RESEARCH COMMUNICATION

Tocopherol synthesis from homogentisate in *Capsicum anuum* L. (yellow pepper) chromoplast membranes: evidence for tocopherol cyclase

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The present study shows for the first time appreciable tocopherol cyclase activities in plastidial membrane preparations of *Capsicum annuum* L. (yellow pepper) fruits. When chromoplast membranes from yellow peppers were incubated with [^{3}H]homogentisate and phytyl pyrophosphate under strictly reducing conditions, all biosynthesis precursors were labelled. The main labelling was found in γ-tocopherol. These observations contradict the hypothesis that assigns a rate-limiting function to tocopherol cyclase in plastidial α-tocopherol biosynthesis. The stoichiometry of α-tocopherol, 2,3-dimethylphytylquinol and γ-tocopherol formation and the inhibition of α-tocopherol synthesis by increasing γ-tocopherol concentrations suggests the regulation of this pathway by its precursors.

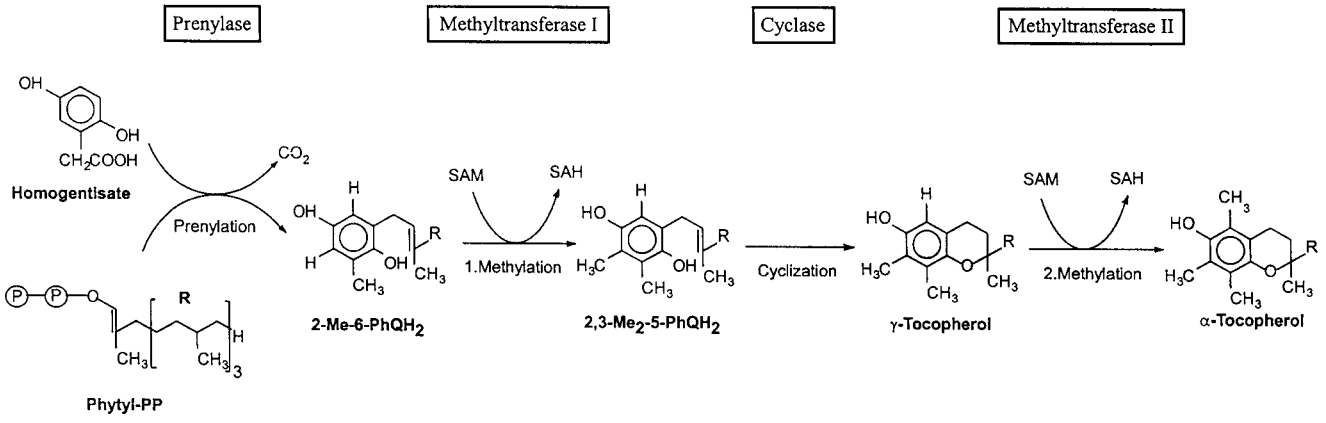
Key words: homogentisate, phytyl pyrophosphate, vitamin E.

INTRODUCTION

α-Tocopherol (commonly known as vitamin E) is the most important lipophilic radical-chain-breaking antioxidant in living tissues. It also participates in the stabilization of biological membranes. The absence of vitamin E in membranes could make them highly permeable and therefore vulnerable to degradation. Vitamin E seems also to influence other important biophysical membrane characteristics, such as fluidity, in a manner similar to that of cholesterol [1].

Earlier investigations showed that the accumulation of carotenoids during chloroplast-to-chromoplast differentiation in the fruit pericarp of *Capsicum annuum* L. (yellow pepper) is accompanied by an increasing α-tocopherol content [2,3]. The enzyme mechanism of the final steps in α-tocopherol biosynthesis was elucidated by tracer experiments using [^{3}H]homogentisate or *S*-adenosyl[*methyl*-^{14}C]methionine ([^{14}C]SAM) [4–6] (Scheme 1). In contrast with these findings, the methylation of constituents of the α-tocopherol pathway in chromoplasts from yellow *Capsicum* fruits with [^{14}C]SAM occurred even in the absence of exogenous precursors [7]. About 80% of the label was found in 2,3-dimethylphytylquinol and 20% in α-tocopherol, while γ-tocopherol formation was at the detection limit, which agrees with the results of previous investigations [4].

Both methylation capacities have been recently separated by fractionation of carefully shocked chromoplasts on continuous sucrose gradients [8]. We suggest, therefore, that α-tocopherol-biosynthetic enzymes in these chromoplasts are localized on different domains (peripheral or integral) of chromoplast membranes. The distinct membrane location of these enzymes and the hydrophobic character of the tocopherol cyclase [9], therefore, requires distinct assay conditions in chromoplast membrane preparations.



Scheme 1 α-Tocopherol synthesis from homogentisate and phytyl-PP

Abbreviations: SAH, *S*-adenosylhomocysteine; 2-Me-6-PhQH$_2$: 2-methyl-6-phytylquinol; 2,3-Me$_2$-5-PhQH$_2$, 2,3-dimethyl-5-phytylquinol.

Abbreviations used: phytyl-PP, phytyl pyrophosphate; SAM, *S*-adenosylmethionine; SAH, *S*-adenosylhomocysteine; 2-Me-6-PhQH$_2$: 2-methyl-6-phytylquinol; 2,3-Me$_2$-5-PhQH$_2$, 2,3-dimethyl-5-phytylquinol.

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The present study describes suitable incubation conditions for measuring α-tocopherol cyclase activities in *Capsicum* chromoplast membranes and produces findings that refute the hitherto existing hypothesis which assigns a rate-limiting function to this enzyme [4].

MATERIALS AND METHODS

Plant material

Mature *Capsicum annuum* L. fruits of the yellow variety were obtained from the local market.

Isolation of broken chromoplasts

Chromoplasts were isolated from 2 kg of *Capsicum annuum* fruits.

Broken chromoplasts were prepared from intact chromoplasts isolated as described by Arango and Heise [7]. The chromoplast pellet resulting from the centrifugation in shock buffer was resuspended in the same medium for the enzymic assays.

Analytical methods

Chemicals

[^{3}H]Homogentisate (sp. radioactivity 28 Ci/mmol) was purchased from Amersham International, Amersham, Bucks., U.K. Unlabelled homogentisate was provided by Fluka, Buchs, Switzerland, and phytyl pyrophosphate (phytyl-PP) from the Polish Academy of Sciences, Warsaw, Poland.

[^{3}H]Homogentisate was reduced for 4 min with solid $NaBH_4$ and immediately used for the enzyme assay.

Standards for the chromatographic analysis were synthesized as described by Soll [10].

Reaction mixtures

Standard incubation mixtures (final vol. 0.5 ml) contained 50 mM Tricine/NaOH, pH 7.7, 4 mM $MgCl_2$, 0.1 mM phytyl-PP, 3.6 μM [^{3}H]homogentisate, 40 μM unlabelled homogentisate, 50 μM SAM or 0–0.72 mM γ-tocopherol. The reaction was started by addition of chromoplast membranes with 0.7–1 mg of protein, determined by the method of Bradford [11]. The enzyme reaction was carried out for 2 h at 25 °C, then stopped with a mixture of chloroform/methanol (1:2, v/v) to give a monophasic solution. NaCl solution (1 ml; 0.9 %) was added with vigorous shaking. The organic layer containing the prenylquinones was evaporated under N_2, streaked on a silica-gel-G-25 chromatoplate and developed with light petroleum (b.p. 40–60 °C/diethyl ether (10:1, v/v).

An aliquot of standard compounds corresponding to the expected reaction products was added to the mixture as a carrier.

The radioactive areas were detected by radioscanning, scraped off the plate and dissolved in 10 ml of scintillation fluid after addition of 0.6 ml of Scint-Gel plus (Packard) [12].

RESULTS AND DISCUSSION

Chromoplast membranes of yellow *Capsicum* fruits were incubated with [^{3}H]homogentisate and phytyl-PP under strictly reducing conditions. In contrast with earlier investigations with chloroplasts [4,5] the whole reaction sequence of α-tocopherol synthesis was labelled. The highest radioactivity was measured in γ-tocopherol, which points to a remarkable cyclase activity.

For the above experiments, [^{3}H]homogentisate was reduced with solid $NaBH_4$ immediately before use in order to maintain the reactive quinol stage. Thus reducing conditions resulting from $NaBH_4$ might be responsible for the protection of the freshly synthesized prenylation products against oxidation and for the maintenance of the tocopherol cyclase reaction centre in the active form (i.e. the reduced stage).

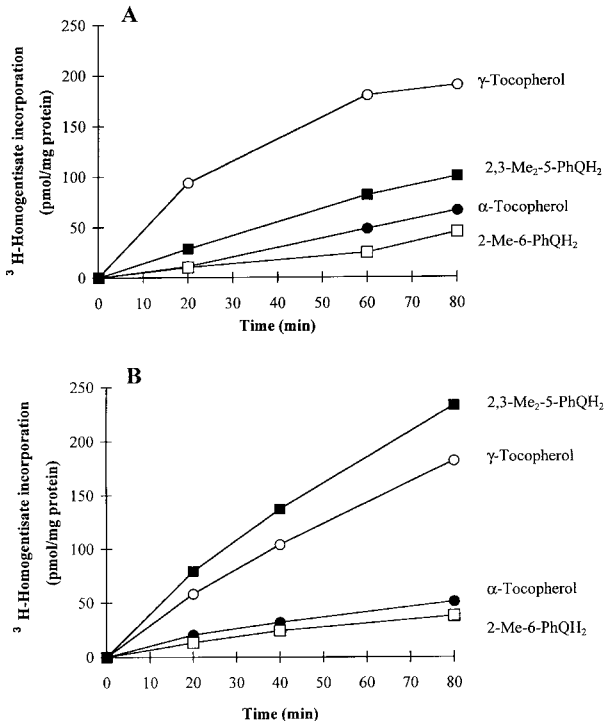

Figure 1 Kinetics of incorporation of α-tocopherol- synthetic-pathway products after prenylation of [^{3}H]homogentisate with phytyl-PP by chromoplast membranes from *Capsicum annuum* fruits

(**A**) Absence of SAM; (**B**) presence of SAM. For abbreviations, see Scheme 1.

An investigation into the mechanism of tocopherol cyclase has recently shown the enzyme to catalyse the stereospecific chromanol-ring closure of 2,3-dimethylphytylquinol to form γ-tocopherol [9,13] in the cyanobacterium (blue-green alga) *Anabaena variabilis* Kuetzing. For optimal turnover the authors converted the hydrophobic substrate in a water-soluble inclusion complex that was kept in the reduced stage by ascorbic acid during incubation.

In the present study, the initiation of tocopherol biosynthesis with water-soluble homogentisate avoided the solubility problems of the hydrophobic precursors and therefore facilitated their incorporation into chromoplast membranes [10]. Stocker [14] showed that weak reducing agents are suitable only for maintaining α-tocopherol-biosynthetic substrates in their reduced form, while strong reductants are necessary to keep the reaction centre of the tocopherol cyclase in its active form. Stocker further suggested that the –SH groups of the reaction centre might be essential for the cyclase activity. Beyer et al. [15] found similar effects for the cyclization of lycopene in chromoplast membranes from daffodil (*Narcissus pseudonarcissus*), which proceeded only under anaerobic conditions.

The observations emphasize the importance of suitable reducing conditions for a successful demonstration of tocopherol cyclase activity in plastidial membranes and may explain the lack of activities in earlier investigations.

The formation of γ-tocopherol (Figure 1A) shows, for the first time, the existence of tocopherol cyclase activities in plastid

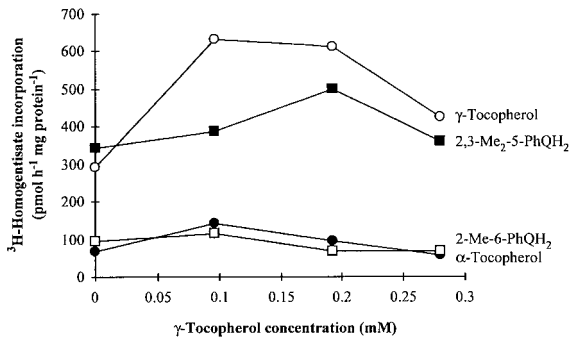

Figure 2 Dependence of tocopherol biosynthesis from [^{3}H]homogentisate and phytyl-PP on the concentration of γ-tocopherol

For abbreviations, see Scheme 1.

membranes of higher plants that are comparable with those of the *Anabaena* enzyme [9]. The methylation activities following the phytylation of [^{3}H]homogentisate were also found to be similar to those observed when [^{14}C]SAM was used [7]. The comparably low extent of labelling in the direct prenylation product of homogentisate (i.e. 2-methylphytylquinol) suggests its rapid turnover by the subsequent SAM-dependent methylation to form 2,3-dimethylphytylquinol [16], which is immediately followed by the cyclization to form γ-tocopherol. The decreased α-tocopherol formation under these conditions (Figure 1) was further increased when the endogenous levels of γ-tocopherol in chromoplast membranes were exogenously supplemented, as shown in Figure 2. In accordance with the incorporation of [^{14}C]SAM [7], the presence of SAM during the phytylation of [^{3}H]homogentisate led to an accumulation of radioactivity in the first methylation product (2,3-dimethylphytylquinol), while α-tocopherol showed only 20% of this label (Figure 1B). Independently of the labelled substrate ([^{14}C]SAM or [^{3}H]homogentisate), total tocopherol biosynthesis by chromoplast membranes was stimulated most (usually by a factor of 2) by the exogenous supply of γ-tocopherol concentrations (up to 100 μM). The constant ratios in the formation of 2,3-dimethylphytylquinol to 2-methylphytylquinol and of γ- to α-tocopherol from [^{3}H]homogentisate and phytyl-PP under different incubation conditions (in the presence or absence of SAM respectively) (Figures 1A and 1B) and the inhibition of tocopherol biosynthesis by higher γ-tocopherol concentrations (Figure 2) suggest a control function of both substrates in this pathway. However, further research has to be done to elucidate the mechanism of regulation of α-tocopherol biosynthesis as well as cyclase activity in higher plants.

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