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SITE OF BIOSYNTHESIS OF α-TOCOPHEROL IN SPINACH CHLOROPLASTS

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1. Introduction

The chloroplast envelope is a continuous boundary of two osmiophilic membranes and has an important role in galactolipid synthesis [1]. It was interesting to determine whether the envelope is the site of synthesis of other lipid components in the chloroplast. We have chosen α-tocopherol for these experiments because it represents >40% of the total prenylquinones of the chloroplast [2]. The biosynthetic origin of α-tocopherol has been studied extensively by several authors [3–12]. In addition, its synthesis in isolated intact spinach chloroplasts has been shown [11]. Here we demonstrate that the chloroplast envelope membranes catalyse the incorporation of 2 methyl groups from *S*-adenosylmethionine (SAM) into the aromatic moiety of the α-tocopherol precursors.

2. Material and methods

2.1. *Radiochemicals*

[*Me*-^{14}C]SAM (spec. act. 50 mCi/mmol) was purchased from Amersham Buchler (Braunschweig) and CEA (Gif sur Yvette). The geranylgeranyl- and phytyl-substituted methylquinols and quinones (fig.1) were prepared from the corresponding methylquinol with *trans*-geranyllinalool and isophytol, according to [11]. γ-T_3 (fig.1) was synthesized according to [13]. α-T_3 (fig.1) and *trans*-geranyllinalool were a gift from Dr F. Weber (Hoffmann-La Roche, Basel).

2.2. *Isolation of chloroplast membranes*

Chloroplasts were isolated from washed spinach leaves by classical methods [14]. Isolation of envelope membranes included selection of intact chloroplasts from a sucrose gradient, gentle osmotic shock and separation of envelope membranes and thylakoids on a second sucrose gradient [14]. From 2 kg spinach leaves the yield of envelope membranes can reach up to 8–10 mg protein. Electron microscopy of the purified envelope fraction shows relatively large vesicles or elongated structures bordered by a single or a double membrane. No plastoglobuli are trapped in the network of the envelope membranes. NADH-cytochrome *c* oxidoreductase activity is negligible in the envelope fraction. The envelope is devoid of *b*-type cytochromes [1,14]. Hence this fraction is essentially free of microsomal and mitochondrial membrane contaminations. Thylakoids were washed twice in Tricine buffer (10 mM (pH 7.6); 4 mM $MgCl_2$) prior to use. The top phase (8–10 ml) of the membrane fractionating gradient [14] was used as chloroplast extract (stroma). The stroma was concentrated by ultrafiltration prior to use (Diaflo, membrane PM 10). Protein determinations were done by the Lowry method.

2.3. *Reaction mixture*

The complete reaction mixture, if not defined otherwise, contained: 10 mM Tricine–NaOH buffer (pH 7.6); 4 mM $MgCl_2$; 70 μM [*Me*-^{14}C] SAM (50 mCi/mmol); 180 μM methylprenylquinol used immediately after reduction of the corresponding quinones [11], and known amounts of chloroplast membranes in 600 μl final vol. Reactions were initiated by addition of SAM, incubation was at 20°C for 80 min in the dark. Aliquots (100 μl) were taken at different times. The reaction was stopped and the lipids extracted according to [15].

2.4. *Chromatography of [^{14}C]methylprenylquinols*

Unlabelled carrier substances identical to the expected labelled products were added to the extraction solution. Methylprenylquinols were oxidized by

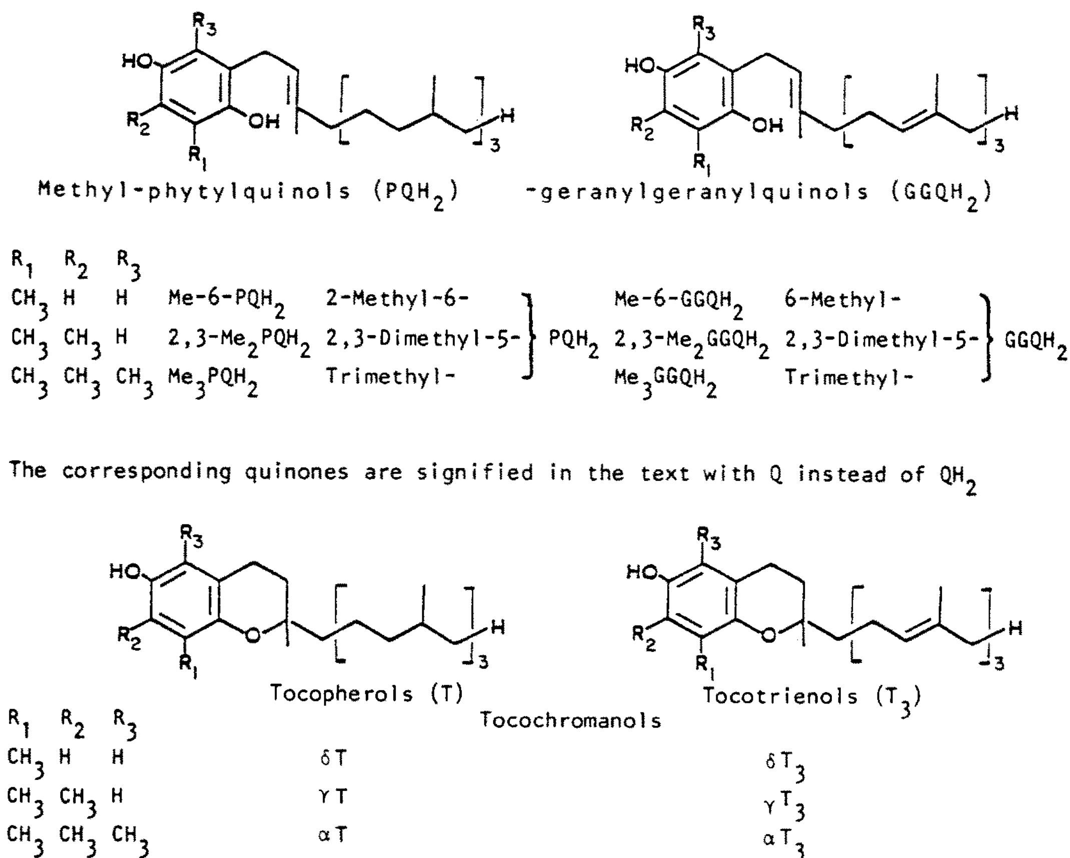


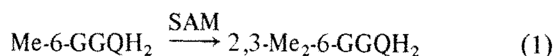
Fig.1. Structures of methylprenylquinols and tocochromanols.

air for better purification and were chromatographed on silicagel thin-layer chromatographic plates. A solvent system of petroleum (b.p. 60–80°C)/Et_2O 10:1 (v/v) was used. The products were rechromatographed on cellulose plates impregnated with 7% paraffin in petroleum (b.p. 100–140°C). A solvent system of Me_2CO/H_2O 85:15 (v/v) was used. All thinlayers (Schleicher and Schüll, Germany) contained a fluorescent indicator, F254. For further details see [13]. The radioactive areas were scraped from the plates, dissolved in 2 ml MeOH and counted in 10 ml scintillation mixture (Aquasol, Amersham Buchler) using an Intertechnique SL 4000 liquid scintillation counter.

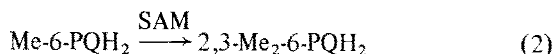
3. Results

3.1. *Synthesis of dimethylprenylquinols*

Me-6-$GGQH_2$ and Me-6-PQH_2 were used as precursors to locate the methyltransferase reaction:



$$\text{Me-6-GGQH}_2 \xrightarrow{\text{SAM}} \text{2,3-Me}_2\text{-6-GGQH}_2 \qquad (1)$$



$$\text{Me-6-PQH}_2 \xrightarrow{\text{SAM}} \text{2,3-Me}_2\text{-6-PQH}_2 \qquad (2)$$

Fig.2 indicates that the envelope membranes are able to catalyse the introduction of a methylgroup from SAM into either Me-6-$GGQH_2$ (1) or Me-6-PQH_2 (2) to yield the dimethyl derivatives. However, in good agreement with a report on intact chloroplasts [12], a strong preference for the geranylgeranyl-compound could be observed (fig.2). No radioactivity could be detected in trimethylprenylquinols or cyclisized tocopherols like γT, γT_3, αT and αT_3. In marked contrast to the envelope fraction, the methyltransferase activity was negligible in the thylakoid and stromal fractions. It is likely that the very low activity found is entirely attributable to a contamination by small envelope vesicles [1,14].

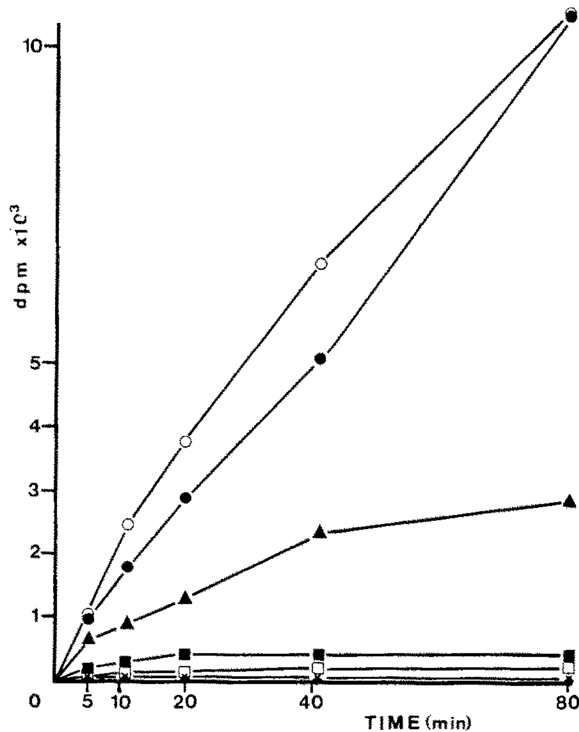


Fig.2. Time course incorporation of Me-^{14}C from [*Me*-^{14}C]-SAM by the different chloroplast fractions into Me-6-GGQH$_2$ and Me-6-PQH$_2$ (both 1.8×10^{-4} M in the test). Incubation mixture and lipid extraction were as in section 2. Incubation mixture plus: (○) Me-6-GGQH$_2$ + 0.2 mg envelope protein; (●) Me-6-GGQH$_2$ + 0.2 mg envelope protein + 3.2 mg concentrated stroma protein; (▲) Me-6-PQH$_2$ + 0.2 mg envelope protein; (□) Me-6-GGQH$_2$ + 3.6 mg thylakoid protein; (■) Me-6-GGQH$_2$ + 1.8 mg thylakoid protein + 4.8 mg concentrated stroma protein; (∗) Me-6-GGQH$_2$ + 3.2 mg stroma protein.

When stromal [14] and membrane fractions (thylakoid and envelope membranes) were combined, the rate of synthesis of dimethylprenylquinols was not stimulated. Consequently, these findings demonstrate not only that the methyltransferase is located in the chloroplast but also that it is bound to the envelope membranes.

3.2. *Dimethyl compounds as substrates*

2,3-Me$_2$-GGQH$_2$ and 2,3-Me$_2$-PQH$_2$ (90 μM) were incorporated neither into αT_3 and αT nor into Me$_3$-GGQH$_2$ and Me$_3$-PQH$_2$. Only γT (90 μM) was a precursor for αT. This second methylation step also occurred exclusively in the envelope fraction (data not shown). However it is interesting to note that the incorporation rate for γT (35 pmol/h . mg envelope protein) was much lower than for Me-6-PQH$_2$ (0.7 nmol/h . mg envelope protein) and Me-6-GGQH$_2$ (2 nmol/h . mg envelope protein).

4. Discussion

The chloroplast envelope isolated from intact purified spinach chloroplasts was shown to be the only site of two important steps in the biosynthesis of α-tocopherol: Me-6-GGQH$_2$ and Me-6-PQH$_2$ are methylated to 2,3-Me$_2$-GGQH$_2$ and 2,3-Me$_2$-PQH$_2$, respectively, by an envelope bound methyltransferase which uses SAM as CH$_3$-donor. The same is true for the conversion of γT to αT. Under the present assay conditions, with isolated envelope membranes (in contrast to the intact chloroplasts [12,13]) the chromanol stage, which is a prerequisite for the second methylation step was not reached. However this might be due to a lack of different cofactors in the medium used. It is also probable that the unnatural process of hypotonic shock to prepare the envelope membranes markedly affects the enzyme involved in the cyclization reaction.

Geranylgeraniol (C$_{20}$-alcohol) and geranylgeranylpyrophosphate were shown synthesized from isopentenylpyrophosphate by a recombined system of chloroplast envelope and chloroplast extract (stroma) [15]. In addition, the reaction products were discharged into the lipid phase of the envelope membranes. Consequently, these results suggest strongly that the enzymatic prenylation which forms Me-6-prenylquinols from homogentisic acid and a C$_{20}$-alcohol (PP) is also located in the envelope fraction. Our preliminary results indicate that the homogentisate decarboxylase–polyprenyltransferase activity is also localized in the envelope membranes.

It seems that the plastid envelope membranes contain the complete sequence of α-tocopherol biosynthesis. Under these circumstances, during thylakoid biogenesis, massive transport of α-tocopherol molecules should occur between the envelope membranes and the thylakoids. A possible solution of this problem may be membrane flow, as proposed [16]. In this case, the α-tocopherol synthesized in the envelope could diffuse laterally very rapidly within a transient membrane network to the outer surface of the thylakoid membranes. For example, observations made during electron microscopic studies of developing plastids (reviewed [1]) indicate that the inner mem-

brane of the plastid envelope invaginates and forms discrete vesicles which could then fuse with the growing internal membrane system.

Acknowledgements

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