Structure and Function of Octaprenyl Pyrophosphate Synthase from Hyperthermophilic *Thermotoga maritima*

Life in extreme environment has to cope with its surrounding in some particular ways. Bacteria in hot spring often possess stable proteins, which are more ameable to a number of biochemical studies including X-ray crystallography. Isoprenoid compounds are widely distributed in nature and serve a variety of important biological functions. The C_{40} product of octaprenyl pyrophosphate synthase (OPPs) constitutes the side chain of bacterial ubiquinone, an essential component in the electron transfer reactions. Because of its importance, this enzyme is a good target for development of new antibiotics by rational design based on its 3D structure. While the OPPs from the mesophilic Escherichia coli is not easily crystallized in a suitable unit cell for structure determination, we have been successful by working on the equivalent enzyme from Thermotoga maritima.

Prenyltransferases catalyze consecutive condensation reactions of isopentenyl pyrophosphate (IPP) with allylic pyrophosphate to generate linear isoprenyl polymers. Starting from IPP and its isomer dimethylallyl pyrophosphate, the C_{15} farnesyl pyrophosphate (FPP) is formed by farnesyl pyrophosphate synthase (FPPs). Using FPP and IPP as substrates, C_{40} octaprenyl pyrophosphate (OPP) is synthesized by octaprenyl pyrophosphate synthase (OPPs) via five IPP condensation reactions with FPP. This polymer serves as the side chain of bacterial ubiquinone or menaquinone, a component involved in electron transfer for oxidative phosphorylation. During each IPP condensation, a new double bond is formed. Thus the prenyltransferases are classified as *cis*- and *trans*-type depending on the stereoisomer of the double bond formed. Two DDxxD motifs in the amino acid sequences were found in *trans*-type prenyltransferases. The first motif is responsible for binding and reaction with FPP and the second motif for IPP binding. OPPs is a *trans*-type enzyme synthesizing the C_{40} long-chain product. The only other *trans*-type enzyme with known structure is FPPs of the short chain type. Based on its 3-D structure and mutagenesis studies, a bulky amino acid residue located in the 5th position before the first DDxxD motif of FPPs appeared to block further elongation of the product FPP. The corresponding amino acid in OPPs is substituted with a small amino acid Ala which may be required to remove the steric obstacle for OPPs to synthesize longer product than FPP. Further elongation is stopped by the large amino acids at the distal end of the active site to form the final C_{40} product.

In the present studies, we have solved the crystal structures for wild-type and several mutants' OPPs. Two identical subunits are associated into a dimer by forming a four-layer helix bundle using helices E and F (Fig. 1A and 1B). The refined

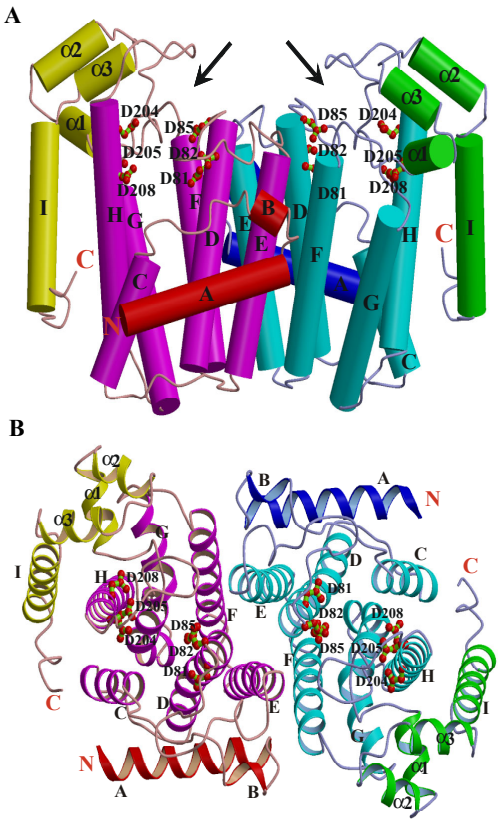

Fig. 1: *In A, the side view model of T. maritima OPPs is shown using a cylinder diagram. Two identical subunits are associated into a dimer by forming a four-layer helix bundle. In B, the top view model of T. maritima OPPs is shown using a ribbon diagram.*

structure of wild-type OPPs in complex with six sulfate ions contains amino acid residues 9-288 in two subunits. The structure contains 12 α helices, 9 of them surrounding a large central cavity (helix A to I). Two conserved DDxxD sequences are located on helices D and H near the opening of this deep cleft of the substrate-binding pocket. Between helix H and I are three short α helices, with helix α1, α2, α3 on the outer surface of the α-cone. Although helices α1, α2 and α3 are not directly involved in the structure of active site, it may pull loop2 to keep the top of the active site tunnel open and allow the IPP and FPP substrates to enter the tunnel.

An elongated tunnel-shaped cavity surrounded by 4 α-helices (Helix C, D, F and H) can be proposed as the active site since the two DDxxD motifs for substrate binding are located in helix D and H, respectively, near the top of the tunnel (Fig. 1A). Hydrophobic side chains of the amino acids cover the entire inner surface of the tunnel, except near the two DDXXD conserve motifs for substrate binding. The amino acids in the region of substrate binding site are displayed in Figure 2. We observed two sulfate ions (S1 and S2) in the active site with S1 near the first DDXXD motif and S2 further down in the active site surrounded by K41, R44 and H74.

Further down the tunnel, two large amino acid residues, F52 and F132, occupy the bottom portion of the tunnel. One of these residues or both may provide the blockage for further chain elongation of C_{40} product. We replaced these large residues with Ala and examined the chain length of the products synthesized by the mutant enzymes by TLC analysis (Fig. 3A). Both mutant enzymes F52A and F132A have similar activity compared to the wild-type. Under the same reaction condition, F52A generated products similar to that of the wild-type OPPs. In contrast, F132A synthesized longer products. Apparently, F132 rather than F52 plays a critical role in product chain length determination.

From the crystal structure, the side chain of F132 is pointed towards the tunnel interior. From the top view, F132 is well positioned to seal the bottom of the hollow tunnel. On the other hand, F52 is located on the other side of the tunnel and F52 cannot block the bottom of the tunnel. We thus identify the single residue for determining the ultimate chain length of OPPs product. This also clearly shows that the chain elongation of FPP in OPPs reaction is along one side (helix D) of the tunnel (Fig. 4).

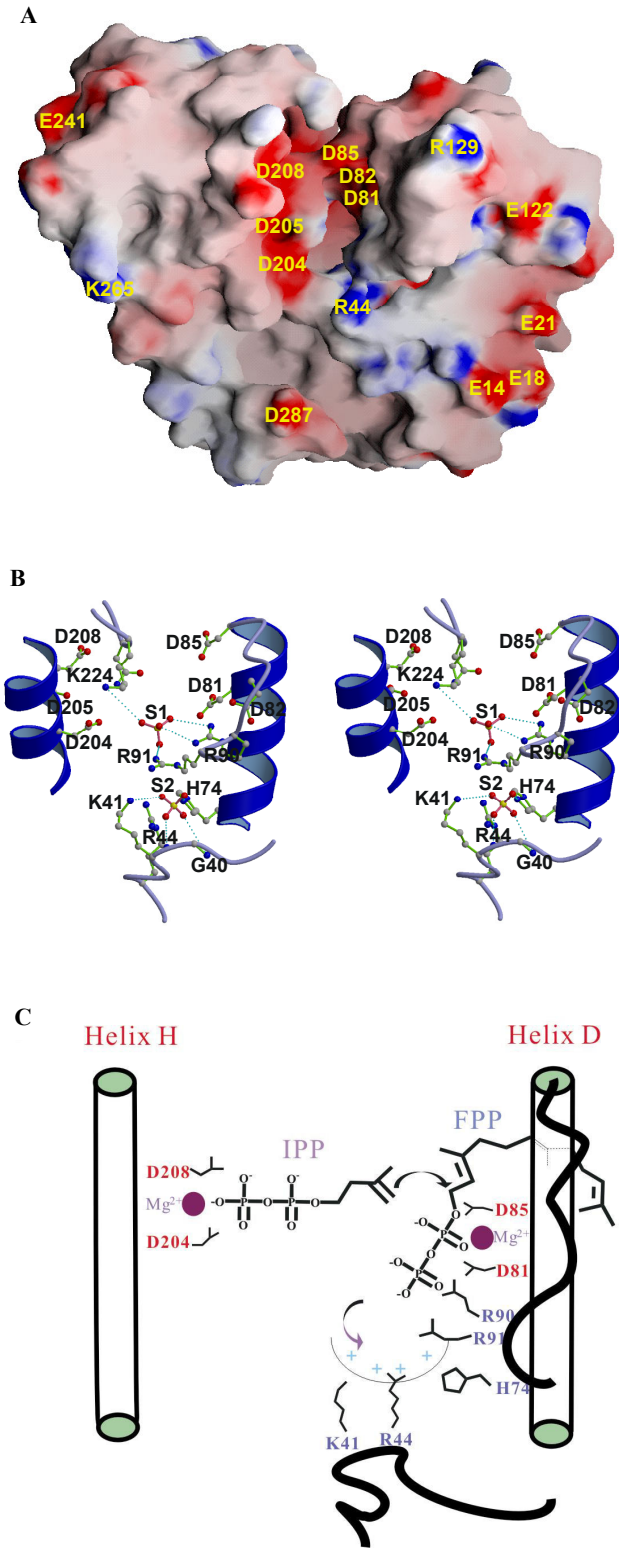

Fig. 2: OPPs active site structure and reaction mechanism. In A, the surface charge of the active site is shown. In B, two sulfate ions in the active site are shown. Fig. 2C is the proposed reaction mechanism.

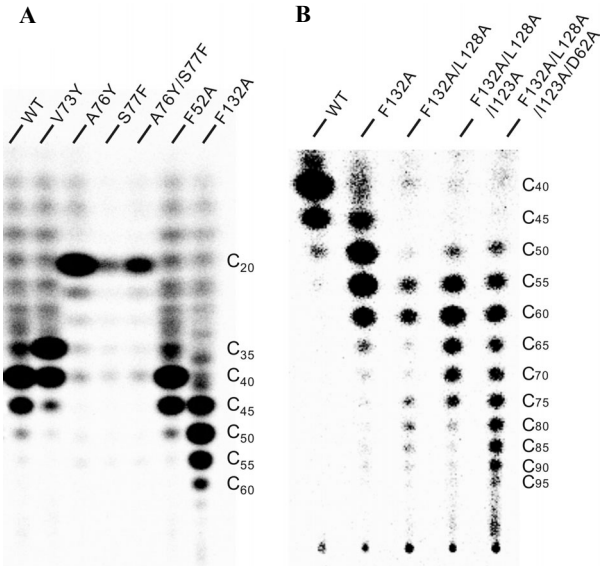

Fig. 3: From the final product synthesized by several mutants. In(A), A76Y, S77F, and A76Y/S77F synthesize shorter product C_{20} and F132A generated longer product (major product = C_{50}) than the C_{40} synthesized by wild type. In (B), F132A/L128A/I123A/D62A can synthesize the largest products (longest product = C_{95}).

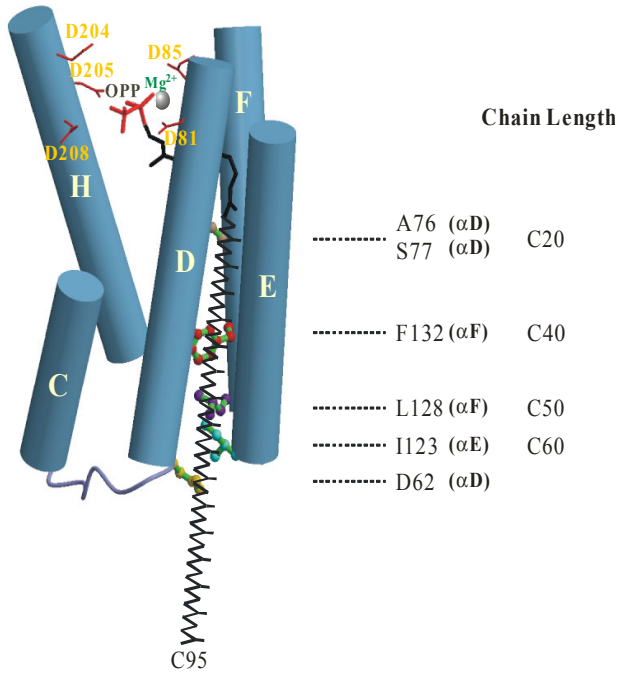

Fig. 4: Proposed mechanism for chain length determination catalyzed by OPPs.

Near where the substrates FPP and IPP bind, several amino acids including V73, A76, and S77 are found. The two amino acids A76Y and S77F are located approximately 10-13 Å beneath the first DDxxD, allowing adequate space to accommodate a FPP molecule. The A76Y and A76Y/S77F mutants produced C_{20} since the substituted bulky residues block the continuous chain elongation of C_{20} (Fig. 3A). Surprisingly, the single mutant S77F generates C_{20} at much lower quantity than the double mutant A76Y/S77F. S77F has similar activity to that of A76Y/S77F, although their k_{cat} values are significantly smaller than that of the wild type. The structure of A76Y/S77F shows that the large Y76 pushes F77 away and leaves a space in between, thereby allowing the penetration of FPP hydrocarbon terminus to wedge between the two aromatic side chains. On the other hand, F77 aromatic side chain of S77F is directly underneath the FPP hydrocarbon tail and could block the FPP chain elongation more effectively so less amount of C_{20} is produced. The determined structure of A76Y shows that the Y76 alone could not completely stop the FPP elongation so larger amount of C_{20} was obtained by this mutant. On the other hand, V73 is located further down the tunnel and away from the FPP site so V73Y has final products more similar to that of the wild-type.

By removing the blockade along the pathway of product chain elongation, we aimed at engineering mutant enzymes which can produce much longer product than C_{40} OPP. We thus produced three OPPs mutants (F132A/L128A, F132A/L128A/I123A, and F132A/L128A/I123A/D62A). Compared to the wild-type OPPs and the F132A mutant which synthesize C_{40} and C_{50} as the major products, respectively (lane 1 and 2 of Fig. 3B), F132A/L128A (lane 3), F132A/L128A/I123A (lane 4) and F132A/L128A/I123A/D62A (lane 5) produce C_{60} as major product. Finally, the quartet mutant synthesizes up to C_{95} product. Apparently, step-by-step removal of the large amino acids near the bottom of tunnel gradually increases the chain length of the final products of the mutants. However, the C_{60} major product shared by F132A/L128A, F132A/L128A /I123A, and F132A/L128A/I123A/D62A indicates that the chain of C_{60} may reach the location of I123, and the I123A mutation is not able to create a sufficiently large opening to allow further chain elongation effectively. The replacement of I123 with even smaller Gly residue in the mutant F132A/L128A/I123G/D62A still failed to provide sufficient space to efficiently "thread" the growing isoprenoid, and the major product remained to be C_{60}. Indeed, the diameter of the tunnel in the section of I123A is ~5.2 Å, the smallest among the areas

beside the four amino acids being mutated to Ala. Therefore, by converting F132 to Ala, the product chain length is extended from the wild-type C_{40} to C_{50} for the mutant. With the alternation of L128 to Ala in addition to the F132A mutation, the major product becomes C_{60}. But further change to I123A fails to extend the chain length of the major product. Based on these results, a molecular ruler for the chain elongation reactions catalyzed by OPPs is proposed, which shows that F132, L128, and I123 are located at the C_{40}, C_{50}, and C_{60} key positions, respectively (Fig. 4). The position of L128 is a bit lower so a significant amount of C_{55} was also produced along with C_{50} (C_{50}:C_{55} = 37:29, lane 2 of Fig. 3B).

In *T. maritima* OPPs, the quartet mutant can generate C_{95} product, record high for *trans*-prenyltransferases, but the chain length is still much shorter than the polymers containing thousands of IPP units produced by the *cis*-type rubber prenyltransferases. The enlargement on the side opening may be required in addition to the bottom opening of the tunnel to produce super long *trans*-type polymers. Very long chain polymers may be obtained by adding some biological accessory apparatus to capture the huge hydrophobic products.

BEAMLINES
17B W20 X-ray Scattering beamline
SP12B Biostructure and Materials Research beamline

EXPERIMENTAL STATION
Protein Crystallography end station

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PUBLICATIONS
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