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INHIBITION OF GERANYLGERANYL DIPHOSPHATE SYNTHESIS IN *IN VITRO* SYSTEMS

ANDREA STEIGER, URSULA MITZKA-SCHNABEL, WERNER RAU, JÜRGEN SOLL and WOLFHART RÜDIGER

Botanisches Institut der Universität München, Menzinger Str. 67, 8000 München 19, W. Germany

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Key Word Index—*Avena sativa*; Gramineae; *Cucurbita pepo*; Cucurbitaceae; etiolated seedlings; endosperm; mevalonic acid; isopentenyl diphosphate.

Abstract—The incorporation of [^{14}C]mevalonate and [^{14}C]isopentenyl diphosphate into geranylgeranyl diphosphate was investigated in *in vitro* systems from *Cucurbita pepo* (pumpkin) endosperm and from *Avena sativa* etioplasts. Mevalonate incorporation was effectively inhibited in the pumpkin system by geranylgeranyl diphosphate and geranylgeranyl monophosphate but less effectively by phytyl diphosphate or inorganic diphosphate. Membrane lipids, geranyllinalool, or lecithin enhanced mevalonate incorporation in the *Cucurbita* system. Incorporation of isopentenyl diphosphate was also enhanced by lecithin and inhibited by geranylgeranyl diphosphate in the *Cucurbita* system. No lipid enhancement was found in the *Avena* system; inhibition by GGPP required a much higher GGPP concentration than in the *Cucurbita* system.

INTRODUCTION

The biosynthesis of GGPP has received considerable attention because it is the precursor of the biologically important diterpenes (e.g. gibberellins, phytol side chain of chlorophylls, phylloquinone, tocopherol) and tetraterpenes (carotenoids). Systems for *in vitro* synthesis of GGPP have been described for various organisms [1–11]. The biosynthetic pathway from MVA to GGPP is catalysed by several enzymes (MVA–kinase, 5-P MVA kinase, IPP isomerase, GG synthase) which are all soluble [2, 6, 8, 10, 12]. The last enzyme (GG synthase) was purified from a bacterial [2] and a plant source [6]; it catalyses the reaction of IPP with either DMAPP, GPP or FPP to GGPP. In none of these papers was the regulation of GGPP biosynthesis studied.

The biosynthesis of phytol which is closely connected to that of GGPP [13] is inhibited by PhPP [14]. This effect is attributed to feed-back inhibition because it has been demonstrated that mevalonate kinase is inhibited by PhPP [15]. PhPP and GGPP are very similar and therefore both are accepted by enzymes like chlorophyll synthetase [16]. GGPP is present in etiolated and green seedlings in about ten fold higher concentration than PhPP [17, 18]. The GGPP pool of etiolated oat seedlings which is depleted during onset of chlorophyll biosynthesis seems to be reestablished only to a well-defined limiting value [18]. In pumpkin endosperm, only a limited accumulation of GGPP is detected *in vitro* [U. Mitzka-Schnabel and W. Rau, unpublished results].

In the study reported in this paper, we have investigated whether GGPP is able to regulate its own biosynthesis in *in vitro* systems from etiolated oat seedlings and from *Cucurbita* endosperm.

RESULTS

A cell-free system (35 KS) from the endosperm of immature seeds of *C. pepo* was able to convert MVA into several lipid compounds (Table 1). The product pattern shows that the bulk of isoprenoid lipids were derived from GGPP (kaurene, GG, carotenes) with only a small part of the lipids being derived from FPP (farnesol, squalene, sterols). In the presence of AMO 1618 the incorporation of MVA was strongly reduced. Although the lipid pattern was shifted by AMO 1618 towards the FPP products the larger part of the isoprenoid lipids were still derived from GGPP. It was to be expected, therefore, that GGPP rather than FPP was the main water-soluble product derived from MVA.

The production of [^{14}C]GGPP from [^{14}C]MVA in the *Cucurbita* system was verified by isolation of GGPP by the method of Benz *et al.* [17] which basically comprises five chromatographic steps. It was more convenient, however, to hydrolyse GGPP in the aqueous layer after pre-extraction of lipophilic compounds with diethylether and then determine free GG and GL (see Experimental). This simple, indirect determination of GGPP could be used in this case since MVA metabolism in this *in vitro* systems does not lead to the production interfering compounds.

The GGPP synthesizing enzymes were localized in the 115 000 *g* supernatant (Fig. 1). The slightly higher enzyme activity in the 35 000 *g* supernatant might have been due to the presence of small amounts of enzymes in the membrane fraction but could equally as well be due to

Abbreviations: GGPP, geranylgeranyl diphosphate; PhPP, phytyl diphosphate; FPP, farnesyl diphosphate; MVA, mevalonic acid; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; GL, geranyllinallool; Hepes, N-2-hydroxyethylpiperazine-N-2-ethanesulfonic acid; AMO 1618, 2-isopropyl-4-dimethylamino-5-methylphenyl-1-piperidin-carboxylate-methylchloride; 35 KS, 115 KS, 35 000 *g* or 115 000 *g* supernatant.

Table 1. Incorporation of [^{14}C]MVA into the unsaponifiable lipid fraction and the product pattern in the 35000 *g* supernatant (3.0 ml) prepared from *Cucurbita* endosperm

Incubation conditions	Incorporation [nmol and (%)]					
	Total	Farnesol	Squalene + sterols	GG	Kaurene	Carotenes
−AMO 1618	13.40	0.01	0.13	0.43	12.76	0.04
	(100.0	0.1	1.0	3.2	95.2	0.3)
+AMO 1618	1.98	0.02	0.26	0.39	1.19	0.03
	(100.0	1.0	15.0	19.9	60.4	1.7)

In the presence of AMO 1618 (2 μM), the conversion of GGPP to kaurene was reduced. The *in vitro* system (final volume 3.75 ml) was incubated after addition of 229 nmol (91.4 kBq) [^{14}C]MVA at 27° for 150 min.

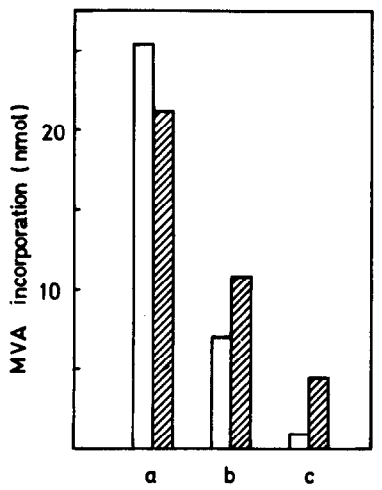

Fig. 1. Incorporation of [^{14}C]MVA into GGPP *in vitro*. The 35000 *g* supernatant (blank columns) or the 115000 *g* supernatant (hatched columns) were prepared from *Cucurbita* endosperm. Each sample containing 3 ml 35 KS or 115 KS fraction of endosperm was incubated in the presence of 498 nmol (83.5 kBq) [^{14}C]MVA ± GGPP (final volume 3.75 ml) at 27° for 150 min. The incubation was started by addition of the endosperm fraction. a, control without GGPP; b, addition of 8.25 nmol GGPP (final conc. 2.2 μM); c, addition of 82.5 nmol GGPP (final conc. 22 μM).

enzyme activation by membrane constituents (see below). In both the 115000 *g* and 35000 *g* supernatants, the addition of GGPP reduced the incorporation of MVA into GGPP (Fig. 1). This effect was more pronounced in the 35000 *g* supernatant. This difference cannot be explained in a straightforward way since only in experiments with the 35000 *g* supernatant was the cyclase inhibitor AMO 1618 added to reduce further reactions of newly formed GGPP [10, 19]. Addition of this inhibitor was not required in experiments with the 115000 *g* supernatant because most of the GGPP consuming membrane-bound enzymes had been removed. All subsequent experiments were performed with the 115000 *g* supernatant.

In order to test the specificity of the inhibitory effect of GGPP upon its own biosynthesis, we also tested some structural analogues of GGPP (Table 2). GGMP had about the same effect as GGPP probably because of a kinase present in the soluble fraction which phosporylates GGMP to GGPP as described for oat etioplasts [16]. Less inhibition was found with PhPP whereas FPP showed a slight stimulation of GGPP synthesis. These results clearly demonstrated the structural specificity of GGPP inhibition. There may be several reasons for the stimulatory effect of FPP which *inter alia* can act as a C-15 precursor of GGPP. In order to elucidate the individual influence of the hydrophilic and the lipophilic part of GGPP, PhPP and FPP, we investigated the effect of inorganic pyrophosphate (PP_i) and the tertiary alcohol GL which does not react with the kinase. The primary alcohol GG was not tested since like GGMP it reacts with the kinase [16]. Addition of PP_i showed a slight but significant inhibition whereas GL exhibited a pronounced stimulation of GGPP biosynthesis from MVA. Apart from the specific effect of GGPP, a general requirement for this inhibition seems to be the pyrophosphate group whereas the lipid part of these molecules seems to stimulate rather than to inhibit GGPP biosynthesis.

Lipid activation of membrane enzymes is a well-known phenomenon (review in ref. [20]). The possibility of lipid activation of GGPP biosynthesis was substantiated by the following experiment (Table 3). Lipids extracted from the membrane pellet obtained by centrifugation of the 35 KS

Table 2. Effect of GGPP and structural analogues upon the biosynthesis of GGPP from [^{14}C]MVA in the 115000 *g* supernatant from *Cucurbita* endosperm

Compound	Concentration (μM)	MVA incorporation (nmol)	MVA incorporation (% control)
—	—	5.52	100.0
GGPP	22	1.18	21.4
GGMP	22	0.76	12.7
PhPP	22	3.21	58.1
FPP	22	7.33	132.8
PP_i	150	3.58	64.8
GL	150	10.50	190.2

Table 3. Effect of lipids upon the biosynthesis of GGPP from [^{14}C]MVA in the 115000 *g* supernatant from *Cucurbita* endosperm. For conditions see Fig. 1

Compound	Concentration (μM)	MVA incorporation (nmol)	MVA incorporation (% control)
—	—	4.1	100
'Membrane pellet'*	†	12.1	295
Lipids from the 'membrane pellet'	†	28.1	685
Lecithin	150	31.2	760
	600	30.1	734

*The 'membrane pellet' was prepared by centrifugation of 9.0 ml of the 35000 *g* supernatant at 115000 *g* for 120 min.

†The 'membrane pellet' from 9.0 ml 35 KS fraction (see *) or the lipids extracted therefrom, respectively, were added to the 115000 *g* supernatant (3.0 ml).

fraction at 115000 *g* strongly enhanced GGPP synthesis. The membrane pellet itself showed less stimulation probably because substantial amounts of GGPP were further metabolized due to the lack of AMO 1618 in this experiment (see [10]). The pellet lipids could be substituted for by lecithin. The stimulation by lecithin was concentration dependent up to a saturation level (Fig. 2).

Under conditions of full enzyme activation, i.e. saturation of lecithin, the influence of GGPP upon its own biosynthesis was reassayed. As shown in Fig. 3, the inhibition of GGPP was more pronounced in the presence than in the absence of lecithin. These data clearly show that GGPP has, besides its specific inhibitory effect, a stimulatory 'lipid' effect.

The biosynthetic pathway from MVA to GGPP comprises several enzymes (MVA-kinase, 5-P MVA kinase, IPP isomerase, GGPP synthase). To determine whether the kinases or the isomerase and synthase respond to GGPP inhibition, the incorporation of IPP into GGPP was investigated (Table 4). GGPP formation from IPP was strongly inhibited by GGPP and enhanced by lecithin in the pumpkin system. Incorporation of IPP into GGPP only requires the enzymes IPP isomerase and GGPP synthase. Our results demonstrate that the site of regulation of overall enzyme activity is at these enzymes.

GGPP formation from IPP had also been demonstrated in chloroplasts [8, 9] and indirectly in etioplasts [21]. We therefore investigated GGPP biosynthesis from IPP in broken etioplasts in more detail. Substrate saturation was reached at 0.14 mM IPP (data not shown). The optimal incubation time was 30 min. Prolonged incubation decreased incorporation of IPP into GGPP probably due to further metabolization of GGPP. The influence of GGPP was tested at 0.14 mM IPP and an incubation time of 30 min (Table 5). No significant difference from the control value was detected between 2.75 and 54.5 μM GGPP. IPP incorporation was reduced by 50% only at the highest GGPP concentration tested (170 μM). Lecithin did not enhance incorporation of IPP into GGPP in the oat etioplast system (Table 5).

DISCUSSION

GGPP an important precursor of di- and tetraterpenes can be labelled from ^{14}C-labelled MVA or IPP using *in vitro* systems from oat etioplasts or *Cucurbita* endosperm. In order to find optimal incubation conditions, a knowledge of the characteristics of regulation of GGPP synthesis in these systems is essential. The results of such investigations might also give an indication of how GGPP synthesis is regulated *in vivo*.

The properties of the oat etioplast system are clearly different from the properties of the pumpkin endosperm system. The last enzymes of GGPP synthesis are not activated by lipids like lecithin. Inhibition by the product occurs only at a high concentration (170 μM) of GGPP. This concentration is presumably higher than the physiological concentration in oat tissue which can be estimated roughly as follows:

The amount of GGPP has been estimated to be 16 nmol/g fr. wt [17] which is nearly identical with the protochlorophyllide content of 15 nmol/g fr. wt [22]. From data obtained with primary leaves of *Phaseolus vulgaris* [23], the etioplast volume can be calculated to be about 0.09 ml per g fr. wt. Such data are not available for primary leaves of oat but it can be calculated from the data

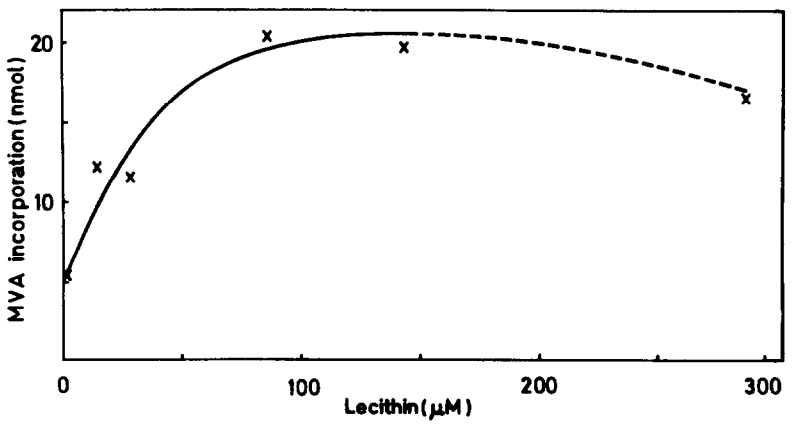

Fig. 2. Enhancement of [^{14}C]MVA incorporation into GGPP in the 115000 *g* supernatant prepared from *Cucurbita* endosperm by lecithin. Conditions of incubation as in Fig. 1.

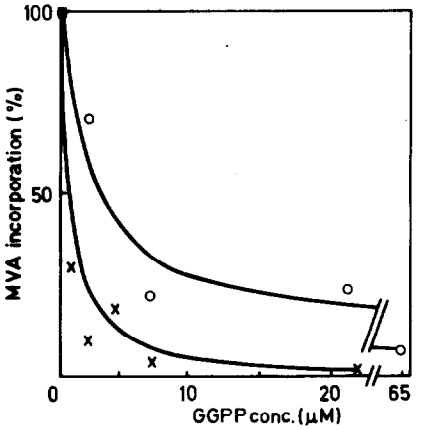

Fig. 3. Inhibition of [^{14}C]MVA incorporation into GGPP in the 115000 *g* supernatant prepared from *Cucurbita* endosperm by GGPP. O—O, without lecithin, 495 nmol (82 kBq) [^{14}C]MVA were added per sample; ×—×, with lecithin (350 nmol per sample, final conc. 93 μM), 468 nmol (37 kBq) [^{14}C]MVA were added per sample. Conditions of incubation as in Fig. 1.

Table 4. Effect of GGPP and lecithin upon the biosynthesis of GGPP from [^{14}C]IPP in the 115000 *g* supernatant from *Cucurbita* endosperm

Compound	Concentration (μM)	IPP incorporation (nmol)	IPP incorporation (% control)
—	—	12.5	100.0
GGPP	2.2	4.8	38.5
GGPP	22.0	1.4	10.9
GGPP/Lecithin	2.2/93	14.2	115.6
GGPP/Lecithin	22.0/93	3.8	30.4

Conditions as in Fig. 1, except that 468 nmol [^{14}C]IPP (37 kBq) per sample were added instead of [^{14}C]MVA.

Table 5. Incorporation of [^{14}C]IPP into GGPP in broken etioplasts from *A. sativa*: influence of GGPP and lecithin

Compound	Concentration (μM)	Incorporation into GGPP (% control)
Control	—	100
GGPP	2.75	96.8
	5.5	117.3
	27.5	97.8
	54.5	104.5
	170.0	50.3
Lecithin	116	85.3

Each sample (final vol 3.0 ml) from the etioplast pellet derived from 10 g (fr. wt) primary leaves at 26° for 30 min.

of Lütz [24] that the total plastid volume per mesophyll cell is comparable in oat (220 μm^3) and bean (190 μm^3, recalculated from ref. [23]). Assuming an etioplast volume of 0.09 ml per g fr. wt, an overall concentration of 177 μM GGPP can be calculated if the GGPP is restricted to the plastid. However, because GGPP can easily penetrate the plastid membrane [25, 26] it will be distributed within a much higher volume, i.e. the actual concentration of GGPP in the etioplasts will be much less than this.

The inefficient inhibition of GGPP formation should result in a high accumulation of GGPP, a property which could be used for preparation of labelled GGPP. However, oat etioplasts contain soluble and membrane-bound phosphatases which produce considerable amounts of free GG from GGPP, up to 30 nmol GGPP/g fr. wt were hydrolysed [G. Jung and J. Benz, unpublished]. The production of large amounts of free GG instead of GGPP was also described for chloroplasts [8, 9].

The *in vitro* system of *Cucurbita* does not hydrolyse GGPP to any great extent; free GG can be detected only in traces. GGPP synthesis is enhanced 4–7 fold (Fig. 2 and Table 3) by polar lipids like lecithin and is inhibited some 80–97% by GGPP (Figs 1 and 3, Tables 2 and 4). The inhibition is specific for GGPP since structural analogues of GGPP show either a decreased inhibition or no inhibition (Table 2). Therefore, it can be assumed that this inhibition is due to 'product' rather than 'feed-back' inhibition since the last enzymes of the biosynthesis chain are concerned (Table 4). We assume that the last enzyme (GGPP synthase = prenyltransferase) is inhibited rather than IPP isomerase because a strong binding of GGPP to GGPP synthase had been described [4, 6] but this question is in need of further investigation.

EXPERIMENTAL

Preparation of the Cucurbita system. Fruits of *Cucurbita pepo* L. ('Gelber Zentner') were harvested when fully grown but still pale, from plants cultivated in the Botanical Garden. From the immature seeds the tips were cut off and the semi-liquid endosperm was squeezed out and frozen immediately at −20°. 15–20 ml endosperm was obtained per fruit; it could be stored frozen for up to 2 years without great loss of activity.

Preparation of the *in vitro* system provided by the semi-liquid endosperm of maturing seeds of pumpkin was essentially as in refs [10, 27]. The thawed liquid endosperm was homogenized gently in a Potter–Elvehjem homogenizer and centrifuged either at 35000 *g* for 20 min or at 115000 *g* for 2 hr. A centrifugation time of 2 hr was necessary to remove the bulk of the membranes. Aliquots (3 ml, each) of the resulting supernatants (35 KS or 115 KS, each containing *ca* 18 mg protein) were used as enzyme sources for the synthesis of [^{14}C]GGPP in the presence of [^{14}C]MVA or [^{14}C]IPP.

Incubation. The standard incubation mixture (total vol. 3.75 ml) contained: 2.6 mM $MgCl_2$, 0.5 mM $MnCl_2$, 0.5 mM NADPH, 5.3 mM ATP, an ATP-regenerating system (0.5 mM PEP; 100 μg pyruvate kinase), 26.3 mM KF, 0.13 mM [2-^{14}C]MVA (0.168 or 0.079 kBq/nmol) respectively [^{14}C]IPP (0.079 kBq/nmol). DL-MVA lactone was converted prior to incubation to its K-salt with aq. KOH according to ref. [28]. Finally 3 ml 35 KS (natural pH 6.5) or 115 KS fraction of *Cucurbita* endosperm homogenate was added. When the 35 KS-fraction was used as enzyme source the assay additionally contained 1 or 10 μM AMO 1618. Incubations were performed in 18 ml gas wash flasks for 150 min at 27° under weak aeration. The incubations were terminated by heating the samples to 70° for 10 min. Any variations in the incubation conditions are given in the Results.

Lipids were extracted with $CHCl_3$ from the 'membrane pellet' obtained by centrifugation of the 35 KS fraction at 115000 *g* for

2 hr (see Table 3). After addition of 50 μl 1% Tween 20, the lipid soln was evaporated to dryness under a stream of N_2 and the incubation mixture was added. For lipid dispersion the mixture was sonicated (Branson, sonic power, 3 times for 5 sec). Lecithin ($CHCl_3$-soln) was added in the same way.

Preparation of the Avena *system.* Seedlings of oat (*Avena sativa* L. cv. Pirol, Bayerische Futter- und Saatbau GmbH, München) were grown on moist vermiculite at 27–28° for 7 days in the dark. The primary leaves (100 g) were homogenized with 200 ml buffer (pH 7.5) containing 0.45 M D-(−)-sorbitol, 0.2% bovine albumin, 1 mM $MgCl_2$, 1 mM KH_2PO_4, 50 mM N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), 1 mM $NaNO_3$, 1 mM NaF and 1 mM ascorbic acid. The homogenate was filtered through a nylon net (30 μm mesh). The filtrate was centrifuged for 20 min at 6000 *g*. The pellet which mainly consisted of etioplasts was suspended in 28 ml buffer (pH 7.5) which contained 50 mM HEPES, 4 mM KH_2PO_4, 10 mM $MgCl_2$, 10 mM NaF, 2 mM ascorbic acid, 0.5 mM $MnCl_2$, 1 mM ATP, 1 mM PEP, 1 mM NADPH and 0.1 mg pyruvate kinase. The etioplasts were disrupted at first in a Potter–Elvehjem homogenizer and then by exposure to ultrasound (Branson, sonic power, 3 times for 5 sec).

Incubation. Aliquots of the suspension (2.8 ml each) were incubated at 26° for 30 min after addition of [^{14}C]IPP and, in some experiments, GGPP, lecithin and other compounds (see Table 5). The final vol was 3.0 ml. The incubation was terminated by heating the samples to 70° for 10 min. All steps were performed under dim-green safelight.

Purification of GGPP was essentially as in ref. [13]. Non-labelled GGPP (0.5 mg) was added to each sample. For hydrolysis of GGPP, the samples were acidified to pH 1.0 with H_2SO_4, heated to 85° for 10 min, and, after cooling, extracted with Et_2O (3 × 3 ml) [13]. The identities of the radioactively labelled products with added GG and GL were determined by TLC on silica gel with C_6H_6–EtOAc (3:1). The radioactive bands in question were recovered from the TLC-plates by repeated extraction with MeOH and the label was quantitatively determined in a liquid scintillation counter. The data presented in the tables and figures are corrected for GG/GL recovery and are given as nmol MVA or IPP converted to GGPP per incubation.

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