Substrate Specificity of cis-Prenyltransferase in Rat Liver Microsomes*

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Long chain cis-prenyltransferase in rat liver microsomes was studied using various allylic isoprenoid substrates. Microsomes could utilize trans-geranyl pyrophosphate, but not cis-geranyl pyrophosphate for polypropenyl pyrophosphate synthase. Both trans, trans-farnesyl pyrophosphate and trans,cis-farnesyl pyrophosphate were used as substrates with $K_m$ values of 24 and 5 $\mu$M, respectively. trans,trans,cis-Geranylgeranyl pyrophosphate could be used as substrate with an apparent $K_m$ of 36 $\mu$M. trans,trans,trans-Geranylgeranyl pyrophosphate was also utilized as substrate, but with a very low affinity. After pulse labeling for 4 min, using $[^3H]$isopentenyl pyrophosphate and trans,trans-farnesyl pyrophosphate, the only product formed was trans,trans,cis-geranylgeranyl pyrophosphate, which, upon chasing, yielded polypropenyl pyrophosphate. Independent of the nature of the substrate used, even in the case of polypropenyl 12-pyrophosphate and all-trans-nonaprenyl pyrophosphate, the chain lengths of the products were identical, i.e. polypropenyl pyrophosphates with 15–18 isopropyl residues. Microsomes were able to synthesize trans,trans-farnesyl pyrophosphate using trans-geranyl pyrophosphate as substrate. The results indicate that rat liver microsomes contain a farnesyl pyrophosphate synthase activity and that the reaction catalyzed by cis-prenyltransferase may consist of two individual steps, i.e. synthesis of trans,trans,cis-geranylgeranyl pyrophosphate and elongation of this product to long chain polypropenyl pyrophosphates.

cis-Prenyltransferase is known to catalyze the condensation reaction in which cis additions of isopentenyl pyrophosphate (IPP)1 to trans,trans-FPP lead to polypropenyl pyrophosphates of appropriate lengths (1). A number of reactions modifying the latter product give rise to dolichol and dolichol phosphate (2, 3). In contrast to the enzymes responsible for the production of FPP, cis-prenyltransferase has been studied to only a limited extent, in spite of the fact that it has a central role in the synthesis of polyprenol lipids.

cis-Prenyltransferases have been isolated and characterized from several bacterial systems (4–6). Using enzyme preparations from hen oviduct, rat liver, rat brain, Ehrlich ascites tumor cells, and spermatogenic cells, certain properties of the enzyme activity have been analyzed using FPP and GGPP as substrates (7–12). In most systems the enzyme was found to be membrane-bound, but activities have also been described in the cytosol (13). The mammalian enzyme has proven difficult to purify, and all attempts so far have been unsuccessful (9, 14). In earlier investigations the enzyme activity in rat liver preparations was low, in spite of long incubation times (10–20 h). These investigations pointed out that the incubation conditions used were of utmost importance for the nature and amount of the products formed. In recent studies Ogura and co-workers (15) analyzed the condensation reactions using FPP, GGPP, and geranylgeranyl pyrophosphate as substrates. After a 17-h incubation of microsomes with FPP and IPP, a product, identified as trans,trans,cis-GGPP, was found in addition to long chain polypropenyl-PP. The question was raised as to whether this product was an intermediate in the biosynthesis of long chain polypropenyl pyrophosphates. These investigators also showed that the enzyme could utilize GGPP as substrate, as well as certain other isoprenoid pyrophosphates, but the affinity of the enzyme for these substrates was not investigated. The low enzyme activity, requiring long incubation times, made it difficult to perform further kinetic studies.

Analysis of cis-prenyltransferase activity requires the presence of high concentrations of detergent. It was recently found that SCP2 both stimulates the enzyme activity and results in the synthesis of polyisoprenyl-PP with natural chain lengths, i.e. 18–21 isopropyl residues in the absence of detergent (16).

The substrate for cis-prenyltransferase in vivo is believed to be trans,trans-FPP, which is synthesized by a cytosolic FPP synthase. This latter enzyme has been studied in great detail in rat and in human, as well as in several procaryote systems (17). According to the established view cytosolic FPP is the common substrate for the processes by which cholesterol, dolichol, and ubiquinone are synthesized.

It has been shown that a soluble enzyme in both pig liver and in various procaryote systems is able to synthesize trans,trans,trans-GGPP (18, 19). The recent finding that a number of proteins participating in the regulation of cell growth is activated by isoprenylation with trans,trans-farnesol or trans,trans,trans-geranylgeraniol has initiated extensive studies on these substances (20–22). Since isoprenylation of proteins is a cytosolic process, the presence of these two isoprenoid substrates in this compartment has attained new importance (23). The question of a possible compartmental-
zation of the FPP and GGPP pools has not yet been investigated.

In the present study we have employed a number of substrates related to the mevalonate pathway in order to analyze the condensation reactions associated with cis-prenyltransferase. Our aim has been to characterize the substrate specificity of the enzyme, as well as to further examine the properties of the reactions involved.

MATERIALS AND METHODS

Chemicals—α-Unsaturated polyprenols were prepared from Sorbus aucuparia as described previously (24). Polypropenyl phosphate and poly-prenyl prophosphorylase were prepared according to Popjik et al. (25). Labeled isoprenol was prepared from commercial isoprenol (Jansen Chimica, Belgium) by oxidation to isopentenol, followed by reduction with [3H]sodium borohydride (Amersham, Great Britain, 16.5 Ci/mmol) according to Keenan and Krucek (26). This labeled isoprenol was subsequently phosphorylated in order to obtain isopentenyl phosphate (1.15 Ci/mmol). All unlabeled propenyl phosphate substrates were synthesized from the corresponding alcohols using the same method (25). The geraniol, farnesol, and geranylgeraniol isomers were a gift from Dr. T. Takegawa of the Kuraray Co., Okayama, Japan. All-trans-nonaprenol was prepared from tobacco leaves of the Virginia type, and polyprenol-12 was prepared from Rhus typhina as described previously (27).

Upon TLC in different solvent systems, all polyprenols and phosphorylated derivatives ran as single spots.

Isolation of Rat Liver Microsomes—Male Sprague-Dawley rats (180-200 g) were used without starvation. Isolation of the microsomal fraction was performed as described earlier (28). The microsomal pellet was suspended in 0.15 M Tris-HCl, pH 8.0, and recentrifuged at 105,000 × g, for 45 min in order to remove adsorbed cytosolic proteins.

Incubations—Enzyme activity was measured in a 300-μl incubation mixture containing 25 mM imidazole-Cl, pH 7.0, 1 mM MgCl₂, 100 mM KF, 0.9 mM dithiothreitol, 1.0% Triton X-100, 10 μM [3H]isoprenyl phosphate, and various amounts of the unlabeled propenylphosphorylated substrates. The reaction was started by the addition of 0.5-1.0 mg of microsomal protein and continued for 60 min at 37 °C. The reaction was stopped by the addition of 300 μl of 0.87 M KOH, and hydrolysis was subsequently performed at 100 °C for 30 min. Finally, the samples were supplemented with 1.5 ml of 2 M KCl and 300 μl of 1 M HCl.

Extraction of Lipids—The reaction mixture was extracted three times with 3 ml of diethylether/petroleum ether (1:1). The pooled organic phases were then washed three times with 2 ml of 0.9% NaCl. The washed organic phase was then transferred to a scintillation vial. The organic solvent was evaporated, the residue dissolved in 10 ml of Optiscint (LKB Scintillation Products, Sweden), and the radioactivity determined by scintillation counting.

When the reaction products were analyzed by TLC or HPLC, the washed organic extract was dried under N₂ and the residue dephosphorylated enzymatically according to the method of Wong and Lennarz (29). For TLC the dephosphorylated products were applied to a Silica Gel 60 plastic sheet (Merck) and developed with benzene/ethyl ether (1:1) and isopropyl alcohol (1:1). The plates were sprayed with EN'HANCE spray (Du Pont-New England Nuclear) and exposed to X-Omat AR film (Eastman Kodak, Rochester) at -80 °C. The radioactive spots were identified by comparison with unlabeled internal standards after visualization with iodine vapor.

The chain length distribution of the enzyme products was analyzed by reversed-phase HPLC using a Hewlett-Packard Hypersil ODS 3-μm C₁₈ column. A convex gradient was employed from the initial methanol/water (9:1) in pump system A to methanol/hexane/2-propanol (2:1:1) in pump system B at a flow rate of 1.5 ml/min with a program time of 35 min. The absorbance at 210 nm and the radioactivity flow detector (Radiomatic Instruments, Tampa, FL), respectively.

Chemical Measurements—Protein concentration was determined by the biuret procedure using bovine serum albumin as standard (30).

RESULTS

Products of the Transferase Reaction—Isolated microsomes were incubated with trans/trans-FPP and [3H]IPP, and the products were dephosphorylated and analyzed by reversed-phase HPLC. After a 4-min incubation, only one product eluting after approximately 2 min was observed in the chromatogram (Fig. 1A). The radioactivity in this peak comigrated with geranylgeraniol added as a standard. When the 4-min pulse was followed by a 20-min chase in the presence of a 1000-fold excess of unlabeled IPP, products eluting after 2 min as well as long chain polyprenols with retention times of 25-30 min were observed (Fig. 1B). When the chase period was extended to 120 min, no product eluting early could be observed, and all the radioactivity was associated with the long chain polyprenols (Fig. 1C). These results indicate that the product after a short incubation is GGPP, which can be metabolized to long chain products containing 15-18 isoprene residues.

The dephosphorylated products from a standard incubation, using FPP and [3H]IPP as substrates, were also analyzed on silica gel TLC in order to determine the trans/cis configuration of the short chain isoprenoid. The resulting autoradiogram revealed three radioactive spots (Fig. 2). One of these spots (RF, 0.75) comigrated with trans,trans,trans-geranylgeraniol, and the other spot (RF, 0.52) comigrated with long chain polyprenol standards. The third spot, migrating between the front and the polyprenol standard, corresponds to isoprenoid hydrocarbons produced during alkaline hydrolysis. Substrate Specificity—The substrate specificity of the microsomal cis-prenyltransferase was investigated in incubations with increasing concentrations of the different allylic substrates (Fig. 3). The substrate concentration is plotted against the total extractable radioactivity. trans-GPP could be utilized as substrate at low concentrations, in contrast to cis-GPP (Fig. 3A). At higher substrate concentrations cis-GPP could be used by the microsomes, but the only product was FPP (not shown). trans,cis-GPP was a better substrate than trans,trans-FPP, with enzyme saturation being achieved at 25 and 70 μM, respectively (Fig. 3B). This finding is

FIG. 1. HPLC chromatogram of the products formed during pulse-chase labeling of rat liver microsomes with [3H]IPP. Rat liver microsomes were incubated in the presence of [3H]IPP and trans,trans-FPP. The products formed were subsequently extracted, dephosphorylated enzymatically, and separated using reversed-phase HPLC. A, 4 min with [3H]IPP; B, a 4-min pulse followed by a 20-min chase; C, a 4-min pulse followed by a 120-min chase. The numbers above the peaks indicate the number of isoprene units and retention time of added isoprenoid standards.
**Substrate Specificity of cis-Prenyltransferase**

**Fig. 2.** Autoradiography of the products formed during incubation of rat liver microsomes with \[^{3}H\]IPP and trans,trans-FPP. The dephosphorylated reaction products were separated on silica gel TLC. After development, the plate was exposed on x-ray film for 48 h. The lines indicate the positions of trans,trans,trans- (t,t,t) and trans,trans,cis- (t,t,c) geranylgeraniol and long chain polyprenol (P) standards.

Interesting, since trans,trans-FPP is considered to be the natural substrate for this enzyme. At high substrate concentrations, both isomers of GGPP resulted in extensive formation of product. As expected, the trans,trans,cis isomer was used more efficiently than the trans,trans,truns isomer (Fig. 3C). It was not possible to saturate the enzyme with trans,trans,truns-GGPP which may indicate that this substrate is not utilized under in vivo conditions. Since microsomes could utilize trans-GPP for polyisoprenyl-PP synthesis in the absence of cytosol, the microsomal membranes appear to contain an FPP synthase.

The data from Fig. 3 were used to calculate $K_m$ and $V_{max}$ values for the different substrates, using Lineweaver-Burk analysis (Table I). Generally, FPP is bound with a higher affinity than is GGPP. The enzyme exhibits a higher affinity for trans,cis-FPP than for the trans,trans-FPP isomer, with the $K_m$ values being 5.2 and 24.3 $\mu$M, respectively. The $V_{max}$ values for trans,trans,cis- and trans,trans,trans-GGPP are high, i.e. 50,500 and 45,500 dpm/mg protein $\times$ h, respectively, but the $K_m$ values for both these GGPP isomers are higher than those for FPP. The kinetic constants for trans,trans,trans-GGPP are uncertain since saturation was not achieved and should only be used for comparison.

The nature of the products formed during incubations with the different substrates was analyzed on reversed-phase HPLC. With all substrates the product distribution was similar (Fig. 4). In all cases polyisoprenes containing between 15 and 18 isoprene units were detected, with the two dominating components having 16 and 17 isoprene units. This is the pattern usually obtained with various in vitro incubations in the presence of detergent. When trans-GPP was used as substrate the dominating peak was not associated with long chain polyprenols but appeared in the beginning of the chromatogram.

The fact that trans-GPP could be used as a substrate by microsomal cis-prenyltransferase in the absence of cytosol was unexpected, and, therefore, the reaction products analyzed in Fig. 4A were also investigated using silica gel TLC (Fig. 5). Two radioactive bands were observed in this system; the first ($R_F = 0.54$) comigrated with trans,trans-farnesol, and the second ($R_F = 0.88$) comigrated with long chain polyprenol standards.

**Elongation of Long Chain Polyprenyl-PP**—Since the products of the cis-prenyltransferase reaction using various sub-

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**Table I**

<table>
<thead>
<tr>
<th>Allylic substrate</th>
<th>$K_m$ $\mu$M</th>
<th>$V_{max}$ dpm/mg protein/h</th>
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</thead>
<tbody>
<tr>
<td>trans-GPP</td>
<td>ND*</td>
<td>ND</td>
</tr>
<tr>
<td>trans,cis-FPP</td>
<td>5.2</td>
<td>25,400</td>
</tr>
<tr>
<td>trans,trans-FPP</td>
<td>24.3</td>
<td>27,400</td>
</tr>
<tr>
<td>trans,trans,cis-GGPP</td>
<td>35.6</td>
<td>50,500</td>
</tr>
<tr>
<td>trans,trans,trans-GGPP</td>
<td>280</td>
<td>45,500</td>
</tr>
</tbody>
</table>

*Not determined.

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**Fig. 3.** Substrate specificity of rat liver cis-prenyltransferase. Microsomes were incubated with increasing amounts of various allylic substrates and saturating amounts of \[^{3}H\]IPP. The amount of product formed (determined as the total extractable radioactivity) was plotted against substrate concentration. A, trans- and cis-GPP; B, trans,trans- and trans,cis-FPP; C, trans,trans,trans- and trans,trans,cis-GGPP. The values are the means ± S.E of five independent experiments.
Substrate Specificity of cis-Prenyltransferase

FIG. 4. Distribution of polyisoprene products after incubation of rat liver microsomes with various allylic substrates. Microsomes were incubated with[^3H]IPP and the allylic substrates indicated. The reaction products were extracted, dephosphorylated, and subsequently separated using reversed-phase HPLC. A, trans-GPP; B, trans,cis-FPP; C, trans,trans-FPP; D, trans,trans,cis-GGPP; E, trans,trans,trans-GGPP. The radioactivity in the effluent was monitored using a radioactivity flow detector. The numbers denote the number of isoprene residues.

Strategies were long chain polyrenols, it appeared of interest to examine whether polyrenols containing more than 4 isoprene units could be employed in condensation reactions. For these experiments we selected trans,trans-poly-cis-prenyl-12-PP and all-trans-nonaprenyl-PP. When the products from these incubations were analyzed on HPLC, it was found that both these substrates gave rise to formation of polyisoprenyl-PP containing 15–18 isoprene units (Fig. 6), which was similar to the pattern found in previous incubations. In the case of nonaprenyl-PP, the individual peaks appeared to be split, probably indicating the presence of several cis-trans isomers. The total radioactivity in products, reflecting the number of IPP units incorporated, after incubation with these substrates was low compared with that obtained in the presence of FPP (Table II). On the other hand, when the enzyme activity was calculated so as to reflect the number of newly synthesized polyrenyl-PP chains, no substantial difference could be observed between trans,trans-FPP and polyrenyl-12-PP. However, the amount of final product obtained using all-trans-nonaprenyl-PP was substantially lower. These experiments indicate that longer polyrenyl-PPs can be elongated by cis-prenyltransferase to a length of 15–18 isoprene residues and that cis-prenyltransferase, at high substrate concentrations, utilizes both cis and trans isomers non-specifically.

FIG. 5. Autoradiography of the reaction products formed using[^3H]IPP and trans-GPP as substrates. After extraction and dephosphorylation, the lipids were separated on silica gel TLC. After development, the plate was exposed on x-ray film for 48 h. The lines indicate the positions of trans,trans- (t,t), and trans,cis- (t,c) farnesol and long chain polyrenol (P) standards.

DISCUSSION

In this study the substrate specificity of the microsomal long chain cis-prenyltransferase activity is characterized. Isolation of this enzyme from mammalian tissues has not yet been achieved, and its substrate specificity has thus been examined to only a limited extent. cis-Prenyltransferase is considered to utilize trans,trans-FPP as substrate. FPP is also the substrate for squalene synthetase and trans-prenyltransferase, i.e. the first committed enzymes in the biosynthesis of cholesterol and ubiquinone, respectively. Utilization of FPP by the different branches of this pathway must therefore be highly regulated. Differences in the affinities of the different branch point enzymes for FPP have been suggested earlier as one possible explanation for the different rates of dolichol, ubiquinone, and sterol synthesis observed under various conditions (31–33). Furthermore, FPP is utilized by specific cytosolic protein-farnesytransferases, which have been reported to have very high affinities for FPP (34). Recently it was shown that isolated rat brain cytosol has the capacity to convert mevalonate into protein-bound all-trans-geranylgera
Fig. 6. Product distribution after incubation of microsomes with [3H]IPP and long chain substrates. The reaction mixture was extracted with organic solvents, and the extracted lipids were dephosphorylated enzymatically. The sample was subjected to reverse-phase HPLC, and the radioactivity of the effluent was monitored using a radioactivity flow detector. A, incubation with poly-prenyl-12-PP; B, incubation with all-trans-nonaprenyl-PP.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Total radioactivity (dpm)</th>
<th>Amount of final product (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poly-prenyl-12-PP</td>
<td>12,000 ± 1,350</td>
<td>1.05 ± 0.12</td>
</tr>
<tr>
<td>All-trans-nonaprenyl-PP</td>
<td>8,310 ± 880</td>
<td>0.43 ± 0.05</td>
</tr>
<tr>
<td>trans,trans-FPP</td>
<td>35,400 ± 3,240</td>
<td>1.03 ± 0.09</td>
</tr>
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Table II

Substrate Specificity of cis-Prenyltransferase

The microsomal cis-prenyltransferase activity showed a much higher affinity for trans,trans,cis-GGPP than for the all-trans-isomer. This was not surprising, since we were able to show, using pulse-chase techniques, that the trans,trans,cis-isomer was produced as an intermediate in the biosynthesis of long chain polyprenyl-PP. After a short incubation with FPP as substrate, only trans,trans,cis-GGPP was produced. If this pulse was followed by a chase with unlabeled IPP, the GGPP was further metabolized to long chain polyprenyl-PP products.

In the chase experiment, no intermediary metabolites could be detected, indicating that the reaction occurs in two separate steps, i.e., the initial synthesis of GGPP, followed by completion of the long chain polyprenyl-PP. It is also possible that the synthetic sequence from FPP involves two separate enzymes or two different active sites in the same protein, one responsible for the first condensation introducing the trans-cis-bond and the second enzyme catalyzing the repetitive cis-cis additions. In line with this hypothesis is the fact that cis-prenyltransferase displayed a higher affinity for trans,cis-FPP than for the all-trans-isomer. This phenomenon may be explained if this former isomer interacts directly with the second enzyme, while trans,trans-FPP must first be converted to trans,trans,cis-GGPP in order to be further elongated. The relatively low affinity found for trans,trans-FPP would then be explained by the fact that the reaction involves two separate steps. If this is the case, one would expect to find lower amounts of short chain products when using trans,cis-instead of trans,trans-FPP as substrate. In fact, it has been shown earlier that distributions of the products formed from these two isomers were different (15). When trans,cis-FPP was used, the ratio of long to short chain products (presumably GGPP) was 5.5, whereas when the all-trans isomer was used, this ratio was only 1.5. The overall incorporation was similar in both cases. Similar results were also obtained in the present investigation (see Fig. 4, B and C). In order to further analyze the condensation process, isolation and characterization of cis-prenyltransferase will be necessary.

It was unexpected that trans-GPP could be used as a substrate to produce FPP and that this FPP could then be used for polyprenyl-PP synthesis by microsomes. These results are, however, supported by earlier observations in which GPP could be utilized by cis-prenyltransferase in microsomes from Ehrlich ascites tumor cells and Saccharomyces cerevisiae (11, 38). On the other hand, the intermediate formation of FPP was not demonstrated in these studies. The fact that we were unable to remove FPP synthase activity from the micro-
some by repeated washing, which removed cytosolic proteins, raises the possibility that this is truly a microsomal activity. On the basis of their earlier experiments, Poulter and Rilling (17) and Ogura and co-workers (39) indeed raised the possibility that this enzyme may be present in microsomal membranes, as well as in mitochondria. Furthermore, Edwards and co-workers (40) have found at least five copies of the FPP synthase gene in rat liver, which may indicate the presence of several isoenzymes.

All of our in vitro incubations gave rise to similar end products, i.e. polyisoprenes containing 15-18 isoprene units. Even when longer polypropenyl pyrophosphates, such as those with 9 and 12 isoprene units, were employed, the end product was the same, implying that product release is governed by the overall length of the product. In recent studies we have found that under in vitro conditions SCP2 stimulates cis-prenyltransferase activity and leads to the production of polyisoprenyl-PPs with the same chain lengths as those found in biological membranes. It will be of interest in the future to elucidate the processes governing the fate of newly synthetized FPP, as well as the regulation of different branch point enzymes utilizing FPP or GGPP.

REFERENCES