Inactivation of Undecaprenylpyrophosphate Synthetase with a Photolabile Analogue of Farnesyl Pyrophosphate†

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ABSTRACT: Two photolabile analogues of the allylic pyrophosphate substrates of prenyltransferases have been prepared and tested as substrates of *Lactobacillus plantarum* undecaprenylpyrophosphate synthetase. (*E,E*)-(2-Diazo-3-trifluoropropionyloxy)geranyl pyrophosphate (DATFP-GPP), a photolabile analogue of *trans,trans*-farnesyl pyrophosphate, was 47% as reactive as the natural substrate *trans,trans*-farnesyl pyrophosphate, whereas DATFP-dimethylallyl pyrophosphate, a geranyl pyrophosphate analogue, was unreactive as a substrate. The product of the enzymic reaction of DATFP-GPP and isopentenyl pyrophosphate was a long-chain DATFP-polyprenyl pyrophosphate with nine isoprene units. The K_m's of the enzyme for DATFP-GPP and *trans,trans*-farnesyl pyrophosphate were 0.17 and 0.13 μM, respectively. *o*-Azidophenethyl pyrophosphate, a photolabile analogue of the homoallylic substrate isopentenyl pyrophosphate, was ineffective as an inhibitor of the undecaprenylpyrophosphate synthetase. DATFP-dimethylallyl pyrophosphate was also ineffective as a substrate for farnesylpyrophosphate synthetase, the chicken liver prenyltransferase. Undecaprenylpyrophosphate synthetase was inactivated by irradiation with ultraviolet light in the presence of DATFP-GPP. However, the shorter chain isoprenologue DATFP-dimethylallyl pyrophosphate was ineffective as an inactivator. The dissociation constant (K_i = 0.22 μM) of the DATFP-GPP–enzyme complex was determined from an analysis of the kinetics of enzyme inactivation. This constant correlated well with the K_m of the enzyme for *trans,trans*-farnesyl pyrophosphate. *trans,trans*-Farnesyl pyrophosphate protected the enzyme from inactivation, when the enzyme was irradiated in the presence of DATFP-GPP. Furthermore, both isopentenyl pyrophosphate and $MgCl_2$ were required for enzyme inactivation in the presence of the photolabile substrate. It appears, therefore, that the photolabile substrate, DATFP-GPP, caused inactivation of this prenyltransferase by reacting at the allylic pyrophosphate binding site.

Undecaprenylpyrophosphate synthetase catalyzes the biosynthesis of the long-chain polyprenyl pyrophosphate precursor of the polyprenol carbohydrate-carrier, undecaprenyl monophosphate. $C_{55}P$[1] is essential for the biosynthesis of a variety of bacterial cell envelope components. The synthetase is a member of the prenyltransferase group of enzymes, which catalyze the condensation of an allylic isoprenyl pyrophosphate with isopentenyl pyrophosphate (IPP). This synthetase has been described in bacteria such as *Salmonella newington* (Christenson et al., 1969), *Micrococcus luteus* (Kurokawa et al., 1971; Baba & Allen, 1980), and *Bacillus subtilis* (Takahashi & Ogura, 1982), but it has been studied most extensively in *Lactobacillus plantarum* (Keenan & Allen, 1974a,b; Allen et al., 1976; Allen & Muth, 1977; Baba & Allen, 1978). The *L. plantarum* enzyme has several features which distinguish it from a more thoroughly studied and better defined prenyltransferase, FPP synthetase (Poulter & Rilling, 1981). Three of these features are (1) the introduction of *cis*-isoprene residues instead of trans residues during product formation, (2) the addition of seven or eight isoprene residues to the allylic pyrophosphate substrate in contrast to the one or two units added with FPP synthetase, and (3) the requirement for detergent or phospholipid for enzymic activity. A desirable goal is to determine what differences in protein structure of the prenyltransferases might account for these different features. The characterization of the substrates' binding sites is likely to show some important differences.

Attempts to identify amino acid residues in the active site of FPP synthetase were partially successful, when the arginine-specific reagent phenylglyoxal was used (Barnard & Popjak, 1980, 1981; Brems et al., 1981). Since phenylglyoxal may also react at arginine residues not associated with the enzyme active site, it is desirable to have more specific reagents for modifying the prenyltransferase active site. One approach is the preparation of photoactivatable substrate analogues. This approach has been successful in one instance when *o*-azidophenethyl pyrophosphate, a photolabile analogue of the homoallylic substrate IPP, was used. Although this analogue was not a substrate, it has been used to photolabel a polypeptide segment of the active site of FPP synthetase (Brems & Rilling, 1979; Brems et al., 1981).

We describe here the preparation of a different class of photolabile analogues related to the allylic pyrophosphate substrate. The activity of one of these analogues as a substrate and photoactivatable inactivator of the $C_{55}PP$ synthetase from *L. plantarum* is described here.

Materials and Methods

Geraniol (99+% pure) and *o*-aminophenethyl alcohol were obtained from Aldrich Chemical Co. The amino alcohol was redistilled before use. *p*-Aminophenethyl alcohol (Eastman) was recrystallized before use. GPP, FPP, and IPP were prepared as previously described (Keenan & Allen, 1974a; Baba & Allen, 1978). [1-^{14}C]IPP was purchased from Amersham/Searle Corp. and diluted to lower specific activities with unlabeled IPP. Kieselguhr G for TLC and silica gel 60 F_{254} on plastic sheets were products of E. Merck. Mass spectra and NMR spectra were taken on a single focusing magnetic sector Du Pont 490F mass spectrometer (70 eV, EI) and a

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[1] Abbreviations: $C_{55}P$, undecaprenyl monophosphate; $C_{55}PP$, undecaprenyl pyrophosphate; *t,t*-FPP, *trans,trans*-farnesyl pyrophosphate; GPP, geranyl pyrophosphate; IPP, Δ^3-isopentenyl pyrophosphate; DATFP, 2-diazo-3-trifluoropropionyloxy; PP, pyrophosphate, Tris, tris(hydroxymethyl)aminomethane; TLC, thin-layer chromatography.

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Varian A60 instrument, respectively.

The preparation of the photolabile analogues and their respective synthetic intermediates is detailed below. In each case the molar equivalence of the reactants is given in parentheses following the reactant.

(E)-3,7-Dimethyl-1-(chloroacetoxy)-2,6-octadiene (Geranyl Chloroacetate). Geraniol (1) and chloroacetic anhydride (3) were reacted together in pyridine (25) at 0 °C for 2 h by the method of Cook & Maichuk (1970). The ester was isolated in 81% yield (0.93 g) after purification by silica gel chromatography with petroleum ether–benzene (2:1 v/v).

3-Methyl-1-(chloroacetoxy)-2-butene (3,3-Dimethylallyl Chloroacetate). 3-Methyl-2-butenol (1), prepared by the reduction of 3,3-dimethylacrylic acid with $LiAlH_4$ (Yuan & Bloch, 1959), was reacted with chloroacetic anhydride (2) in pyridine (10), and the resulting ester was isolated in 78% yield (2.15 g) as described above for the geranyl derivative.

(E,E)-3,7-Dimethyl-1-(chloroacetoxy)-2,6-octadien-8-ol. Geranyl chloroacetate (1) was oxidized with *tert*-butyl hydroperoxide (3.6) in the presence of catalytic quantities of H_2SeO_3 (0.02) and salicylic acid (0.1) in the solvent CH_2Cl_2 for 20 h at room temperature by an adaption of the method of Umbreit & Sharpless (1977). The trans ω-allylic alcohol was obtained in 47% yield (0.99 g) after purification by silica gel chromatography with benzene–ethyl acetate (10:1 v/v). NMR shifts (ppm, $CDCl_3$) were 1.63 (3 H, s), 1.72 (3 H, s), 2.08 (4 H, m), 2.26 (1 H, s), 3.90 (2 H, s), 4.00 (2 H, s), 4.62 (2 H, d), and 5.28 (2 H, t).

(E)-3-Methyl-1-(chloroacetoxy)-2-buten-4-ol. Dimethylallyl chloroacetate (1) was treated with *tert*-butyl hydroperoxide (3.6), H_2SeO_3 (0.1), and salicylic acid (0.1) for 93 h at room temperature as described above. The trans alcohol was obtained in 37% yield (0.71 g). NMR shifts (ppm, $CDCl_3$) were 1.72 (3 H, s), 2.26 (1 H, s), 4.01 (4 H, s), 4.70 (2 H, d) and 5.57 (1 H, t).

The NMR measurement of each of the last two products confirmed the trans configuration of the ω-hydroxyl moiety. The chemical shifts showed a good coincidence with those described by Büchi & Wüest (1967), Bhalerao et al. (1970), and Chan et al. (1968).

The 2-diazo-3-trifluoropropionyloxy derivatives of the diol monoesters were prepared with 2-diazo-3-trifluoropropionyl chloride. The latter was prepared by the method of Chowdhry et al. (1976).

(E,E)-8-DATFP-3,7-dimethyl-1-(chloroacetoxy)-2,6-octadiene. (E,E)-3,7-dimethyl-1-(chloroacetoxy)-2,6-octadien-8-ol (1) was esterified with 2-diazo-3-trifluoropropionyl chloride (1.2) in pyridine (20) at room temperature for 2 h. The diester was isolated in 74% yield (2.64 g) after purification by silica gel chromatography in benzene–petroleum ether (1:2 v/v): IR (neat) 2150, 1725 (br) and 1140 cm^{-1} (br).

(E)-4-DATFP-3-methyl-1-(chloroacetoxy)-2-butene. (E)-3-Methyl-1-(chloroacetoxy)-2-buten-3-ol (1) was esterified with 2-diazo-3-trifluoropropionyl chloride (1.2) in pyridine (2) for 2 h at room temperature. The diester was isolated in 71% yield (2.13 g) as described above with the geranyl analogue: IR (neat) 2150, 1725 (br) and 1140 cm^{-1} (br).

(E,E)-8-DATFP-3,7-dimethyl-2,6-octadien-1-ol (DATFP-geraniol). (E,E)-8-DATFP-3,7-dimethyl-1-(chloroacetoxy)-2,6-octadiene (1) was hydrolyzed with 0.1 M NH_3 (2) in aqueous methanol (90% MeOH, v/v) at room temperature for 90 min by an adaption of the method of Reese & Stewart (1968) to give the DATFP ester. The yield was 87% (1.12 g) after purification by silica gel chromatography in benzene–ethyl acetate (5:1 v/v). NMR chemical shifts (ppm, $CDCl_3$) were 1.65 (6 H, s), 2.08 (4 H, m), 4.11 (2 H, d), 4.59 (2 H, s), and 5.38 (2 H, t). Principal IR bands (neat) were at 2150 and at 1710 (br) and 1140 cm^{-1} (br). High-resolution mass spectral analysis gave the highest mass component as the ion (parent – N_2 – H_2O) of mass 261.1058 ($C_{13}H_{16}O_2F_3$). Other significant masses were at 221.05 ($C_8H_8N_2O_2F_3$) and at 152.12 ($C_{10}H_{16}O$).

(E)-4-DATFP-3-methyl-2-buten-1-ol (DATFP-dimethylallyl Alcohol). (E)-4-DATFP-3-methyl-1-(chloroacetoxy)-2-butene (1) was hydrolyzed to the monoester with 0.1 M NH_3 (2) in aqueous methanol at room temperature for 60 min and purified as described above for the geranyl derivative in 79% yield (1.27 g). NMR chemical shifts (ppm, $CDCl_3$) were 1.68 (3 H, s), 4.14 (2 H, d), 4.59 (2 H, s), and 5.59 (1 H, t). Principal IR bands (neat) were at 2140 and at 1740 (br) and 1140 cm^{-1} (br). Electron ionization (70 eV) high-resolution mass spectral analysis gave an ion (parent – H_2O) at mass 220.0516 corresponding to $C_8H_8N_2O_2F_3$.

Each of the DATFP isoprenols was phosphorylated by the method of Popjak et al. (1962). Purification of the pyrophosphate esters was accomplished by an adaption of the procedures of Holloway & Popjak (1967) where Amberlite XAD-2 and DEAE-cellulose chromatography was used. The phosphorylated products were applied to the 20 × 210 mm Amberlite XAD-2 (20–30 mesh) column previously equilibrated with 1 mM NH_4OH. Pure DATFP-GPP or DATFP-dimethylallyl PP, free of any inorganic phosphate, was eluted with 1 mM NH_4OH–50% methanol or with 1 mM NH_4OH, respectively. The phosphate content was determined by the method of Chen et al. (1956).

The *o*- and *p*-azidophenethyl pyrophosphates were prepared by the general procedure described by Brems & Rilling (1979).

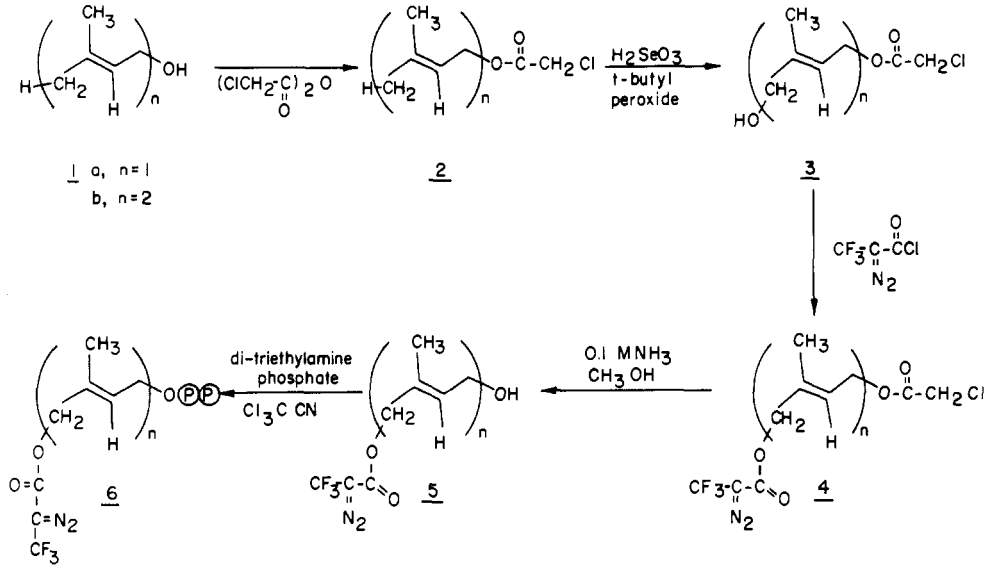
Enzyme Preparations and Assays. The chicken liver FPP synthetase was a 30–60% ammonium sulfate precipitate prepared by the method of Reed & Rilling (1975). This enzyme was assayed by the method of Holloway & Popjak (1967).

The C_{55}PP synthetase was prepared from *L. plantarum* through the hydroxylapatite purification step as previously described (Allen et al., 1976) except that no Triton X-100 was used in any step of the procedure. The enzyme assays contained, in a final volume of 0.5 mL, 9–22 μg of protein, 0.10 M Tris-HCl buffer (pH 7.5), 0.5% Triton X-100, 200 μM $MgCl_2$, 8.7 μM [^{14}C]IPP (3.1 μCi/μmol, 30 000 dpm) unless otherwise indicated, 10 μM *t,t*-FPP (except as indicated), and/or photolabile analogue. The reaction mixtures were incubated at 35 °C for 30 min, and the products were analyzed after acid hydrolysis as previously described (Allen et al., 1976). The radioactivity of all samples was determined in a toluene scintillation fluid containing 4 g/L 2,5-diphenyloxazole and 80 mg/L bis(*o*-methylstyryl)benzene.

Preparation of Polyprenyl Phosphates, Polyprenols, and Acetyl Esters. Larger quantities of the polyprenyl pyrophosphates were prepared in 3-mL incubation mixtures, containing 340 μg of protein, under the normal enzyme assay conditions. The radiolabeled products were extracted with 1-butanol as previously described (Allen et al., 1976) for TLC analysis.

The isoprenoid monophosphates were prepared by treating the pyrophosphates with a wheat germ phosphatase at pH 6.2 for 4 h by the method of Carson & Lennarz (1981). Hydrolysis can be accomplished either in the presence or in the absence of octyl glucoside. This is probably due to the presence of small quantities of Triton X-100 in the isoprenoid pyrophosphate preparation. Little free isoprenol was obtained under these conditions.

Scheme I



The isoprenoid pyrophosphates were hydrolyzed to the free alcohols with potato acid phosphatase at pH 5.0 in 60% methanol for 3 h by the method of Fujii et al. (1982). The products were extracted from the reaction mixture with $CHCl_3$–MeOH (2:1 v/v).

The polyprenols, prepared by dephosphorylation of the biosynthesized [^{14}C]polyprenyl phosphates with potato acid phosphatase, were partially purified by passing them through a column of silica gel 60 (300 mg) in $CHCl_3$. These [^{14}C]-polyprenols were acetylated by the addition of 50 μL of a pyridine solution containing 47.5 μmol of [^{3}H]acetic anhydride (808 μCi) and incubation at room temperature for 150 min with occasional shaking. Then 0.1 mL of absolute ethanol was added to the reaction solution, and this mixture was allowed to stand for 30 min at room temperature. The solvent and volatile components were then removed by evaporation in vacuo. The resulting residue was dissolved in benzene, and the acetylated polyprenols were purified by chromatography on silica gel 60 (300 mg) in benzene–ethyl acetate (10:1 v/v). These doubly labeled polyprenyl acetates were analyzed by reverse-phase TLC (5% liquid paraffin impregnated Kieselguhr G).

The radioactive areas were scraped from the reverse-phase TLC sheets and extracted twice with 1 mL of $CHCl_3$ and 3 times with 1 mL of acetone. The pooled extracts were freed of Kieselguhr G by centrifugation. The organic phase was removed by evaporation under a N_2 stream and the residue analyzed for the amount of the ^{14}C and ^{3}H isotopes with a Triton X-100/toluene-based scintillation mixture.

Photolysis Conditions. Photolysis was conducted at 4 °C for 5 min except as otherwise described in a 1-cm path-length quartz cuvette situated at 1-cm distance from the surface of an 8-W GE germicidal lamp (G8T5). Ninety-five percent of the output of the lamp is at 254 nm.

Photolysis mixtures, containing 125 mM Tris-HCl buffer, 250 μM $MgCl_2$, 11 μM [^{14}C]IPP (3.1 μCi/μmol) and different concentrations of photoanalogue and/or *t,t*-FPP in a volume of 1.2 mL, were cooled to 4 °C in the cuvettes. A solution of the C_{55}PP synthetase at 0 °C was added to the contents of each cuvette and mixed well. This mixture was then irradiated for the desired time. Triton X-100 was omitted from the irradiation mixtures to prevent both the absorption of irradiating light and product formation. Following irradiation, Triton X-100 and *t,t*-FPP were added to bring the concentration of all components to the level that was used in the standard assay conditions. Two 0.5-mL aliquots from each of these mixtures were then transferred to an assay tube and assayed by the standard assay procedure described above. The average of these duplicate determinations is reported.

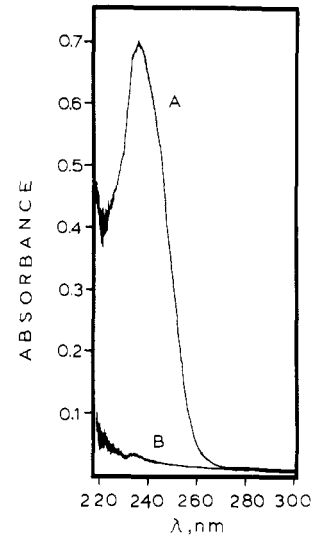


FIGURE 1: UV spectrum of DATFP-GPP before and after irradiation. (A) Spectrum before irradiation. (B) Spectrum after irradiation. DATFP-GPP (50 μM) in 1 mM NH_4OH was irradiated at room temperature for 15 min in a quartz cell as described under Materials and Methods.

The enzyme exhibited a gradual loss in enzymic activity in dilute solutions in the absence of Triton X-100; therefore, to ensure reproducible and meaningful results, it was necessary to minimize the time between enzyme addition and photolysis to less than 2 min and to add Triton X-100 immediately after irradiation was completed. Alternatively, a constant time interval was maintained between the time of enzyme addition and time of Triton X-100 addition for each test mixture. This time interval was 10–15 min, depending upon the size of the experiment.

Results

The DATFP derivatives of dimethylallyl pyrophosphate (**6a**) and GPP (**6b**) were synthesized by the reaction outlined in Scheme I, for use as photolabile analogues of the allylic

Table I: Kinetic Properties of Photolabile Substrates with C_{55}PP Synthetase

substrate[a] (concn)	nmol of IPP incorporated	% control
control, *t,t*-farnesyl pyrophosphate (10 μM)	1.37	100
DATFP-GPP, **6b** (10 μM)	0.65	47
DATFP-GPP, **6b**, radiationally decomposed (10 μM)[b]	0.50	36
DATFP-dimethylallyl PP, **6a** (100 μM)	0	0

[a] Each compound was tested as a substrate with 20 μM [^{14}C]IPP (2 μCi/μmol) as cosubstrate under standard incubation conditions for 30 min at 35 °C. [b] DATFP-GPP was decomposed by irradiation in 1 mM NH_4OH in a quartz cuvette at 4 °C for 1 h with a mineral light before incubation with the enzyme.

Table II: Effect of DATFP-dimethylallyl PP on Farnesyl PP Synthetase

DATFP-dimethylallyl PP (μM)	pmol of IPP incorporated[a] −GPP	+GPP
0	0	86
0.8	1.6	
5	0.9	
10	0.64	
100	3.5	106

[a] Enzyme was incubated for 5 min at 30 °C in 200-μL mixtures containing 8.5 mM KPO_4 buffer, pH 7.0, 0.85 mM $MgCl_2$, 10.6 μM [^{14}C]IPP (24 μCi/μmol) with or without 1 μM GPP, and varying concentrations of DATFP-dimethylallyl PP.

substrates GPP and *t,t*-FPP. DATFP-GPP (**6b**) has an absorption maximum at 236 nm (ϵ = 14 000) and a shoulder at 340 nm (ϵ = 27) (Figure 1). Irradiation of this compound in 1 mM NH_4OH in quartz cells at room temperature resulted in its decomposition with a $t_{1/2}$ of 1.5 min as measured by the loss in absorbance at 236 nm.

Photolabile Analogues as Prenyltransferase Substrates and Inhibitors. The photolabile analogues were tested as either substrates or inhibitors of prenyltransferase activity. The diazo derivatives were compared with *t,t*-FPP as substrates for the *L. plantarum* C_{55}PP synthetase (Table I). DATFP-GPP, the *t,t*-FPP analogue, was about 47% as reactive as a substrate as FPP, when the enzyme was saturated with the substrate analogue. The photodecomposition product (uncharacterized) of DATFP-GPP was also a substrate, but it was less reactive than DATFP-GPP. The K_m values for DATFP-GPP and its photodecomposition product were 0.17 and 0.53 μM, respectively. These values compare favorably with that for *t,t*-FPP, which exhibited a K_m of 0.13 μM for this enzyme preparation. DATFP-dimethylallyl PP (**6a**), a GPP analogue, was not a substrate, which is consistent with the poor reactivity of GPP as a substrate (Baba & Allen, 1978).

DATFP-dimethylallyl PP was also tested as a substrate of *t,t*-FPP synthetase, the chicken liver prenyltransferase, which uses GPP as a natural substrate. The photolabile derivative had no significant activity as a substrate with this enzyme and gave no inhibition of activity when the enzyme was assayed in the presence of 100-fold molar excess of analogue compared to that of GPP (Table II).

The photolabile analogues of IPP, *o*- and *p*-azidophenethyl pyrophosphate, were also tested as inhibitors of the C_{55}PP synthetase. In contrast to the inhibition shown when the ortho derivative was tested with the chicken liver prenyltransferase, neither of these photolabile analogues was inhibitory toward the bacterial long-chain prenyltransferase (Table III), even when they were present at 10–100 times the concentration of IPP in the reaction mixture.

Table III: Effect of Azidophenethyl Pyrophosphate (AP-PP) on Prenyltransferase Activity

AP-PP (μM)	pmol of IPP incorporated: C_{55}PP synthetase *L. plantarum*[a] *o*-AP-PP	*p*-AP-PP	C_{15}PP synthetase chicken liver[b] *o*-AP-PP	*p*-AP-PP
0	382	382	91	91
50	366	369	51	91
100	391	362	18	89

[a] Enzyme was assayed under the usual conditions described under Materials and Methods except that 1.0 μM [^{14}C]IPP (24 μCi/μmol), 5 μM *t,t*-FPP, and varying concentrations of AP-PP were used. [b] Enzyme was assayed as described in footnote *a* of Table II with 1 μM GPP and varying concentrations of AP-PP.

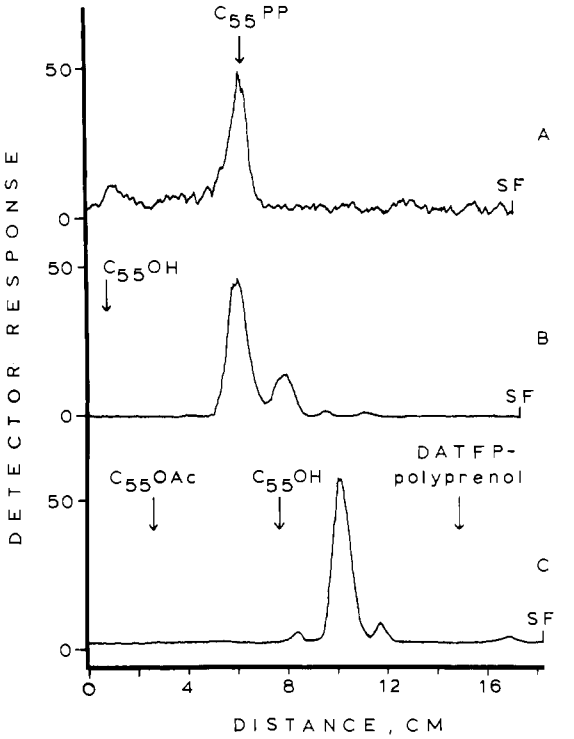


FIGURE 2: TLC of DATFP-polyprenyl products. (A) The enzymic product from incubation mixtures containing DATFP-GPP and [^{14}C]IPP was extracted with 1-butanol and chromatographed on silica gel 60 in solvent A. The arrow represents the position of migration of C_{55}PP. (B) The free DATFP-polyprenol prepared by treatment of the enzymic product with potato acid phosphatase was chromatographed by reverse-phase TLC. (C) The [^{3}H]acetyl ester of [^{14}C]DATFP-polyprenol was chromatographed by reverse-phase TLC. The arrows indicate the position of migration of the free DATFP-polyprenol and acetylated C_{55}OH.

TLC of the Reaction Products. Thin-layer chromatographic analysis of the photolabile product obtained from the action of the C_{55}PP synthetase on DATFP-GPP and IPP showed that the product migrated with an R_f = 0.35 on silica gel 60 TLC in diisobutyl ketone–acetic acid–H_2O (60:37.5:7.5 v/v/v), solvent A (Figure 2A). Treatment of the enzyme product with wheat germ phosphatase at pH 6.2 for 4 h yielded products whose principal component chromatographed on silica gel with an R_f = 0.48 in solvent A. These mobilities are comparable to the R_f values of 0.35 and 0.51 for C_{55}PP and C_{55}P, respectively, in the same TLC system. Complete hydrolysis of the enzymic product to the free polyprenol was obtained by treatment with potato acid phosphatase in 60% methanol at pH 5.0 for 3 h. The polyprenyl phosphates were

Table IV: Number of Isoprene Units Added by C_{55}PP Synthetase[a]

	expt	dpm of [^{3}H]acetate incorporated (A)	dpm of [^{14}C]IPP incorporated (B)	B/A	isoprene units added
undecaprenyl acetate	I	10 778	16 667	1.55	(8)
	II	1 199	1632	1.36	(8)
DATFP-polyprenyl acetate	I	18 868	26 238	1.39	7.20
	II	3 316	4 005	1.21	7.11

[a] The C_{55}PP synthetase was incubated with unlabeled 15 μM *t,t*-FPP or 50 μM DATFP-GPP and 60 μM [^{14}C]IPP (specific activity 2.1 μCi/μmol) under the standard incubation conditions. The free polyprenols, prepared from the products by treatment with potato acid phosphatase, were acetylated with [^{3}H]acetic anhydride. The acetylated products were separated by reverse-phase TLC (see Figure 2C). In experiment I the acetylated products were first purified by silica gel chromatography as described under Materials and Methods before TLC. The major radioactive product on the TLC sheet was then processed as described under Materials and Methods to determine the ratio of ^{14}C/^{3}H. The number of isoprene units, added in the formation of the analogue product, was determined by setting the ratio of ^{14}C/^{3}H in experiments I and II for undecaprenyl acetate to eight, since it has been unequivocally determined that eight isoprene units were added in its biosynthesis, and then normalizing the ^{14}C/^{3}H ratio found in the analogue product to that value.

not hydrolyzed in the absence of phosphatase under the conditions described. The R_f values of the resulting DATFP-polyprenol and undecaprenol on silica gel were 0.85 and 0.89, respectively, in solvent A and 0.51 and 0,53, respectively, in benzene–ethyl acetate (10:1 v/v), solvent B. Reverse-phase chromatography of the free polyprenol was carried out as described by Allen et al. (1976) on paraffin-coated Kieselguhr G plates in acetone–H_2O (80:20 v/v). The DATFP-polyprenol product gave a major component with an R_f value of 0.35 and minor components with R_f values 0.46, 0,50, and 0.65 (Figure 2B). Undecaprenol chromatographed in this system with an R_f = 0.04. Whereas the DATFP polyprenol and its mono- and pyrophosphorylated derivatives chromatographed similarly to the analogous C_{55} compounds on normal-phase TLC, the DATFP-polyprenols chromatographed quite differently from C_{55}OH on reverse-phase chromatography. The higher mobilities of the DATFP-polyprenols were indicative of a compound which was either more polar or shorter in chain length than the C_{55}OH. Several control experiments were carried out to explore the effect of the polar DATFP moiety on the TLC mobilities of the free polyprenols. The polar DATFP moiety had a small effect on the relative mobility of the DATFP-geraniol, compared to the similar chain length polyprenol farnesol, on normal-phase TLC. The R_f values of DATFP geraniol and farnesol on silica gel 60 in solvent B were 0.19 and 0.30, respectively. On the other hand, DATFP-geraniol migrated with an R_f of 0.64, whereas farnesol migrated with an R_f of only 0.10 on reverse-phase chromatography in acetone–H_2O (40:60 v/v). Since the polar DATFP group has a marked effect on the mobility of polyprenol on reverse-phase TLC, it was impossible to assess the exact chain length of the photoanalogue product by simply comparing the mobility of the DATFP-polyprenol of that of the standard C_{55}OH. However, analysis by normal-phase TLC indicated that the DATFP products were long-chain polyprenols, similar to C_{55}OH, rather than shorter chain length polyprenologues.

Product Chain Length. The chain length of these analogue polyprenols was determined by the use of a double-label radioisotopic technique. The analogue polyprenyl pyrophosphates were biosynthesized from unlabeled DATFP-GPP and [^{14}C]IPP. The free polyprenols were obtained by hydrolysis of the ester with the potato acid phosphatase. The resulting free alcohols were acetylated with [^{3}H]acetic anhydride and the esters separated by reverse-phase TLC in acetone–H_2O (92:8 v/v) (Figure 2C). The ratios of ^{14}C/^{3}H found in the separated esterified products were compared to those of the acetylated C_{55}OH prepared from *t,t*-FPP and [^{14}C]IPP and isolated in a similar manner (Table IV). Since the chain length of the enzymatically synthesized C_{55}OH was known and confirmed by its comigration with C_{55}OH, purified

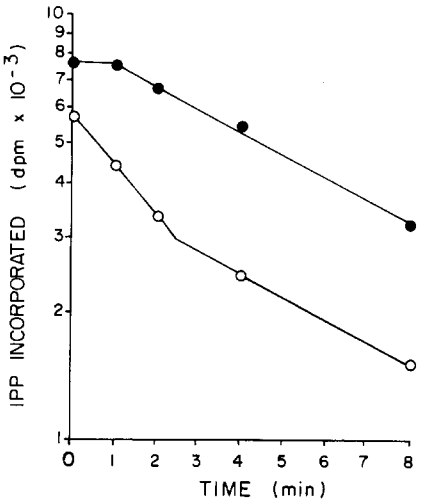


FIGURE 3: Time course of enzyme inactivation on irradiation with DATFP-GPP. C_{55}PP synthetase was irradiated at 4 °C in the presence (○) or absence (●) of 0.63 μM DATFP-GPP under conditions described in the text. Aliquots were taken at the indicated time intervals and assayed under standard conditions for the enzymic activity remaining.

and characterized from *L. plantarum*, the number of isoprene units added during C_{55}PP biosynthesis is known to be eight. Normalization of the ^{14}C/^{3}H ratios observed for the major esterified analogue polyprenol, with the ratio observed for the standard acetylated C_{55}OH, showed that the analogue was formed by the addition of seven isoprene units. The use of the control in which C_{55}OH was biosynthesized under the same experimental conditions as the analogue polyprenol overcame the experimental errors in the estimation of the specific activities of the two radiolabeled substrates.

Photoinactivation of C_{55}PP Synthetase. C_{55}PP synthetase was incubated with 0.63 μM DATFP-GPP in the presence of 11 μM IPP and 250 μM $MgCl_2$ and irradiated with UV light at 4 °C. No product was formed under these conditions because Triton X-100 was omitted from the reaction mixture. The enzymic activity, which remained after irradiation, was measured by using saturating concentrations of the natural substrate *t,t*-FPP. The time-dependent loss of enzyme activity in the presence and absence of the 0.63 μM DATFP-GPP is shown in Figure 3. A $t_{1/2}$ of 2.6 min was calculated from the first and more rapid phase of the plot shown in Figure 3, where enzyme inactivation due to the analogue-independent process was minimal. This rate of inactivation was comparable to the rate of decomposition ($t_{1/2}$ = 1.5 min) of DATFP-GPP, when it was irradiated in the absence of enzyme. Neither DATFP-GPP nor its photodecomposition product caused a decrease in enzyme product formation, when it was used in

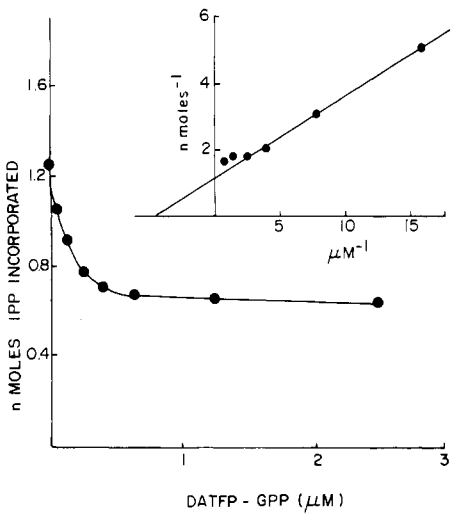


FIGURE 4: Effect of the concentration of DATFP-GPP on the irradiation-dependent inactivation of $C_{55}PP$ synthetase. The $C_{55}PP$ synthetase activity remaining after irradiation of the enzyme for 5 min at 4 °C at different concentrations of DATFP-GPP, under the conditions described under Materials and Methods, was measured with saturating *t,t*-FPP (10 μM). The insert is a reciprocal plot of the inactivator concentration vs. the difference in the activity of the irradiated control (no DATFP-GPP present) minus the activity remaining after irradiation in the presence of inactivator.

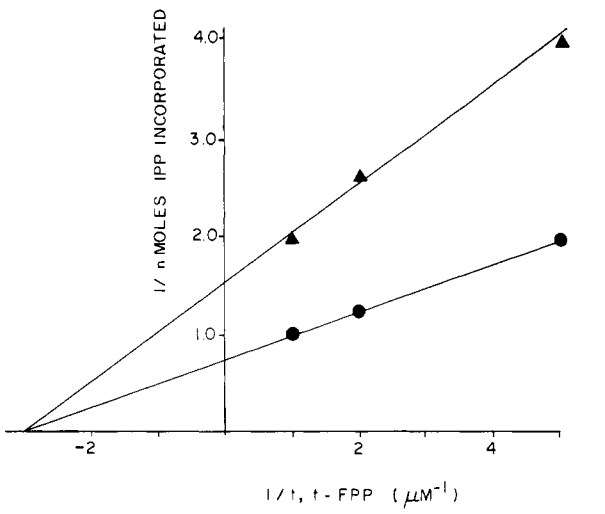


FIGURE 5: Effect of photoinduced inactivation on the kinetics of the synthetase. $C_{55}PP$ synthetase was irradiated under standard conditions with (▲) or without (●) 0.63 μM DATFP-GPP. The remaining activity was determined with varying concentrations of *t,t*-FPP.

2 μM concentration in the presence of 10 μM *t,t*-FPP (data not shown). Therefore, the analysis of these and subsequent experiments presented here did not require a consideration of the inhibitory effect of the photodecomposition product or any remaining undecomposed DATFP-GPP on the assay for the remaining enzyme.

The binding of the inactivator (I) to the enzyme was evaluated by a modification of the methods used by Meloche (1967) and Baker (1975). It was assumed that enzyme and DATFP-GPP form a complex prior to the irradiation-dependent inactivation (eq 1) and that k_1 and k_2 are very large

$$E + I \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E\text{---}I \xrightarrow[h\nu]{k_3} E\text{–}I^* \qquad (1)$$

relative to k_3. I* is the molecular species after irradiation.

The enzyme exhibited saturation kinetics for inactivation by DATFP-GPP during UV irradiation (Figure 4). The K_i for the interaction of the enzyme with DATFP-GPP was determined to be 0.22 μM by the derived relationship

$$\frac{1}{v_{in}} = \frac{k_i}{f(V_{in})(I)} + \frac{1}{f(V_{in})} \qquad (2)$$

where (I) = inactivator concentration, V_{in} = maximum rate of inactivation, v_{in} = rate of inactivation, K_i = (E)(I)/(E---I), and f = (E–I*)/(E---I) (after irradiation). Since the enzyme was inactivated by about 50% even when saturated with inactivator, the factor f was introduced to represent the ratio (E–I*)/(E---I) or the fraction of enzyme–DATFP-GPP complex (E---I) inactivated on irradiation.

This partial inactivation may be accounted for by the nonproductive loss of the irradiation-generated carbanion of the inactivator by the reaction of the carbanion with the solvent or the Tris buffer instead of its reaction with enzyme. These scavenging reactions led to an irreversible loss of the inactivator. This was well illustrated by the observation that inclusion of the scavenger dithiothreitol (12.5 mM) in the enzyme–inactivator reaction mixture resulted in no loss of enzymic activity on irradiation (data not shown).

The K_m of the active enzyme remaining after the photolysis was also determined. The activity of the enzyme was measured at different *t,t*-FPP concentrations after irradiation of the enzyme with a fixed level (0.63 μM) of DATFP-GPP (Figure 5). The double-reciprocal plot for the reaction velocity as a function of *t,t*-FPP concentration showed that the K_m value (0.33 μM) for the remaining activity was unchanged. The experimental data fitted a linear curve described by the derived relationship shown in eq 3. If K_i is 0.22 μM as determined

$$\frac{1}{v} = \frac{K_m}{V_{max}} \frac{K_i + I}{K_i + I(1-f)} \frac{1}{S} + \frac{1}{V_{max}} \frac{K_i + I}{K_i + I(1-f)} \qquad (3)$$

above, then f = 0.69. This value for f should also correspond to the ratio of $V_{max,in}/V_{max}$, where $V_{max,in}$ is the maximum velocity of the enzyme reaction after irradiation with the inactivator at saturating concentrations and V_{max} is the maximum velocity of the untreated enzyme. At the highest inactivator concentration tested (2 μM), this ratio was 0.51 (Figure 4). So, the agreement with the observed value of f was quite good.

Another experiment was performed to confirm the equilibrium constant, K_i, and f values determined in the previous experiment and reestablish the validity of our assumption that there is an equilibrium condition established before inactivation of the enzyme.

Various concentrations of enzyme were irradiated with a fixed concentration of DATFP-GPP (1.25 μM), and the remaining uninactivated enzyme was assayed with saturating levels of *t,t*-FPP. A comparison of these activities with a control, where enzyme was irradiated under the same conditions without inactivator, showed that there was a linear increase in enzyme activity with increasing enzyme concentration with or without inactivator added but that the activity of the enzyme remaining after irradiation with inactivator was reduced by a constant factor (Figure 6). This factor or reduction in the slope of the line should reflect the magnitude of K_i and the factor f. It can be calculated that the reduction in the slope of the experimental line compared to the control, where the enzyme was irradiated without inactivator, should be represented by the relationship

$$\frac{K_i + I(1-f)}{K_i + I}$$

For K_i = 0.22 μM and I = 1.25 μM, f was calculated to be 0.63 for the slope reduction of 0.47 observed. Again this value of f is consistent with the other data presented above.

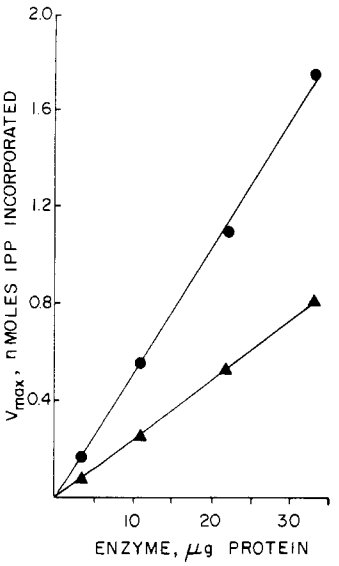


FIGURE 6: Effect of photoinduced inactivation on the V_{max} at different enzyme concentrations. C_{55}PP synthetase was irradiated under standard conditions with (▲) and without (●) 1.25 μM DATFP-GPP and varying amounts of enzyme protein. The remaining activity was determined with 10 μM *t,t*-FPP.

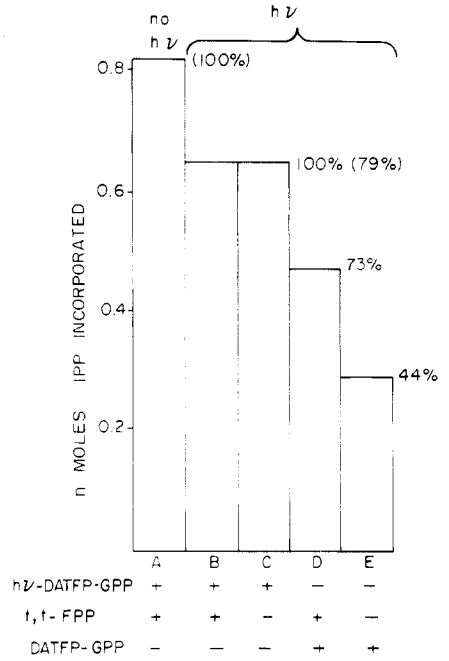


FIGURE 7: Protection of synthetase from inactivation by DATFP-GPP in the presence of *t,t*-FPP. C_{55}PP synthetase was irradiated under standard conditions with various combinations of photodecomposed DATFP-GPP (*hν*-DATFP-GPP), DATFP-GPP, or *t,t*-FPP present at 12.5 μM concentration each. The remaining activity was then determined with 10 μM *t,t*-FPP.

Experiments have been carried out to investigate the ability of substrate *t,t*-FPP to protect the enzyme from inactivation by DATFP-GPP. The extent of enzyme activity remaining after irradiation of enzyme with 12.5 μM effector in the presence or absence of 12.5 μM FPP is compared in Figure 7. Irradiation of the enzyme with previously decomposed DATFP-GPP (*hν*-DATFP-GPP), in the presence or absence of *t,t*-FPP, resulted in a 21% decrease in activity (column B and C). This illustrates the lability of the enzyme to denaturation during irradiation. Irradiation of the enzyme in the presence of DATFP-GPP, without *t,t*-FPP present (column E), resulted in 56% loss in activity relative to controls B and C, whereas irradiation with inactivator, in the presence of 12.5 μM *t,t*-FPP (column D), gave only a 27% decrease in activity. A similar observation was made when the enzyme was inactivated with only 1.25 μM DATFP-GPP and protected with 1.25 μM *t,t*-FPP except that less light-induced inactivation (34%) was observed, whereas nearly complete protection (97%) was offered by the substrate *t,t*-FPP. This illustrates the protective effect of *t,t*-FPP toward DATFP-GPP mediated inactivation. The concentration dependence of this protective effect by *t,t*-FPP is illustrated in Figure 8. There was a concentration-dependent protection due to *t,t*-FPP, with activity increasing from 62% to 99% of the control, which was irradiated without *t,t*-FPP and DATFP-GPP. These experiments involving substrate protection of inactivation were, however, complicated by the observation that the enzyme was also shown to be inactivated by irradiation in the presence of *t,t*-FPP. This effect is minimized, however, by the presence of the photodecomposed DATFP-GPP, which protects the enzyme from nonspecific photoinactivation.

The possibility that the photoanalogue of the substrate was acting in a nonspecific manner was also examined. The GPP analogue DATFP-dimethylallyl PP, which had no activity as a substrate, was tested as a photoinactivator of the enzyme. Figure 9 shows that DATFP-dimethylallyl PP did not sig-

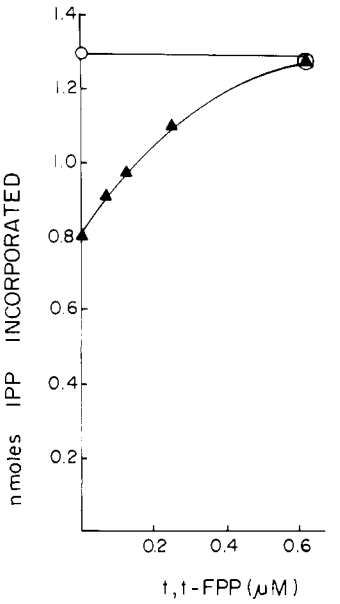


FIGURE 8: Concentration-dependent protective effect of *t,t*-FPP on the photoinactivation of C_{55}PP synthetase. The enzyme was irradiated with 1.25 μM DATFP-GPP and various concentrations of *t,t*-FPP (▲), and the remaining activity determined as in Figure 7. The control (○) indicates the activity observed after irradiation in the absence of DATFP-GPP.

Table V: Effect of IPP and $MgCl_2$ on Irradiation-Dependent Inactivation of C_{55}PP Synthetase in the Presence of DATFP-GPP[a]

component present during irradiation	product formed (dpm)	% activity relative to control
$MgCl_2$ + IPP	1973	60
IPP	3408	104
$MgCl_2$	3379	103
no IPP, no $MgCl_2$	3607	110
$MgCl_2$ + IPP (no DATFP-GPP), control	3291	100

[a] The enzyme was irradiated for 2 min at 4 °C in the presence or absence of 1.67 μM DATFP-GPP, 33 μM IPP, and 333 μM $MgCl_2$ as indicated. The enzymic activity remaining after irradiation was measured under standard conditions.

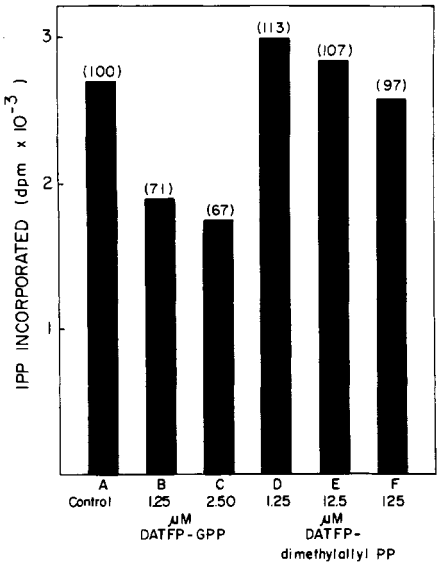


FIGURE 9: Effectiveness of DATFP-dimethylallyl PP as an enzyme inactivator. The enzyme was irradiated with varying concentrations of DATFP-GPP (B and C) or DATFP-dimethylallyl PP (D, E and F) for 2 min at 4 °C in the presence of 250 μM $MgCl_2$ and 25 μM IPP. The enzymic activity remaining after irradiation was measured under standard conditions. The values in parentheses represent the percentage of the activity relative to the control (A), which was irradiated in the absence of the photoanalogue.

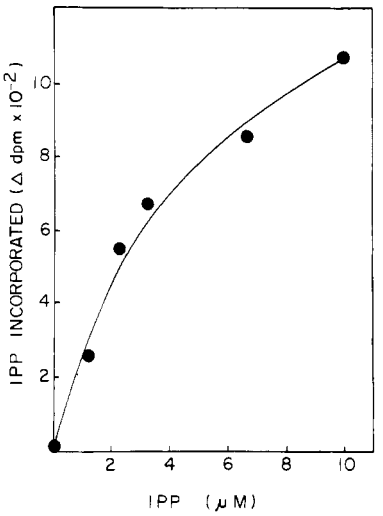


FIGURE 10: Effect of varying IPP concentration on the irradiation-dependent inactivation of the enzyme by DATFP-GPP. The enzyme was irradiated for 2 min in the presence of 1.67 μM DATFP-GPP, 333 μM $MgCl_2$, and variable IPP, as indicated. The activity remaining was then assayed as described in the legend to Table V. The difference in the activity of the irradiated control (no IPP present) minus the activity remaining after irradiation in the presence of IPP was plotted.

nificantly inactivate the enzyme even at photoanalogue concentrations 50-fold higher than those shown to inactivate with DATFP-GPP.

Since IPP and $MgCl_2$ were present in the irradiation mixtures described thus far, it was of interest to determine if either or both of these components were required to achieve the photoinactivation by DATFP-GPP. Table V illustrates that both IPP and $MgCl_2$ were required for inactivation, since if either or both were omitted from the irradiation mixtures, there was no enzyme inactivation. The dependences of the inactivation of the enzyme on the concentration of IPP and $MgCl_2$ are shown in Figure 10 and Figure 11A. In both experiments the nonvariable component (i.e., IPP or $MgCl_2$) was present at saturating concentrations. A $K_{i,IPP}$ value[2] of 2.4 μM was

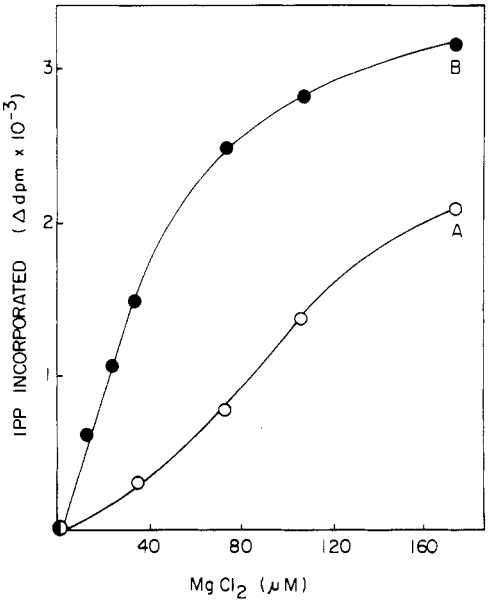


FIGURE 11: Effect of varying $MgCl_2$ concentration on the irradiation-dependent inactivation of the enzyme. The enzyme was irradiated with (○) or without (●) DATFP-GPP as described in Table V in the presence of 33 μM IPP and varying concentration of $MgCl_2$. When the enzyme was inactivated by DATFP-GPP (A), the concentration of $MgCl_2$ was adjusted to the standard assay conditions before determining the remaining enzymic activity. The difference in the activity of the irradiated control (no $MgCl_2$ present) minus the activity remaining after irradiation in the presence of $MgCl_2$ was plotted. In experiment B, where no DATFP-GPP was present, no additional $MgCl_2$ was added before measuring the enzyme activity. In this case the enzymic activity observed in the absence of any added $MgCl_2$ was subtracted from each experimental value.

obtained for IPP from a reciprocal plot of the data in Figure 10. This value compares favorably with the K_m (1.92 μM) of the enzyme for IPP. The dependence of enzymic activity on $MgCl_2$ is shown in Figure 11B.

Discussion

Two basic approaches have been used to probe the substrate binding sites of FPP synthetase, the most thoroughly studied prenyltransferase. One is the use of arginine-specific reagents. Barnard & Popjak (1980) found biphasic kinetics for the inhibition of the pig liver enzyme by phenylglyoxal. Two arginyl residues were modified per subunit. Both substrates partially protect the enzyme from inhibition by phenylglyoxal. Brems et al. (1981) reacted the avian liver enzyme with [^{14}C]phenylglyoxal and observed that at least 10 arginyl residues were labeled. We, however, have been unable to effectively protect *L. plantarum* C_{55}PP synthetase from inhibition by phenylglyoxal by preincubation of the enzyme with either of its substrates, which were present either singularly or in combination.

A second approach has been the use of a reagent which is specifically targeted to one of the substrate binding sites of prenyltransferase. The impetus for this aspect of our work was the success of others (Brems & Rilling, 1979; Brems et al. 1981) in designing an effective photolabile substrate analogue of the homoallylic substrate (IPP) for FPP synthetase, the avian liver prenyltransferase. They showed that photoirradiation of the avian enzyme with *o*-azidophenethyl PP gave greater than 90% loss of enzyme activity after successive

[2] A treatment similar to that used in eq 1 to describe the binding of DATFP-GPP to the enzyme can also be used to describe the binding of IPP to the enzyme. $K_{i,IPP}$ represents the dissociation constant of the IPP–enzyme complex in the presence of Mg^{2+} and DATFP-GPP.

treatments with the inhibitor. *t,t*-FPP, the product of the reaction, completely protected the enzyme against photoinactivation. Brems et al. (1981) analyzed peptides derived from the avian liver enzyme photolabeled with [^{3}H]-*o*-azidophenethyl PP and showed extensive labeling of CNBr and tryptic fragments. The modification of many amino acids, instead of just a few, showed that this reagent is a useful tool in probing an extensive area of the active site domain. It would be of interest to compare peptide fragments obtained from different prenyltransferases such as FPP synthetase and C_{55}PP synthetase, which were labeled with photolabile substrate analogues. Unfortunately, neither *o*- nor *p*-azidophenethyl PP serves as an inhibitor of the C_{55}PP synthetase. It was the purpose of this work to design, prepare, and test photolabile analogues of the allylic pyrophosphate substrate of prenyltransferases, particularly with the intent of studying the effectiveness of this analogue as a substrate, inhibitor, or covalent-modifying agent for C_{55}PP synthetase, the long-chain prenyltransferase.

The logic of the design of the photolabile substrate was to add a small photolabile branched chain moiety on the ω-isoprene unit of the allylic pyrophosphate substrate to give a trans-substituted product. The substitution at the ω-isoprene unit leaves the reactive center at C_1 free for the enzymically catalyzed condensation reaction with IPP. The synthesis of the trans product instead of the cis or trans/cis mixture was desirable because of the expectation that the C_{55}PP synthetase would have a stereochemical preference for the trans residue in this position as seen with most prenyltransferases. Allylic substrates with large cis substituents have been shown to be poor substrates with other prenyltransferases (Popjak et al., 1969; Ogura et al., 1970; Nishino et al., 1973).

The photolabile DATFP group selected was first described by Chowdhry et al. (1976). It has been used previously in ester linkage to phospholipids and has proven to be an effective reactant in studies on the interactions of phospholipid with lipids and proteins in membranes (Radhakrishnan et al., 1980).

The addition of this photolabile moiety to the prenol group required the limited oxidation of the methyl substituent of the ω-isoprene unit in a stereospecific manner. This was accomplished by the use of H_2SeO_3 and *tert*-butyl peroxide, which previously had been shown to cause the exclusive oxidation of the *trans*-methyl group in allylic compounds (Umbreit & Sharpless, 1977). The trans configuration in the products described here was established by NMR spectroscopy.

The selective addition of the DATFP moiety to the desired alcoholic group in later steps of the synthesis required that the original prenol hydroxyl group be derivatized before introduction of the second hydroxyl function. Derivatization was accomplished by esterification of the starting prenol with chloroacetic anhydride. This derivative was chosen to permit its selective hydrolysis in mild alkali, which is a condition where the DATFP moiety was shown to be stable.

Phosphorylation of the DATFP-geraniol gave the farnesyl analogue DATFP-GPP, which was a good substrate of the C_{55}PP synthetase. On the other hand, the geranyl analogue DATFP-dimethylallyl PP was not a substrate. The observation that the farnesyl analogue was a substrate supports our previous results (Baba & Allen, 1978), which indicated that C_{15}PP and C_{20}PP were the best substrates for the enzyme compared to shorter chain substrates, C_5PP and C_{10}PP. It also illustrated, however, the considerable tolerance the enzyme permits toward the character of the long-chain allylic substrate, particularly toward the nature of the ω-terminal residue. We previously have shown that the enzyme will also actively elongate the C_{15}PP and C_{20}PP which have either cis or trans residues in the α-isoprene unit.

This represents the first description of the synthesis and utilization of a photolabile analogue of an allylic isoprenoid pyrophosphate as a substrate for prenyltransferase. Furthermore, we have shown here that the photoanalogue DATFP-GPP was also an excellent reagent for the photoinactivation of the enzyme. After a single exposure to DATFP-GPP, the enzyme lost as much as 50–60% of its activity. This loss compares favorably with other enzymes such as prenyltransferase (Brems & Rilling, 1979) and ATPase (Cosson & Guillory, 1979), which were maximally inactivated to about 60% of the original activity in a single photolysis. The kinetics of UV-induced inactivation of the C_{55}PP synthetase by DATFP-GPP were consistent with a rapid reaction of the DATFP-GPP photolysis product with the enzyme, since the rates of chemical decomposition of the diazo derivative and enzyme inactivation were comparable. Binding of the photodecomposition product with the enzyme, however, has only been established indirectly by assaying the enzymic activity remaining after irradiation with the analogue. Studies are in progress to obtain direct evidence for binding with ^{3}H-labeled diazo derivatives.

There are several lines of evidence that show that the inactivation process was directed toward a specific functional domain of the prenyltransferase. First, the diazo derivative DATFP-GPP was a substrate for the C_{55}PP synthetase with a K_m (0.17 μM) similar to that of the natural substrate *t,t*-FPP (K_m = 0.13 μM). On the other hand, DATFP-dimethylallyl PP, a substrate analogue of GPP, failed to serve as a substrate. This analogue also failed to inactivate the enzyme on irradiation.

Second, the irradiation-induced enzyme inactivation showed saturation kinetics with increasing DATFP-GPP concentration, giving a K_i of 0.22 μM, which was similar to the K_m value for DATFP-GPP. This would indicate that equilibrium binding was established before inactivation. An alternative possibility to the establishment of equilibrium binding between DATFP-GPP and enzyme before inactivation is that a nonspecific bimolecular interaction between the enzyme and DATFP-GPP led to inactivation. If such an event happened, the linear curves shown in Figure 6 obtained in the presence of inactivator would not have intersected the x axis at the origin but at some positive point along the x axis. our results showed clearly that this was not the case. If, however, an equilibrium condition was established for the binding of DATFP-GPP to the enzyme at some nonspecific site, then the predicted intercepts would have been indistinguishable from our results (Baker, 1975). This latter possibility, however, can be ruled out on the basis of the structure–activity comparisons; i.e., DATFP-GPP is a good substrate, and the natural substrate *t,t*-FPP protects the enzyme from inactivation by the DATFP-GPP.

Third, the same K_m values of the enzyme for FPP were observed with enzyme UV irradiated either in the presence or in the absence of the inactivator. This indicates no major change in the catalytic activity of the surviving enzyme but complete inactivation of some of the enzyme.

Fourth, the substrate *t,t*-FPP protects the enzyme from inactivation in the presence of its photolabile analogue DATFP-GPP in a concentration-dependent manner. This strongly suggests competition of the natural substrate and photolabile analogue for a common binding site.

Two additional observations concerning the enzyme's requirements for binding the substrates were also apparent from

these studies. Although no direct binding determinations were made, the nature of the experimental design made it possible to assess, indirectly, the requirements for the presence of other components for the binding of the probe to the enzyme active site.

First, both cosubstrate, IPP, and divalent cation, Mg^{2+}, must be present to enable DATFP-GPP to inactivate the enzyme on irradiation. This absolute requirement for IPP during the photolysis indicates that, under the conditions tested, there seems to be no competition between IPP and DATFP-GPP at the allylic binding site in the presence of divalent metal cation. Since IPP and Mg^{2+} elicit their effects over a similar concentration range in which they function during enzyme catalysis, their mode of the association with the enzyme in the inactivation process appears closely related to the association occurring during the catalytic process in the presence of the detergent. Therefore, it can be inferred that that both IPP and Mg^{2+} are required for the binding of the natural substrate FPP to the active site. This situation is different than the observations made with the avian liver FPP synthetase where it was shown directly that either the allylic or homoallylic substrate bound to the FPP synthetase in the absence of the other substrate (Reed & Rilling, 1976). Furthermore, this binding occurred even in the absence of the divalent cation (King & Rilling, 1977). Nevertheless, they observed that the presence of the divalent cation enhanced the binding of the substrates (GPP and IPP) as much as 6–8-fold and permitted the binding of IPP to the allylic PP binding sites. In addition, studies on the binding of Mn^{2+} and IPP to the prenyltransferase revealed a strong cooperation between Mn^{2+} and IPP in the binding process (King & Rilling, 1977). Since our experiments only measured binding that resulted in enzyme inactivation (i.e., activity remaining after the photolysis), it is of course possible that the photoanalogue of FPP binds to the C_{55}PP synthetase in the absence of IPP and divalent cation but not in a manner which resulted in enzyme inactivation.

Second, the detergent Triton X-100, which is required for the formation of the polymerized product (Keenan & Allen, 1974a; Allen & Muth, 1977), was not required for the inactivation of the enzyme by DATFP-GPP. Therefore, detergent is apparently not required for the binding of the substrates and divalent cation to their respective active sites of the C_{55}PP synthetase. Detergent therefore seems only important in the process of polymerization which leads to the long-chain *cis*-isoprenoid.

Apparent differences in the results obtained for the C_{55}PP synthetase and FPP synthetase may be partly explained in terms of the differences in the chain length and the stereochemistry of the substrate and the final product. There is only a C_5 or C_{10} difference in chain length between the substrate and the product of the FPP synthetase, whereas there is a corresponding difference of C_{40} for the C_{55}PP synthetase. Furthermore, the FPP synthetase catalyzes the addition of *trans*-isoprene units to an allylic PP substrate having only trans residues, whereas the C_{55}PP synthetase catalyzes the addition of only *cis*-isoprene residues to an allylic PP substrate which may have either trans residues or both cis and trans residues. It has been suggested that the lack of strict geometric specificity in the binding of the allylic PP substrate to the C_{55}PP synthetase may be caused by a requirement of the allylic PP binding site to accommodate both cis and trans residues during polymerization (Baba & Allen, 1978). The present observations, taken in conjunction with the previous results, indicate that the binding of the allylic PP substrate to the active site of the C_{55}PP synthetase may require a more extensive cooperation of the homoallylic substrate and divalent cation than that observed with FPP synthetase. The cooperative interaction may be necessary to ensure a tight binding of the allylic PP substrate and intermediates to the catalytic site, which must associate with intermediates having both cis and trans stereochemistry. Furthermore, since the product is much larger with the C_{55}PP synthetase than with FPP synthetase, the assistance of IPP and Mg^{2+} in the binding of the shorter chain starting substrates to the C_{55}PP synthetase could ensure that it binds at the catalytic site instead of at an alternative site in the larger binding domain of the active site.

The availability of these photolabile prenyltransferase substrates now offers a number of other opportunities for their application in studying prenyltransferases. The ability to prepare long-chain photolabile polyprenyl phosphates such as photoactivatable undecaprenyl and dolichyl monophosphates would be of significant value in providing a tool to probe enzymes or enzyme complexes, which use the parent prenyl phosphates as glycosyl carriers in bacterial cell wall biosynthesis or mammalian glycoprotein biosynthesis. The probes will also be used to specifically radiolabel prenyl transferases, so that information concerning their polypeptide composition can be obtained even in partially purified preparations. They may also be useful in selectively inhibiting one or more prenyltransferases in a mixture of such activities.

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Registry No. **1a**, 556-82-1; **1b**, 106-24-1; **2a**, 88887-91-6; **2b**, 60758-60-3; **3a**, 88887-92-7; **3b**, 88887-93-8; **4a**, 88887-94-9; **4b**, 88887-95-0; **5a**, 88887-96-1; **5b**, 88887-97-2; **6a**, 88887-98-3; **6b**, 88887-99-4; *t,t*-FPP, 372-97-4; *o*-azidophenethyl PP, 69258-91-9; *p*-azidophenethyl PP, 69258-90-8; C_{55}PP synthetase, 52350-87-5.

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Mechanism-Based Inactivation of Mitochondrial Monoamine Oxidase by *N*-(1-Methylcyclopropyl)benzylamine†

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ABSTRACT: Three different radioactively labeled *N*-(1-methylcyclopropyl)benzylamines [N-(1-Me)CBA] were synthesized and used to show which atoms of the inactivator remain bound to monoamine oxidase (MAO) after inactivation. Organic chemical reactions were employed to elucidate the structure of the enzyme adduct and clarify the mechanism of inactivation. Following inactivation and dialysis, the benzyl substituent is lost, but the methyl group and cyclopropyl carbons remain attached to the enzyme even after further dialysis against solutions containing 1 mM benzylamine or 8 M urea. Treatment of inactivated enzyme with sodium cyanoborohydride prior to dialysis results in the retention of the benzyl group, suggesting an imine linkage. One hydride from sodium boro[^{3}H]hydride is incorporated into the dialyzed inactivated enzyme consistent with a ketone functional group. When Pronase-digested N-(1-Me)CBA-inactivated MAO is treated with basic potassium triiodide, iodoform is isolated, indicating the presence of a methyl ketone. During inactivation, the optical spectrum of the covalently bound active site flavin changes from that of oxidized to reduced flavin. After urea denaturation, the flavin remains reduced, suggesting covalent linkage of the inactivator to the cofactor. On the basis of previous results [Silverman, R. B., Hoffman, S. J., & Catus, W. B., III (1980) *J. Am. Chem. Soc. 102*, 7126-7128], it is proposed that the mechanism of inactivation involves transfer of one electron from N-(1-Me)CBA to the flavin, resulting in an amine radical cation and a flavin radical. Then, either the cyclopropyl ring is attacked by the flavin radical or the cyclopropyl ring opens, and the radical generated is captured by the flavin radical. The product of this mechanism is the imine of benzylamine and 4-flavinyl-2-butanone, the proposed enzyme–inactivator adduct.

Mitochondrial monoamine oxidase (MAO,[1] EC 1.4.3.4), an enzyme containing covalently bound FAD, is one of the enzymes responsible for the catabolism of biogenic amines. Compounds that inhibit MAO are used clinically as antidepressant agents (Baldessarini, 1977; Berger & Barchas, 1977; Tyrer, 1976); many of the known inhibitors of MAO have been shown to be mechanism-based inhibitors (Maycock et al., 1976; Krantz & Lipkowitz, 1977; Kenney et al., 1979; Silverman & Hoffman, 1980). A mechanism-based inhibitor is an unreactive compound that is converted by an enzyme via its

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[1] Abbreviations: MAO, monoamine oxidase; N-CBA, *N*-cyclopropylbenzylamine; N-(1-Me)CBA, *N*-(1-methylcyclopropyl)benzylamine; FAD, flavin adenine dinucleotide; TLC, thin-layer chromatography; NMR, nuclear magnetic resonance; Tris, tris(hydroxymethyl)-aminomethane.

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