] → 1,4-dihydroxy-2-naphthoate (**1**) → 2-prenyl-1,4-naphthoquinol → 2-methyl-3-prenyl-1,4-naphthoquinol (vitamin K) [2–4] An identical mechanism evidently exists in phylloquinone (quinone of **3**) (vitamin K_1) synthesis of higher plants [5, 6] The prenylation of **1** to yield 2-phytyl-1,4-naphthoquinol (**2**) is solely localized in the envelope membranes of chloroplasts (Fig 1) As shown in this paper, the methylation of **2**, which represents the last step in phylloquinone synthesis, occurs, however, in the thylakoid membranes of chloroplasts

RESULTS

Substrate specificity and kinetics of methylation in phylloquinone formation in chloroplasts

Vitamin K in prokaryotes contains an unsaturated prenyl side chain whereas that in photosynthetic eukaryotes has a partly saturated one (phytol) Therefore, it was of interest to clarify the specificity of the phylloquinone forming enzymes in chloroplasts with regard to different prenyl side chains As shown in Table 1, in both the prenylation reaction and the methylation reaction the phytyl derivatives were formed in preference to the geranylgeranyl derivatives By using S/v vs S and $1/v$ vs I plots, the K_m for SAM* was estimated to be in the range 3–5μM SAH acted as a strong competitor, the K_i was 1–2μM In both cases the concentration of **2** was 1 5 $\times 10^{-4}$ M Since a determination of the amount of quinol in solution in the reaction mixture was not possible, corresponding kinetic values for the quinol could not be obtained, however, the rate seemed to increase almost linearly from 10^{-6} M to 10^{-3} M of the added quinol

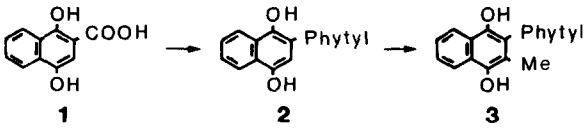

Fig 1 Prenylation and methylation reactions in phylloquinone synthesis

Methylation of 2-phytyl-1,4-naphthoquinol (**2**) *by chloroplast subfractions*

Experiments using envelope membranes in the methylation of **2** to form **3** were unsuccessful Combinations of envelope membranes and stroma protein (method I, see Experimental) were also ineffective An inhibitory effect on the methylation reaction by the *cis*-isomer of the chemically synthesized *cis,trans*-mixture of **2** was ruled out by the following experiment (Fig 2) The envelope membranes were allowed to synthesize the natural *trans*-isomer of **2** for the methylation reaction using phytyl diphosphate plus **1** [7] This endogenously formed precursor was also not methylated in the two-step reaction when SAM was exogenously supplied (Fig 2, reaction B) Neither envelope membranes nor stroma protein (method I) nor the recombined system were active The results in Fig 2 demonstrate that only the prenylation reaction (Fig 2, reaction A), but not the methylation reaction (Fig 2, reaction B), in phylloquinone synthesis is localized in the envelope membranes

As can be seen in Table 2, only thylakoid membranes in combination with stroma protein (method II, but also method I) were active in the methylation of **2** to form **3** The stroma protein fraction was probably needed for supplying soluble cofactors since this fraction was inactive *per se* Under these conditions similar rates were obtained as those measured with 'permeabilized chloroplasts' Experiments were done with [phytyl-1-^{14}C]-**2** plus un-

*Abbreviations SAH, *S*-adenosylhomocysteine, SAM, *S*-adenosylmethionine, HEPES, 4-(2-hydroxyethyl)-piperazine ethane sulfonic acid, Tricine, *N*-(2-hydroxy-1,1-bis(hydroxymethyl)ethyl)-glycine

Table 1 Specificity for prenyl moiety in vitamin K synthesis by 'permeabilized chloroplasts'

Substrates, conditions	Product: Identity	Product: Rate of formation (pmol/mg chlorophyll/hr)
1 (5×10^{-6} M) + [U-^{14}C]phytol ($1\,2 \times 10^{-5}$ M), light*	[Phytyl-^{14}C]-**2**	50 4
1 (5×10^{-6} M) + [1-^{14}C] geranylgeraniol (10^{-5} M), light*	2-[1-^{14}C]Geranylgeranyl-1,4-naphthoquinol	5
2 (3×10^{-4} M) + [methyl-^{14}C]SAM ($1\,5 \times 10^{-5}$ M), dark	[Methyl-^{14}C]-**3**	6 5
2-Geranylgeranyl-1,4-naphthoquinol (5×10^{-4} M) + [methyl-^{14}C]SAM ($1\,5 \times 10^{-5}$ M), dark	2-[Methyl-^{14}C]menaquinol-4	2 5

*The required diphosphates of phytol and geranylgeraniol, respectively, are formed by a kinase in the chloroplast stroma [8] prenol + ATP (from photophosphorylation) → prenyl diphosphate In the reactions studied diphosphate formation was not rate-limiting (cf [8])

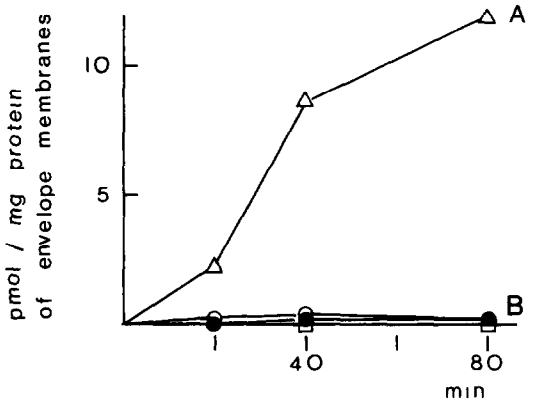

Fig 2 Comparison of prenylation (A) and methylation (B) reactions in phylloquinone synthesis using envelope membranes (E), stroma protein (S) or combinations of both fractions (E + S) (A) **1** + ATP + [U-^{14}C]phytol → [phytyl-^{14}C]-**2** For formation of prenyl diphosphates see Table 1 △, 1 mg E + 4 mg S/ml, substrates **1** (10^{-5} M) + [U-^{14}C]phytol ($2\,5\ 10^{-5}$ M) + ATP (10^{-4} M) (B) **1** + phytyl PP + [methyl-^{14}C]SAM → [methyl-^{14}C]-**3** ○, 1 mg E/ml, ●, 1 mg E + 4 mg S/ml, □, 4 mg S/ml, substrates **1** (10^{-5} M) + phytyl diphosphate (10^{-4} M) + [methyl-^{14}C]SAM ($4\,5 \times 10^{-5}$ M) For further details see Experimental

Table 2 Rate of formation of [^{14}C]-**3** from **2** (10^{-4} M) and [methyl-^{14}C]SAM ($3\,5 \times 10^{-5}$ M) by chloroplast fractions For details see Experimental

Chloroplast fractions	Rate of formation of [methyl-^{14}C]-**3** (pmol/mg chlorophyll/hr)*
Thylakoid membranes (10)†	0
Stroma protein (method II) (11)‡	0‡
Thylakoid membranes and stroma protein (method II) (1 1)	2 7
'Permeabilized chloroplasts' (12 2)†	2 75

*After subtraction of the blank value (on average 0 65 pmol/mg chlorophyll/hr)

†mg protein/mg chlorophyll in parentheses

‡Calculated from the chlorophyll contents of the corresponding chloroplast suspension

labelled SAM (data not shown) or with unlabelled **2** plus [methyl-^{14}C]SAM (Table 2) to eliminate a possible cross-contamination from products of tocopherol synthesis [8, 9]

DISCUSSION

In higher plants as in prokaryotes, vitamin K is present only in small amounts (1 mol phylloquinone per 100 mol chlorophyll *a* in photosystem I [10]) The activity in vitamin K synthesis is comparatively low and amounts to one-fifth to one-tenth of the sum of all prenylquinone synthesis (tocopherol [8, 9] plus plastoquinone [8] plus phylloquinone [7]) in this organelle From an evolutionary point of view some features in the biosynthesis of bicyclic prenylquinones like vitamin K do not seem to be as complicated as in monocyclic ones Because of the restricted regioselectivity in the reactions of naphthoquinones, 1,4-dihydroxy-2-naphthoate is prenylated exclusively at C-2 and methylated at C-3 [2–6] in an electrophilic substitution reaction Thus, the synthesis can be performed by enzymes lacking a pronounced specificity This might explain the early occurrence of vitamin K in prokaryotes besides ubiquinones, which are also formed from chorismate (via 4-hydroxybenzoate) in these organisms (for review on recent work see ref [11]) by a more complicated pathway

EXPERIMENTAL

Radiochemicals and chemical syntheses [Methyl-^{14}C]SAM (2070 GBq/mol) and ethylbromo[1-^{14}C]acetate (208 GBq/mol) were purchased from Amersham–Buchler (Braunschweig, West Germany [U-^{14}C]Phytol (2960 GBq/mol) was obtained from NEN (Dreieich, West Germany) All-*trans* farnesylacetone (6,10,14-trimethyl-5,9,13-pentadecatrien-2-one) and geranylgeraniol were generous gifts from Dr F Weber (Hoffmann-La Roche, Basel, Switzerland), and Dr W Hoffmann (BASF,

Ludwigshafen, West Germany) respectively 1,4-Dihydroxy-2-naphthoic acid was prepared from 1-hydroxy-2-naphthoic acid according to ref [3] [1-^{14}C]Geranylgeraniol was synthesized from ethylbromo[1-^{14}C]acetate and all-*trans*-farnesylacetone [12] [1-^{14}C]Phytol was prepared in the same manner except that hexahydrofarnesylacetone (6,10,14-trimethyl-pentadecan-2-one, made by ozonolysis as in ref [13]) was used 2-Phytyl-1,4-naphthoquinone and 2-geranylgeranyl-1,4-naphthoquinone were synthesized from naphthoquinol plus phytol and geranylgeraniol, respectively, in tetrahydrofuran and BF_3 [13] as modified in ref [7] without alkalization of the product The quinones were reduced to quinols just prior to use as in ref [7]

Isolation of 'permeabilized chloroplasts' and chloroplast subfractions (a) '*Permeabilized chloroplasts*' Intact chloroplasts isolated according to ref [14] were purified on a linear Percoll gradient as in ref [15] They were then hypotonically swollen in a small vol (*ca* 1 ml/mg chlorophyll) of HEPES medium (20 mM HEPES, 2 mM $MgCl_2$, 1 mM $MnCl_2$, 20 mM NaCl, adjusted to pH 7 6 using KOH) As indicated by marker enzymes, the chloroplasts were of *ca* 95% purity (in terms of residual activity of the peroxisomal hydroxypyruvate reductase) These 'permeabilized chloroplasts' take up prenols but not ATP, $NADP^+$, etc

(b) *Thylakoid membranes, stroma protein and envelope membranes (method I)* purified chloroplasts were hypotonically shocked in Tricine medium (10 mM Tricine, 4 mM $MgCl_2$, pH 7 6) at 0° for 10 min and fractionated on a sucrose gradient and tested for purity as described in ref [16] Additionally the homogentisate–phytyl diphosphate–phytyltransferase of tocopherol formation [8] was used as a marker for envelope membranes

Thylakoid membranes and stroma protein (method II) To obtain a stroma fraction higher in protein concn and free of sucrose, 'permeabilized chloroplasts' were centrifuged (10 000 *g* at 0° for 10 min) The pellet was washed twice in HEPES medium (30 ml/mg chlorophyll) and used as the thylakoid fraction The stroma protein free of membranes was prepared by centrifugation of the supernatant at 105 000 *g* at 0° for 30 min

Reaction mixtures in chloroplast and subfraction expts The reaction mixtures contained 'permeabilized chloroplasts' and subfractions (1 mg chlorophyll/ml), respectively, suspended in 1 ml HEPES medium and substrates as stated in the figures and tables The mixtures were incubated at 20° for 80 min either in the dark or in the light (0 7 J/cm^2 per s) Aliquots (200–300 μl) were taken at different times The reaction was stopped and the lipids were extracted as described in ref [8] Protein contents were determined by the method of Lowry and chlorophyll by the method of Arnon

Purification and identification of labelled products The chromatographic methods were as described in ref [7]

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