Original Article

In Silico study of the conformational flexibility of point mutated *Geobacillus stearothermophilus* farnesyl diphosphate synthase

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Abstract

Farnesyl diphosphate synthase from Geobacillus stearothermophilus (*Gs*FPPS) is a thermophilic enzyme which belongs to the prenyltransferase family. We found that the point mutation of an amino acid located on the fifth position upstream of the first aspartate-rich motif (FARM) in *Gs*FPPS (tyr81) affects its flexibility and activity. Enzymatic activities of wild-type *Gs*FPPS, Y81D, Y81R, and Y81S were measured by a ^{14}C radioactivity assay. Their flexibilities were assessed by monitoring the fluorescence intensity changes due to the presence of a collisional quencher and also by coarse-grained molecular dynamics (MD) simulations. Y81R and Y81S showed enhanced activities of up to 1.7-fold, while activity of Y81D was reduced by 1.2-fold. The differences in activity between these enzyme variants are caused by the differences in conformational flexibility, especially in terms of the dynamics of the areas near the active site.

Key words

conformational flexibility, enzymatic activity, farnesyl diphosphate synthase, point mutation, coarse-grained MD simulation

1. Introduction

Prenyltransferases are a family of enzymes that catalyze condensation reaction between isopentenyl diphosphate (IPP) and allylic diphosphates, which is the fundamental reaction of the isoprenoid pathway. These enzymes can be classified based on the stereochemistry of their double bonds and the chain length of the final product (Ito et al., 1987; Ogura and Koyama, 1998).

Farnesyl diphosphate synthase (FPPS; EC 2.5.1.10) belongs to the group of short-chain prenyltransferases and serves as the central enzyme in the isoprenoid chain elongation pathway. It catalyzes the consecutive condensation of two molecules of IPP (C5) with dimethylallyl diphosphate (DMAPP, C5) to give geranyl diphosphate (GPP, C10) and then (*2E*, *6E*)-farnesyl diphosphate (FPP, C15) as the final product. FPPS is the most studied enzyme among *E*-prenyltransferases because of its important role (Poulter, 2006), thermostable FPPSs especially have attracted much attention.

Koyama et al. (1993) have successfully carried out gene cloning, efficient overproduction in *Escherichia coli* cells and purification of *Gs*FPPS. By comparing the amino acid sequences of many *E*-prenyltransferases, they suggested that prenyltransferase family share seven conserved regions, two of which are characteristic aspartate-rich motifs DDXXD. Ohnuma et al. (1996) have found that tyr81, which is located at the fifth position before the FARM, is responsible for the regulation of the product chain length. Substitution of this tyrosine caused the mutated *Gs*FPPS to catalyze the synthesis of geranylgeranyl diphosphate (GGPP, C20) or even longer prenyl diphosphates. The replacement of the amino acid having a hydrophilic side chain alter the substrate specificities of prenyltransferase (Maki et al., 2002). Based on these findings, we expected that these variants would also change their activities toward natural FPPS substrates. Thus, in this study, we examined three variants in which tyr81 was replaced with aspartic acid (Y81D), with arginine (Y81R), and with serine (Y81S). Here, we describe the change of activity of the variants when GPP was used as the initial substrate. We also examined the conformational flexibilities using a fluorescence analysis method and in silico approaches, to investigate the effect of single amino acid substitution on the affinity/activity of *Gs*FPPS.

2. Materials and methods

2.1 Enzyme and chemicals

Alkaline phosphatase, with a specific activity of 10 DEA unit/mg and GPP ammonium salt was purchased from Sigma-Aldrich Co. [1-^{14}C] IPP was purchased from Amersham Corp. Succinimide was from Tokyo Chemical Industry Co. All other chemicals and solvents used were purchased from commercial suppliers.

2.2 Purification of wild-type and mutated *Gs*FPPSs

Purification of wild-type *Gs*FPPS, Y81D, Y81R, and Y81S were carried out according to the previously reported method (Ohnuma et al., 1996), with some modifications. *E. coli* cells harboring expression plasmids of wild-type or mutated *Gs*FPPS genes were cultured overnight at 37 °C in a LB medium containing 100 μg ampicillin/ml. The bacterial cells were transferred to 100 volumes of the same fresh medium and were again grown at 37 °C to an OD_{600} of approximately 0.6. Protein

expression was then induced with 0.1 mM IPTG, and cells were incubated overnight. The cultured cells were harvested and disrupted by sonication in 10 mM Tris/HCl buffer (pH 8.0). The homogenate was heated at 55 ℃ for 1 h, fractionated with 35-60 % ammonium sulfate solution, and purified using a TOYOPEARL DEAE-650M anion exchange chromatography column (Tosoh Corporation, Japan). Purity of the protein fractions was analyzed by SDS–PAGE. The three mutated enzymes showed chromatographic properties similar to those of the wild-type. Protein concentration was determined using a Bradford Assay Kit (Takara, Japan) with BSA used as the standard.

2.3 Measurement of enzymatic activities of wild-type and mutated *Gs*FPPSs

Enzymatic activities were measured by determination of the amount of [1-^{14}C] IPP incorporated into 1-butanol-extractable hydrolysates derived from the enzymatic products. The assay mixture contained, in a final volume of 1 ml, 50 mM Tris/HCl buffer (pH 8.5), 5 mM $MgCl_2$, 50 mM NH_4Cl, 50 mM 2-mercaptoethanol, 50 μM GPP, 25 μM [1-^{14}C] IPP (specific activity 37 GBq mol^{-1}) and 1.6 μM wild-type or mutated *Gs*FPPS. After incubation at 55 °C for 2 h, the reaction mixture was treated with alkaline phosphatase, extracted with 1-butanol and the radioactivity of the butanol extract was measured using an LSC-5100 liquid scintillation counter (Aloka, Japan).

2.4 Fluorescence quenching measurement

Fluorescence steady-state measurements were performed using an F-2000 spectrofluorometer (Hitachi, Japan). The cuvette contained 5 mM Tris/HCl buffer, 3.8 μM wild-type or mutated *Gs*FPPS, and various concentrations of succinimide (0-0.07 M), in a total volume of 1 ml. All samples were incubated with continuous shaking at 25 ℃ for 10 min before measurement. Samples were excited at 280 nm (excitation of tyrosine residues) and the emission spectra recorded from 300 nm to 400 nm. Quenching data in this study were analyzed by the Stern-Volmer equation as follows:

$$\frac{F_0}{F} = 1 + K_{sv}[Q] \quad (1)$$

Where F_0 and F are the fluorescence intensities in the absence and presence of quencher, [*Q*] is the quencher concentration, and K_{SV} is the collisional quenching constant (Stern-Volmer constant).

2.5 Missing loops modeling, point mutation modeling, and coarse-grained MD simulation

The crystal structure of *Gs*FPPS was previously determined at 2.31 Å (PDB ID: 5AYP) (Samori et al., 2017). The missing loop region between residues 224 and 256 in 5AYP was inserted as an unstructured loop of 33-residues length using MODELLER v9.18 (Fiser et al., 2000). The best model according to the MODELLER energy function and Ramachandran plot statistic was then used to construct the 3D models of point-mutated

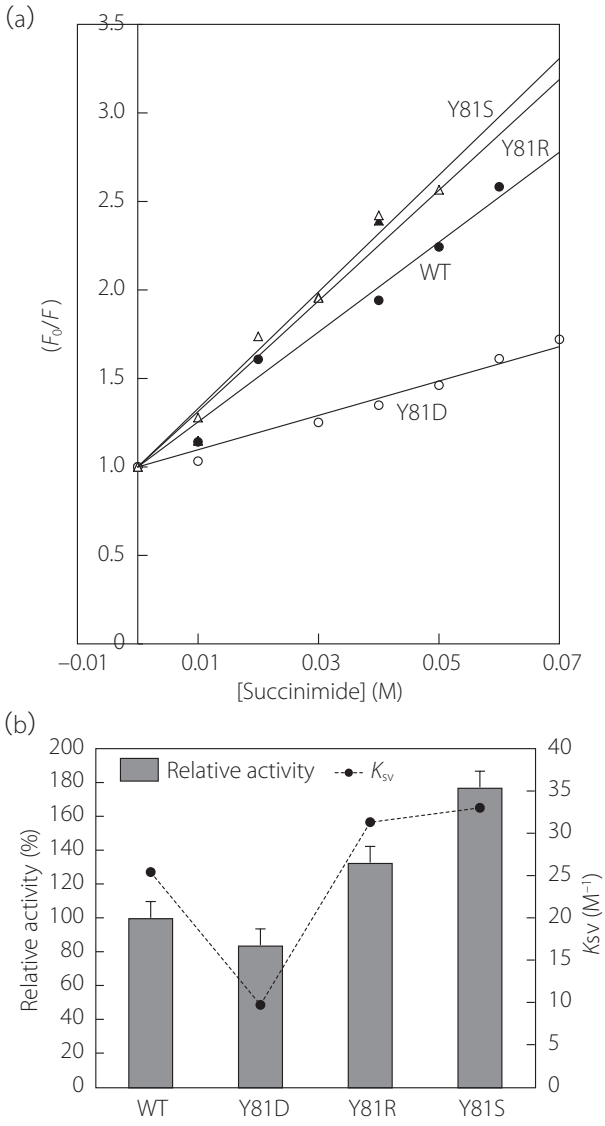

Figure 1: Conformational flexibilities and enzymatic activities of wild-type and mutated *Gs*FPPSs

Notes: A. Stern-Volmer plots (Eq. 1) of succinimide quenching of tyrosine fluorescence for wild-type *Gs*FPPS (●), Y81D (○), Y81R (▲) and Y81S (△). Linear regression lines were calculated for each enzyme. For wild-type *Gs*FPPS: $y = 25.4x + 1$ ($r = 0.98$). For Y81D: $y = 9.7x + 1$ ($r = 0.98$). For Y81R: $y = 31.3x + 1$ ($r = 0.93$). For Y81S: $y = 33.0x + 1$ ($r = 0.99$). B. Enzymatic activities of *Gs*FPPS and the variants when GPP was used as the substrate. Activity of the wild-type (WT) was set to 100 %. K_{SV} values were also plotted to show the relation between activity and flexibility.

enzymes Y81D, Y81R, and Y81S. Structural flexibilities of *Gs*FPPS and the variants were assessed by the coarse-grained MD simulation implemented in the CABS-flex server (http://biocomp. chem.uw.edu.pl/CABS flex/) (Jamroz et al., 2013).

3. Results and discussion

3.1 Activity-flexibility relationship

The 3D structure of *Gs*FPPS shows that it has eight tyrosine residues, with five of the residues located at the surface of the enzyme. The remaining three, including tyr81, are located in-

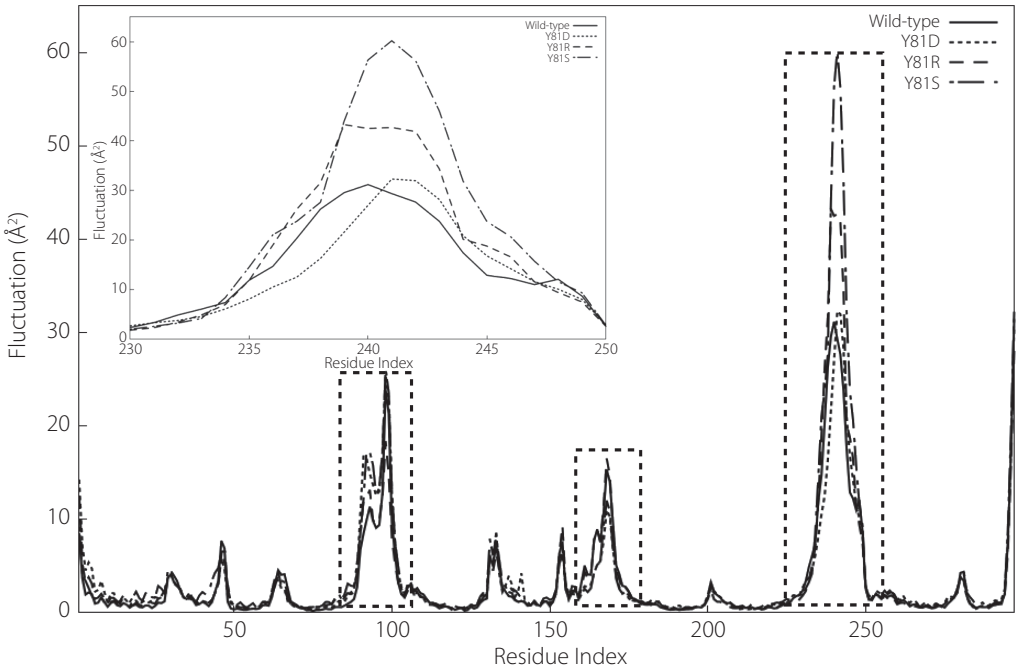

Figure 2: Fluctuation profiles of wild-type *Gs*FPPS and the variants

Notes: Three loop regions that are located on the top of the active site are indicated by bold black-dashed lines. Fluctuations of the active site gatekeepers are shown in the inset.

side the pocket or buried inside the protein (Figure 3). Quenching experiments were conducted to monitor the accessibility of these tyrosine residues, which can be used as an indicator of the conformational flexibility. Succinimide was used because it was reported to be a useful quencher for proteins which contain tyrosine and no tryptophan (Eftink and Ghiron, 1984). In this study, the quenching process of wild-type *Gs*FPPS and the variants were analyzed using a simple collisional quenching model, which is expressed by the Stern-Volmer relation (Eq. 1) (Figure 1 (a)). We obtained K_{SV} values of 25.4 M^{-1}, 9.7 M^{-1}, 31.3 M^{-1}, and 33.0 M^{-1} for the wild-type, Y81D, Y81R, and Y81S, respectively. This result indicates that replacement of tyr81 with

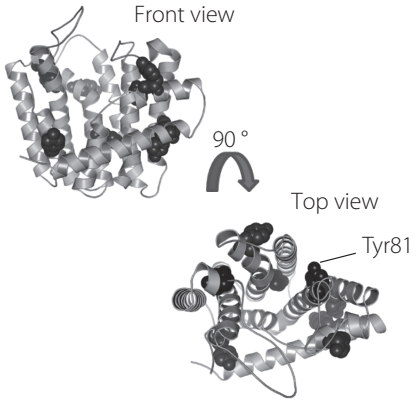

Figure 3: 3D structure of wild-type *Gs*FPPS

Notes: Five tyrosine residues located at the surface of the enzyme, and three tyrosine residues located inside the pocket are shown as spheres. Three important loop regions are shown as wire.

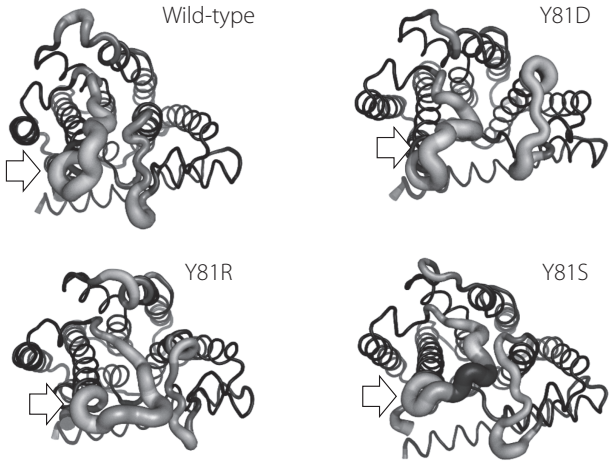

Figure 4: Fluctuation profiles of wild-type *Gs*FPPS and the variants visualized as 3D structures

Notes: Fluctuation values are shown as thickness of tubes. A big tubes indicate that the large fluctuations. Active site gatekeepers of each enzyme are marked with arrows.

arginine and serine increased the flexibility of *Gs*FPPS structure, while replacement with aspartic acid caused the opposite effect. We also measured the relative activities of *Gs*FPPS and the variants toward GPP. Activities of Y81R and Y81S enhanced by 1.3-fold and 1.7-fold, respectively, compared to the wild-type. On the other hand, activity of Y81D was reduced by 1.2-fold. The relative activities and the K_{SV} values showed a good relationship (Figure 1 (b)), where the activity of the enzyme increased with increasing conformational flexibility.

3.2 Dynamics of the active site gatekeeper

Coarse-grained MD simulations were performed on wild-type *Gs*FPPS, Y81D, Y81R, and Y81S on the CABS-flex server to simulate the near-native dynamics of these enzymes. Mean-square-fluctuations of calculated trajectories are shown in Figure 2. All the three variants possess similar fluctuation characteristics with the wild-type, except for three regions (indicated with bold black-dashed lines in Figure 2). These regions are the three loops that located on the top of the active site (Figure 3). Comparison of fluctuations of the three regions reveals that Y81S has the highest flexibility, followed by Y81R, wild-type *Gs*FPPS and Y81D with the lowest flexibility (Figure 2 and 4). Flexibility of these regions, especially the loop between residues 229 and 256, may be crucial for the reaction, based on the molecular mechanism of the FPPS reaction (Hosfield et al., 2004, Kavanagh et al., 2006). In order to catalyze a condensation reaction, FPPS must undergo an open-to-closed conformational change induced by allylic substrate binding. In this step, the loop (residues 229-256) may act as an active site gatekeeper. Increasing in flexibility of this loop makes it easier for the enzyme to undergo the conformational change. Based on this fact, one can assume that tyr81 is responsible not only for the regulation of the product chain length, but also the dynamics of the gatekeeper (conformational flexibility).

4. Conclusion

Two variants in this work, Y81R and Y81S, showed some improvements in flexibilities and activities toward GPP, which revealed that conformational flexibility is important for the reaction. This fact is also supported by the simulation results. Each enzyme which belongs to the short-chain prenyltransfease group has a bulky amino acid (like phenylalanine or tyrosine) at the fourth or fifth position before the FARM, thus, we believe that our results also provide useful information for protein engineering involving this enzyme group.

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