Fluorescent substrate analog for monitoring chain elongation by undecaprenyl pyrophosphate synthase in real time

Kuo-Hsun Teng a,b, Annie P.-C. Chen a, Chih-Jung Kuo a, Yu-Chin Li c, Hon-Ge Liu a, Chao-Tsen Chen c, Po-Huang Liang a,b,*

a Institute of Biological Chemistry, Academia Sinica, Taipei 115, Taiwan, ROC
b Institute of Biochemical Sciences, National Taiwan University, Taipei 106, Taiwan, ROC
c Department of Chemistry, National Taiwan University, Taipei 106, Taiwan, ROC

A R T I C L E   I N F O

Article history:
Received 6 April 2011
Received in revised form 25 May 2011
Accepted 27 May 2011
Available online 7 June 2011

Keywords:
Prenyltransferase
Fluorescent probe
Single turnover
Isotope assay
Inhibitor

A B S T R A C T

Farnesyl pyrophosphate (FPP) is a common substrate for a variety of prenyltransferases for synthesizing isoprenoid compounds. In this study, (2E,6E)-8-O-(N-methyl-2-aminobenzoyl)-3,7-dimethyl-2,6-octadien-1-pyrophosphate (MANT-O-GPP), a fluorescent analog of FPP, was synthesized and demonstrated as a satisfactory substrate for Escherichia coli undecaprenyl pyrophosphate synthase (UPPS) with a \( K_m \) of 1.5 \( \mu M \) and a \( k_{cat} \) of 1.2 \( s^{-1} \) based on \([14C]IPP \) consumption. Interestingly, we found that its emission fluorescence intensity at 420 nm increased remarkably during chain elongation, thereby useful for real-time monitoring kinetics of UPPS to yield a \( K_m \) of 1.1 \( \mu M \) and a \( k_{cat} \) of 1.0 \( s^{-1} \), consistent with those measured using radiolabeled substrate. Using this assay, the IC50 of a known UPPS inhibitor farnesyl thiophosphosphate (FsPP) was confirmed. Our studies provide a convenient and environmentally friendly alternative for kinetics and inhibition studies on UPPS drug target.

© 2011 Elsevier Inc. All rights reserved.

Isoprenoids are natural products composed of 5-carbon isopentenyl pyrophosphate (IPP) as the building block [1,2]. These compounds are ubiquitous in Eukarya, Bacteria, and Archaea and serve a variety of biological functions. To increase structural diversity, IPP is converted to its isomer dimethylallyl pyrophosphate (DMAPP) by the isomerase. Condensation of DMAPP with 2 IPP yields a 15-carbon farnesyl pyrophosphate (FPP) catalyzed by farnesyl pyrophosphate synthase (FPPS), which serves as an outlet point for a variety of different natural isoprenoids. These include linear isoprenyl polymers with designated chain lengths by consecutive condensation of FPP with specific numbers of IPP [3,4].

The enzymes responsible for the synthesis of linear isoprenyl pyrophosphates are classified as cis and trans types according to the stereochemical outcome of the double bonds formed by IPP condensation. Undecaprenyl pyrophosphate synthase (UPPS), a cis-prenyltransferase, catalyzes FPP to the \( E \),\( E \)-form 55-carbon undecaprenyl pyrophosphate (UPP) via consecutive condensation reactions with eight molecules of IPP [5–7], which serves as the lipid carrier in the biosynthesis of bacterial peptidoglycan [8]. Thus, this enzyme is an attractive antibacterial drug target and the inhibitors from high-throughput screening and rational design have been discovered [9,10].

Several FPP analogs have been synthesized for probing the cellular location and protein–ligand interaction of prenyltransferases [11,12]. We previously synthesized a fluorescent analog of FPP, 7-(2,6-dimethyl-8-pyrophospho-2,6-octadienyloxy)-8-methyl-4-trifluoromethyl-chromen-2-one, named TFMC-GPP, by appending a fluorescent group, trifluoromethyl-chromen-2-one (TFMC), to \( C_{10} \)-geranyl pyrophosphate (GPP) to probe UPPS–ligand interactions using a stopped-flow apparatus [13,14]. However, this compound failed to be elongated, probably due to the large size of TFMC and its fluorescence decreases once it is bound. For improvement, we synthesized here a probe \((2E,6E)-8-O-(N\text{-}methyl\text{-}2\text{-}aminobenzoyl)\)3,7-dimethyl-2,6-octadien-1-pyrophosphate (MANT-O-GPP) with the smallest fluorescent MANT ester-linked to GPP to minimize the size and found it can be successfully elongated by UPPS. These two probes are called FPP analogs because a fluorescent group is added into the 10-carbon GPP moiety, making them approximately the size of a 15-carbon FPP. However, emission of MANT-O-GPP at 420 nm approaching visible light gave a better optical signal and fluorescence increase during chain elongation.
elongation which can be used to monitor the UPPS reaction and screen the inhibitor in real time without the need of using isotope assays as reported herein.

**Materials and methods**

**Materials**

Wild-type and D26A and S83A$_5$ mutant UPPS were purified as previously described [15,16]. MANT-O-GPP was synthesized according to reported procedures [17–20] with modifications as shown in Supplementary data. Radiolabeled $[^{14}C]IPP$ (55 mCi/mmol) was purchased from Amersham Pharmacia Biotech. FPP and IPP were obtained from Sigma. Geraniol, 3,4-dihydroxyran (DHP), pyridinium p-toluenesulfonate (PPTs), tert-butyl hydroperoxide, selenium dioxide, salicylic acid, dimethylaminopyridine (DMAP), phthalimide, triphenylphosphine, disisoproplazodicarboxylate, methylamine, $N$-methylisatoic anhydride, triethylamine, $N$-chlorosuccinimide, dimethyl sulfide, and $\text{tris}$(tetra-$n$-butylammonium) hydrogen pyrophosphate were purchased from ACROS. Potato acid phosphatase (2 units/mg) was obtained from Roche Molecular Biochemicals. All commercial buffers and reagents were of the highest grade.

**General methods**

NMR measurements were performed on a VARIAN spectrometer operating at 400 MHz. Mass analysis was carried out using a Jeol-SX102A instrument. FTIR spectra were recorded on Nicolet ter operating at 400 MHz. Mass analysis was carried out using a General methods of the highest grade. Molecular Biochemicals. All commercial buffers and reagents were of the highest grade.

Photospectroscopy and quantum yields of MANT-O-GPP

The absorbance spectra of 1 $\mu$M MANT-O-GPP in ethanol, H$_2$O, and dimethyl sulfoxide (DMSO) were taken on a UV–Vis spectrophotometer (HP 8453, Hewlett Packard). Its corresponding fluorescence emission spectra were recorded on a Hitachi F-4500 spectrophotometer from 360 to 600 nm by using 352 nm as an excitation wavelength. Quantum yields of the compound were determined using 1 $\mu$M coumarin as the reference [21].

Fluorescence titration

A Hitachi F-4500 spectrophotometer was employed to monitor the fluorescence changes. The fluorescence spectra of 1 $\mu$M MANT-O-GPP in the buffer containing 100 mM Hepes–KOH (pH 7.5), 0.5 mM MgCl$_2$, 50 mM KCl at 25 ºC were measured prior to and after the sequential addition of UPPS, respectively. The spectra also were recorded after adding up to 35 $\mu$M FPP into the preformed binary complexes of UPPS/MANT-O-GPP to observe the decreasing fluorescence by FPP replacement.

**Isotope-based kinetic analysis**

A 0.1 $\mu$M UPPS was used in the reaction mixtures containing 100 mM Hepes–KOH (pH 7.5), 0.5 mM MgCl$_2$, 50 mM KCl, 0.1% Triton X-100, and various concentrations of MANT-O-GPP and $[^{14}C]IPP$ as specified below at 25 ºC by following the isotope-based kinetic assay as reported previously [22]. A 0.1% Triton X-100 was included in the reaction so that the product release was not limiting the reaction to allow the measurement of IPP condensation rate at each substrate concentration. For the MANT-O-GPP K$_m$ measurements, 0.5–5 $\mu$M MANT-O-GPP was used along with 50 $\mu$M IPP. For the $[^{14}C]IPP$ K$_m$ and k$_{cat}$ determination, 3.5 $\mu$M MANT-O-GPP was utilized to saturate the enzyme and $[^{14}C]IPP$ concentrations from 1 to 50 $\mu$M were employed. The 40 $\mu$L portions of reaction mixture were periodically withdrawn and mixed with 10 mM EDTA to stop the enzyme reaction. The enzyme initial rate at each substrate concentration was determined within the first 10% substrate depletion. The plot of initial rates vs concentrations of the substrate fitted with the Michaelis–Menten equation (Eq. (1)) gave K$_m$ and k$_{cat}$ (V$_{max}$/[E]) values as described [23].

$$v_0 = V_{max}[S]/(K_m + [S]).$$  

In Eq. (1), $v_0$ is the initial velocity, [E] is the enzyme concentration, [S] is the substrate concentration, V$_{max}$ is the maximum velocity, and K$_m$ is the Michaelis constant.

**Scheme 1.** Synthesis of MANT-O-GPP. (a) DHP, PPTs, CH$_2$Cl$_2$, r.t., 4 h, 90%; (b) SeO$_2$, $^{1}$BuOOH, salicylic acid, CH$_2$Cl$_2$, r.t., 12 h, 40%; (c) N-methylisatoic anhydride, Et$_3$N, DMAP, DMF, 65 ºC, 4 h, 70%; (d) PPTs, EtOH, 60 ºC, 3 h, 80%; (e) NCS, Me$_2$S, CH$_2$Cl$_2$; (f) $^{1}$Bu$_4$N)$_3$HP$_2$O$_7$, CH$_3$CN, r.t., 6 h, 20%.
Fluorescence-based kinetic analysis

Fluorescence change was monitored with time by using a F-4500 fluorescence spectrophotometer (Hitachi) utilizing an excitation wavelength of 352 nm (5 nm slit) and emission wavelength of 420 nm (10 nm slit) during the chain elongation of MANT-O-GPP. A 0.1 μM UPPS was used in the reaction mixtures containing 100 mM Hepes–KOH (pH 7.5), 0.5 mM MgCl₂, 50 mM KCl, 0.1% reduced Triton X-100, and various concentrations of MANT-O-GPP and cold IPP as specified below at 25 °C. For the cold IPP concentration ranging from 0 to 35 μM, the initial rates were obtained at zero and different concentrations of inhibitor and the IC₅₀ value of the inhibitor was determined by fitting the plot of reaction rates versus the inhibitor concentrations using the following equation [23].

\[
A(I) = A(0) \times \left\{ 1 - \frac{I}{IC_{50}} \right\}
\]  

(2)

In this equation, A(I) is the enzyme activity with inhibitor concentration I, A(0) is enzyme activity without inhibitor, and I is the inhibitor concentration.

Fluorescence-based IC₅₀ measurement using MANT-O-GPP

Fluorescence change was monitored by using a F-4500 fluorescence spectrophotometer (Hitachi) utilizing an excitation wavelength of 352 nm (5 nm slit) and emission wavelength of 420 nm (10 nm slit). IC₅₀ of FsPP for 0.1 μM UPPS was performed in a reaction mixture containing 3.5 μM MANT-O-GPP and 50 μM cold IPP in a buffer of 100 mM Hepes–KOH (pH 7.5), 50 mM KCl, 0.5 mM MgCl₂, and 0.1% Triton X-100 in the presence of various concentrations of FsPP ranging from 0 to 35 μM. The initial rates were obtained at zero and different concentrations of inhibitor and the IC₅₀ value of the inhibitor was determined by fitting the plot of reaction rates versus the inhibitor concentrations using the following equation [23].

\[
A(I) = A(0) \times \left\{ 1 - \frac{I}{IC_{50}} \right\}
\]

(2)

Isotope-based IC₅₀ measurement using MANT-O-GPP

IC₅₀ of farnesyl thiopyrophosphate (FsPP) for 0.1 μM UPPS was performed in a reaction mixture containing 3.5 μM MANT-O-GPP and 50 μM [¹⁴C]IPP in a buffer of 100 mM Hepes–KOH (pH 7.5), 50 mM KCl, 0.5 mM MgCl₂, and 0.1% Triton X-100 in the presence of various concentrations of FsPP ranging from 0 to 35 μM. The initial rates were obtained at zero and different concentrations of inhibitor and the IC₅₀ value of the inhibitor was determined by fitting the plot of reaction rates versus the inhibitor concentrations using the following equation [23].

\[
A(I) = A(0) \times \left\{ 1 - \frac{I}{IC_{50}} \right\}
\]

(2)

In this equation, A(I) is the enzyme activity with inhibitor concentration I, A(0) is enzyme activity without inhibitor, and I is the inhibitor concentration.

Synthesis and spectroscopic properties of MANT-O-GPP

As shown in Scheme 1, synthesis of MANT-O-GPP started from commercially available geraniol which was first protected by DHP to generate tetrahydropyranyl ether 2 in good yield. The resulting ether was selectively oxidized with tert-butyl hydroperoxide in the presence of a catalytic amount of SeO₂ to give allylic alcohol 3. The N-methylisatoic anhydride was introduced to 3 via an ester linkage catalyzed by DMAP. Deprotection of DHP group with PPTs yielded 5, which was further converted to the corresponding chloride with NCS/Me₂S, followed by nucleophilic substitution with tris(tetra-n-butylammonium) hydrogen pyrophosphate to give the desired compound MANT-O-GPP.
The spectroscopic properties of MANT-O-GPP were characterized. In aqueous solution, MANT-O-GPP absorbed at \( \lambda_{\text{max}} = 352 \text{ nm} \) \((e_{352} = 5433 \text{ M}^{-1} \text{ cm}^{-1})\) and emitted at \( \lambda_{\text{em max}} = 444 \text{ nm} \) with a quantum yield of 0.23 (Fig. 1A). To test the susceptibility of MANT-O-GPP to the polarity of the surrounding environment, fluorescence quantum yields were measured in ethanol and DMSO. The results showed that the fluorescence intensity of MANT-O-GPP was 1.5 and 4 times larger in ethanol and in less polar DMSO, respectively, compared with that in aqueous solution (Fig. 1A).

**Fluorescence titration**

The fluorescence spectra of 1 \( \mu \text{M} \) MANT-O-GPP was measured prior to and after the sequential addition of 1 \( \mu \text{M} \) UPPS. As shown in Fig. 1B, the fluorescence intensity of 1 \( \mu \text{M} \) MANT-O-GPP was increased by 1.8 times on addition of 4 eq of UPPS and the \( \lambda_{\text{em max}} \) was concomitantly shifted to shorter wavelengths by 30 nm. Adding the cognate substrate FPP into the preformed UPPS/MANT-O-GPP complex decreased the fluorescence intensity back to the original level, indicating that both FPP and MANT-O-GPP bind to the same site.

**MANT-O-GPP served as an alternative substrate of UPPS**

As shown in Fig. 2, MANT-O-GPP was successfully elongated in a UPPS single-turnover reaction with a time course similar to that of FPP, indicating that it can serve as an alternative substrate, but totally 7 IPP were incorporated (one less IPP than those react with FPP). Under steady-state conditions, the kinetic constants were measured to be MANT-O-GPP \( K_m = 1.5 \pm 0.2 \mu \text{M}, \) IPP \( K_m = 23.2 \pm 5.6 \mu \text{M}, \) and \( k_{\text{cat}} = 1.2 \pm 0.2 \text{ s}^{-1} \) (Table 1) using the \([14\text{C}]\text{IPP} \) isotope assay. These values were similar to those measured using FPP with 4-fold larger in MANT-O-GPP \( K_m, \) 6-fold larger in IPP \( K_m, \) and 2-fold smaller in \( k_{\text{cat}}. \)

**Fluorescence-based UPPS kinetic measurements**

As shown in Fig. 3A, the emission fluorescence intensity at 420 nm increased remarkably during the chain elongation of MANT-O-GPP by the wild-type UPPS but not the inactive mutants D26A and S83A5, indicating the feasibility of using fluorescence change to monitor the UPPS reaction in real time. By following the initial rates from the fluorescence increase under different concentrations of MANT-O-GPP and IPP (Fig. 3B and C), the kinetic constants were measured to be MANT-O-GPP \( K_m = 1.1 \pm 0.2 \mu \text{M} \) and IPP \( K_m = 27.9 \pm 0.1 \mu \text{M} \) (Table 1). Using the standard curve as

<table>
<thead>
<tr>
<th>Condition</th>
<th>( k_{\text{cat}} ) (s(^{-1}))</th>
<th>( K_m ) (( \mu \text{M} ))</th>
<th>IPP ( K_m ) (( \mu \text{M} ))</th>
</tr>
</thead>
<tbody>
<tr>
<td>FPP + [( ^{14}\text{C}]\text{IPP} ) (isotope)</td>
<td>2.5 ± 0.1</td>
<td>0.4 ± 0.1</td>
<td>4.1 ± 0.3</td>
</tr>
<tr>
<td>MANT-O-GPP + [( ^{14}\text{C}]\text{IPP} ) (isotope)</td>
<td>1.2 ± 0.2</td>
<td>1.5 ± 0.2</td>
<td>23.2 ± 5.6</td>
</tr>
<tr>
<td>MANT-O-GPP + cold IPP (fluorescence)</td>
<td>1.0 ± 0.1</td>
<td>1.1 ± 0.2</td>
<td>27.9 ± 0.1</td>
</tr>
</tbody>
</table>

Fig. 3. Monitoring UPPS reaction using MANT-O-GPP in real time. (A) The fluorescence increased remarkably during the condensation reactions in wild-type UPPS (●), but not in the D26A (○) and S83A5 (▲) inactive UPPS in the solution containing 0.1 \( \mu \text{M} \) enzyme, 3.5 \( \mu \text{M} \) MANT-O-GPP, and 50 \( \mu \text{M} \) IPP. (B) Measurements of fluorescence increase using 0.1 \( \mu \text{M} \) UPPS, 0.5 \( \mu \text{M} \) (●), 1 \( \mu \text{M} \) (○), 2 \( \mu \text{M} \) (▲), 3.5 \( \mu \text{M} \) (Δ), 5 \( \mu \text{M} \) (■) MANT-O-GPP, and 50 \( \mu \text{M} \) IPP. The results indicate enzyme concentration-dependent increase of activity. (C) Measurements of fluorescence increase using 0.1 \( \mu \text{M} \) UPPS, 3.5 \( \mu \text{M} \) MANT-O-GPP, and 1 \( \mu \text{M} \) (●), 2 \( \mu \text{M} \) (○), 5 \( \mu \text{M} \) (▲), 10 \( \mu \text{M} \) (Δ), 20 \( \mu \text{M} \) (■), and 50 \( \mu \text{M} \) (□) IPP. (D) The standard curve correlates the fluorescence increase with \([^{14}\text{C}]\text{IPP} \) consumption.
shown in Fig. 3D, to correlate fluorescence increase with $[^{14}\text{C}]$IPP consumption, the $k_{\text{cat}} = 1.0 \pm 0.1$ s$^{-1}$ (Table 1). These kinetic constants calculated from the fluorescence increase were consistent with MANT-O-GPP $K_m$ = 1.5 ± 0.2 µM, IPP $K_m$ = 23.2 ± 5.6 µM, and $k_{\text{cat}} = 1.2 \pm 0.2$ s$^{-1}$ (Table 1) obtained from isotope assay.

Fluorescence-based inhibition constant measurements

Since the fluorescence properties have been demonstrated useful for measuring enzyme kinetics, we have further used the probe to measure inhibition constants. Using the fluorescence assay under different concentrations of FsPP (Fig. 4A), a FPP thiol analog used as an inhibitor, gave IC$^{50} = 3.9 \pm 0.2$ µM (Fig. 4B), which was similar to IC$^{50} = 2.4 \pm 0.2$ µM measured by the isotope assay (Table 2).

Discussion

Fluorescent probes are indispensable tools for visualizing molecular recognition events temporally. A natural substrate judiciously appended with environmentally sensitive fluorophores could act as a fluorescent probe. On binding with the targeted enzymes or proteins, the fluorophores would experience the polarity of the local environmental changes and result in changes in fluorescence properties. MANT fluorophore was chosen here not only because it is the smallest fluorophore reported so far, having small fluorescence properties. MANT fluorophore was chosen here not only because it possesses a unique excited-state intramolecular proton transfer (ESIPT) [24–26] with a 6-member ring type of MANT group when it reaches the bottom of the UPPS active site by chain elongation. Importantly, on binding of MANT-O-GPP with UPPS, the fluorophore experiences a polarity of local environmental changes (more hydrophobic) and results in increased fluorescence intensity. As shown, chain elongation by reacting with IPP further causes the increase of emitted fluorescence.

By taking advantage of the fluorescence changes on chain elongation, MANT-O-GPP can be used to measure the UPPS kinetics and inhibition constants without the need of using the isotope substrate. Although the EnzChek pyrophosphate assay kit (Invitrogen), a commercial coupling enzyme method, is available for detecting released pyrophosphate through the formation of a chromophoric product for monitoring prenyltransferase reactions [29], our method is less tedious and less expensive. Moreover, our method is more environmentally friendly and easier to use by saving the extraction and radiocounting steps. Moreover, since MANT-O-GPP can be elongated to a larger product, this probe and its long-chain product may be potentially used to screen allosteric inhibitors such as the tetramic acid [30], which should not exclude MANT-O-GPP but its product from the UPPS active site. In summary, our study provides a more convenient and environmentally friendly assay for high-throughput screening, as well as kinetics and inhibition studies on UPPS drug target.

Acknowledgment

The authors thank the National Science Council, Taiwan, for financial support.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ab.2011.05.043.

References


