Drastic changes in the substrate specificity of geranylgeranyl diphosphate synthase from *Sulfolobus acidocaldarius* by a single amino acid substitution

Takeshi Nakamura, Masahiko Nagaki* and Norimasa Ohya
Department of Material and Biological Chemistry, Faculty of Science, Yamagata University, Kojirakawa-machi, Yamagata 990-8560, Japan
Fax: 81-23-628-4583, e-mail: ohy@sci.kj.yamagata-u.ac.jp
*Graduate School of Science and Technology, Hirosaki University, 3 Bunkyo-cho, Hirosaki, Aomori 036-8561, Japan

Geranylgeranyl diphosphate (GGPP) synthase catalyzes the condensation of isopentenyl diphosphate with dimethylallyl diphosphate, geranyl diphosphate or farnesyl diphosphate to produce GGPP as the final product. GGPP synthase in the thermophilic bacterium *Sulfolobus acidocaldarius* can hardly accept substrate analogs possessing oxygen atoms in their prenyl chains. We have prepared several point-mutated *S. acidocaldarius* GGPP synthases in which phenylalanine was substituted with glycine (F77G) or serine (F77S). Interestingly, the reactivity of the mutated GGPP synthase was enhanced with respect to substrate analogs possessing \(\omega\)-oxygen atoms in their prenyl chains.

Key words: prenyltransferase, geranylgeranyl diphosphate, substrate specificity, substrate analog

1. INTRODUCTION

Prenyltransferase stereospecifically catalyzes the condensation of isopentenyl diphosphate (IPP) with an allylic diphosphate, and the condensation precisely terminates when the prenyl chain reaches a certain length depending on the individual specificity of the enzyme. These activities of the enzymes can be classified into two major types: \((E)\)- or \((Z)\)-prenyl chain elongation reactions [1,2]. Geranylgeranyl diphosphate (GGPP) synthase (EC. 2. 5. 1. 29) is a short prenyl diphosphate synthase that is classified as an \((E)\)-type prenyltransferase. It catalyzes the “head-to-tail” condensation of IPP with dimethylallyl diphosphate (DMAPP), geranyl diphosphate (GPP), or farnesyl diphosphate (FPP) to produce all-\((E)\) GGPP [1-3]. Koyama et al. [4] performed gene cloning to facilitate the efficient overproduction in *Escherichia coli* cells and purification of FPP synthase from a thermophilic bacterium, *Bacillus stearothermophilus*. By comparing the primary structures of multiple \((E)\)-type prenyltransferases, they suggested that prenyltransferases have seven conservative motifs in their amino sequences [4], two of which are aspartate-rich motifs. Subsequently, Ohnuma et al. [5] reported that a tyrosine located at the fifth position upstream of the first aspartate-rich motif regulates prenyl chain elongation by hydrophobic interaction between the \(\omega\)-terminal of the reaction product and the side chain of tyrosine. Amino acid substitution for this tyrosine caused the mutated FPP synthase to catalyze the synthesis of GGPP and longer prenyl diphosphates [5]. On the basis of the findings of Ohnuma et al. [5], we anticipated that some mutated GGPP synthases would exhibit altered substrate specificities and accept substrate analogs possessing hydrophilic moieties in their alkyl chain if the phenylalanine at position 77 (which corresponds to the fifth residue upstream of the first aspartate-rich domain) of the thermostable GGPP synthase was replaced with other amino acid residues possessing hydrophilic side chains. Thus, we have examined two mutated GGPP synthases from *S. acidocaldarius*, F77G and F77S, in which the phenylalanine at position 77 was substituted with glycine (F77G) or serine (F77S). Interestingly, the reactivity of the mutated GGPP synthase was enhanced with respect to substrate analogs possessing \(\omega\)-oxygen atoms in their prenyl chains.

2. EXPERIMENTAL

2.1 Chemicals

All-\((E)\) GPP, all-\((E)\) FPP and DMAPP were prepared as described previously [5, 6]. Substrate analogs 1 - 8 (Fig. 2) of GPP were synthesized according to previously reported method [7-9]. Diphosphorylation of the corresponding alcohol was performed by the method of Davison et al. [10]. \([1-14C]\) IPP was purchased from Amersham Corp. The point-mutated enzymes F77G and F77S were prepared as described previously [5].
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2.2 Enzymatic reaction conditions
The activity of each enzyme was measured by determining the amount of [1-14C]IPP incorporated into hexane-extractable hydrolysates derived from the enzymatic product after acid hydrolysis. The incubation mixture for the GGPP synthase reaction contained the following materials in a final volume of 1 mL: 50 mM phosphate buffer (pH 5.8), 5 mM MgCl₂, 50 mM ammonium chloride, 50 mM 2-mercaptoethanol, 0.01% Triton X-100, 25 μM GPP or the substrate analog being examined, 25 μM [1-14C] (specific activity 37 GBq/mol), and a suitable amount of the wild-type or a mutant GGPP synthase. After incubation at 37°C for 15 min, the reaction was terminated by the addition of HCl to the mixture. Then, the reaction mixture was treated with hexane, and the radioactivity of the hexane extract was measured using a liquid scintillation counter.

2.3 Purification of wild-type GGPP synthase and the mutants F77G and F77S
E. coli DH5α cells harboring the wild-type or mutant S. acidocaldarius gene that had been incubated and treated with isopropyl-β-D-thiogalactosylpyranoside to overproduce the prenyltransferase were disrupted by sonication. After heat treatment to inactivate endogenous proteins from E. coli, the cell-free extract was collected by ultracentrifugation (100,000 × g) and fractionated with ammonium sulfate, followed by two chromatographies (Butyl- and DEAE-Toyopearl) [4]. The point-mutated GGPP synthases F77G and F77S exhibited chromatographic properties similar to that of the wild-type GGPP synthase during purification.

3. RESULTS

3.1 Enzyme activities of GPP substrate analogs possessing oxygen atoms in the prenyl chains (1)
Using wild-type GGPP synthase, 1, 2, and 3 exhibited lower reactivities than GPP (Table 1). However, 2 and 3 exhibited high reactivities (exceeding 100%) with F77G GGPP synthase (Table 1). Moreover, 1, 2, and 3 exhibited much higher reactivities with F77S GGPP synthase than with GPP (Table 1), with 2 exhibiting the highest reactivity (283%) (Table 1). Using wild-type GGPP synthase, 3, possessing the same alkyl side chain as FPP, exhibited the highest reactivity; conversely, using F77G and F77S, 2, possessing carbon chains shorter than FPP, exhibited the highest reactivity (Table 1).

3.2 Enzyme activities of GPP substrate analogs possessing oxygen atom in the prenyl chain (2)
Using wild-type GGPP synthase, 5 exhibited lower reactivity than GPP; however, 4, which has an oxygen atom at position 11 of the alkyl chain, exhibited high activity (Table 1). Using F77G, 4 and 5 exhibited the same reactivity as GPP; however, using F77S, 4 and 5 exhibited much higher reactivity than GPP, with 5 exhibiting the highest reactivity (Table 1). Next, we examined 6, 7, and 8, which have longer alkyl chains than 3, 4, and 5. Using wild-type GGPP synthase, 6 and 8 exhibited lower reactivities than GPP; however, 7, which has an oxygen atom at position 11 of the alkyl chain, exhibited high reactivity similar to that of 4 (Table 1). Conversely, using F77G, 6, 7, and 8 exhibited the same trend as 3, 4, and 5; however, using F77S, 6, 7, and 8 exhibited high reactivity, with 8 exhibiting the highest reactivity (Table 1).
4. DISCUSSION

It might be seen that recognition site of prenyl chain in GGPP synthase is cavity contained walls of hydrophobic acids, which was accepted on hydrophobic substrate. With wild-type GGPP, the GPP substrate analogs 1, 2, and 3 exhibited lower reactivity than GPP, the natural substrate of wild-type GGPP. However, using two point-mutants of GGPP synthase, F77G and F77S, all substrate analogs exhibited much higher reactivities than wild-type GGPP, suggesting that the substrate specificity was largely changed. The hydrophobicity of F77G was weaker than that of wild-type GGPP, and the substrate specificity of F77G was changed from phenylalanine to glycine. In F77S, the hydrophobic cavity was changed to a hydrophilic cavity, and as a result, hydrophilic amino acid side chains interacted and formed hydrogen bonds with oxygen atoms in the alkyl chains of substrate analogs. Substrate analogs, which have oxygen in the alkyl chain, were changed to maximum of activity to interact hydrophilic amino acid with hydrogen bond, so we suggested that 2, which is shorter than FPP, showed peak count of activity.

Using wild-type GGPP synthase, 3 and 6, which have oxygen atoms at position 9 of their alkyl side chains, or 5 and 8, which have two oxygen atoms in their alkyl side chains, exhibited much higher reactivities with wild-type GGPP synthase than with F77G and F77S. We suggest that the stability of the substrate analogs allowed them to interact with GGPP at the hydroxyl group of serine at position 148, which is located in the outer hydrophobic cavity, when repulsion between phenylalanine, located at position 77 of GGPS, and oxygen, located at position 11 of substrate analog, occurred (Fig. 3). Whereas, 3 and 6 exhibited repulsion of the hydrophobic cavity in GGPP synthase through an oxygen atom at position 9 in their alkyl side chains, 5 and 8 exhibited much stronger repulsion of the hydrophobic cavity through an oxygen atom at position 9 in their alkyl side chains despite the presence of an oxygen atom at position 11.

The point-mutant F77G exhibited low reactivity with each substrate analog. We suggest that F77G tended to incorporate substrates due to the wider space of its hydrophobic cavity or the higher hydrophobicity induced by the point-mutation.

The point-mutant F77S exhibited high reactivity with each substrate analog. In particular, 5 and 8, which have two oxygen atoms in their alkyl side chain, exhibited over 2-fold higher reactivity than 3, 4, 6, and 7, which have only a single oxygen atom in their alkyl side chain. We suggest that interactions occur between both the oxygen at position 11 in the alkyl chain and the hydroxyl group of serine at position 11, and between the oxygen at position 9 in the alkyl chain and the other hydroxyl group of serine at position 148.

5. CONCLUSION

In this study, we have demonstrated that a single amino acid substitution dramatically altered the substrate specificity of GGPP synthase from S. acidocaldarius. Although wild-type GGPP synthase can hardly accept substrates possessing a hydrophilic moieties in their

Table 1. Relative activities of wild-type and mutated GGPP synthase (F77G and F77S) with GPP or the analogs (1-8) as allylic substrate. Counts indicate relative activities that the activity of wild-type GGPP synthase with GPP is shown 100.

<table>
<thead>
<tr>
<th>Analog</th>
<th>W.T.</th>
<th>F77G</th>
<th>F77S</th>
</tr>
</thead>
<tbody>
<tr>
<td>GPP</td>
<td>100.0</td>
<td>24.6</td>
<td>24.4</td>
</tr>
<tr>
<td>1</td>
<td>44.9</td>
<td>48.0</td>
<td>155.8</td>
</tr>
<tr>
<td>2</td>
<td>65.0</td>
<td>116.0</td>
<td>283.0</td>
</tr>
<tr>
<td>3</td>
<td>69.7</td>
<td>103.7</td>
<td>155.4</td>
</tr>
<tr>
<td>4</td>
<td>267.8</td>
<td>90.0</td>
<td>157.3</td>
</tr>
<tr>
<td>5</td>
<td>39.0</td>
<td>100.5</td>
<td>527.6</td>
</tr>
<tr>
<td>6</td>
<td>56.9</td>
<td>89.1</td>
<td>128.2</td>
</tr>
<tr>
<td>7</td>
<td>169.0</td>
<td>78.0</td>
<td>95.3</td>
</tr>
<tr>
<td>8</td>
<td>34.3</td>
<td>81.6</td>
<td>465.6</td>
</tr>
</tbody>
</table>

Figure 3. The hypothesis of reaction mechanism of wild-type GGPP synthase and 4, substrate analog. D, Asp; S, Ser; F, Phe
prenyl chains, point-mutated GGPP synthases, such as F77G and F77S, can easily accept these substrates. These results suggest that the mutated GGPP synthases would be more applicable to the organic synthesis of bioactive substances possessing $\omega$-oxygen atoms in their prenyl chain compared to the wild-type enzymes.

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7. REFERENCES


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