Mechanism of Product Chain Length Determination and the Role of a Flexible Loop in *Escherichia coli* Undecaprenyl-pyrophosphate Synthase Catalysis

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Tzu-Ping Ko‡‡, Yi-Kai Chen‡‡, Howard Robinson‡, Pei-Chun Tsai‡, Yi-Gui Gao‡, Annie P.-C. Chen‡, Andrew H.-J. Wang‡‡‡, and Po-Huang Liang‡‡‡‡

From the ‡Institute of Biological Chemistry, Academia Sinica, Taipei 115 and ‡Institute of Biochemical Sciences, National Taiwan University, Taipei 106, Taiwan

The *Escherichia coli* undecaprenyl-pyrophosphate synthase (UPPs) structure has been solved using the single wavelength anomalous diffraction method. The putative substrate-binding site is located near the end of the βA-strand with Asp-26 playing a critical catalytic role. In both subunits, an elongated hydrophobic tunnel is found, surrounded by four β-strands (βA-βB-βD-βC) and two helices (α2 and α3) and lined with large residues Ile-62, Leu-137, Val-105, and His-103. The product distributions formed by the use of the I62A, V105A, and H103A mutants are similar to those observed for wild-type UPPs. Catalysis by the L137A UPPs, on the other hand, results in predominantly the formation of the C50 polymer rather than the C55 polymer. Ala-69 and Ala-143 are located near the top of the tunnel. In contrast to the A143V reaction, the C50 intermediate is formed to a greater extent and is longer lived in the process catalyzed by the A69L mutant. These findings suggest that the small side chain of Ala-69 is required for rapid elongation to the C55 product, whereas the large hydrophobic side chain of Leu-137 is required to limit the elongation to the C55 product. The roles of residues located on a flexible loop were investigated. The S71A, N74A, or R77A mutants displayed 25–200-fold decrease in *K*<sub>m</sub> values. W75A showed an 8-fold increase of the FPP *K*<sub>m</sub> value, and 22–33-fold increases in the IPP *K*<sub>m</sub> values were observed for E81A and S71A. The loop may function to bridge the interaction of IPP with FPP, needed to initiate the condensation reaction and serve as a hinge to control the substrate binding and product release.

Prenyltransferases catalyze consecutive condensation reactions of isopentenyl pyrophosphate (IPP)<sup>1</sup> with allylic pyrophosphate to generate linear isoprenyl polymers. The isoprenylates undergo further modification to form a variety of isoprenoid structures including steroids, terpenes, the side chains of respiratory quinones, carotenoids, natural rubber, the glycosyl carrier lipid, and prenyl proteins (1, 2). E- and Z-type prenyltransferases synthesize trans and cis double bonds, respectively, through the condensation reactions of IPP (3). Each of the E-type enzymes catalyzes the formation of a product having a specific chain length ranging from C10 to C50 (4).

Two conserved DDXXD motifs are observed in E-type enzymes (5–7). X-ray structural (8) and site-directed mutagenesis studies (9–12) of farnesyl-pyrophosphate synthase (FPPs) have shown that the first aspartate-rich motif binds the allylic substrate, whereas the second DDXXD binds IPP via Mg<sup>2+</sup>. Mutagenesis studies indicate that the 5th amino acid residue (Phe-77) upstream from the first DDXXD plays a critical role in controlling the chain length of the final product formed in the reaction catalyzed by E-type geranylgeranyl-pyrophosphate synthase from archaeabacterium (13). By substituting this large residue with the smaller Ser, product synthesis was shifted from the production of C20 product to C25 and C30 products (14). Double (P77G/L74G) and triple (P77G/L74G/I71G) mutants were observed to catalyze the formation of even longer chain products (15). Replacing the active site aromatic residues Phe-112 and Phe-113 with smaller amino acids produced FPPs mutants that synthesize C20 geranyl pyrophosphate (F112A), C25 geranylgeranyl pyrophosphate (F113S), and longer products (F112A/F113S) (16). Analysis of the F112A/F113S structure shows that it contains an enlarged active site that is possibly related to increased product size.

On the other hand, Z-type isoprenyl-pyrophosphate synthases such as undecaprenyl-pyrophosphate synthase (UPPs) and dehydrodolichyl-pyrophosphate synthase catalyze the formation of long chain products ranging from C50 to C100 (17, 18). UPPs, the subject of this paper, generates a C<sub>55</sub> product by catalyzing the chain elongation of farnesyl pyrophosphate (FPP) via sequential condensation reactions of eight IPP, which serves as a carrier in transporting lipid II across the membrane in bacterial cell wall biosynthesis (19, 20). Despite close similarities in the chemical catalyzed, no sequence homology exists between Z-type and E-type enzymes (21, 22). To understand better the Z-type enzyme catalysis, we have studied the reaction mechanism and kinetics of *Escherichia coli* UPPs (23),

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† Present address: Dept. of Biochemistry, School of Molecular and Cellular Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801.

‡ Both authors contributed equally to this work.

¶ Present address: Dept. of Biochemistry, School of Molecular and Cellular Biology, University of Illinois at Urbana-Champaign, Urbana, IL 61801.

** To whom correspondence may be addressed. Tel.: 886-2-2788-1981; Fax: 886-2-2788-9759; E-mail: ahjwang@gate.sinica.edu.tw.

†† To whom correspondence may be addressed. Tel.: 886-2-2785-5696 (ext. 6070); Fax: 886-2-2788-9759; E-mail: phliang@gate.sinica.edu.tw.

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1 The abbreviations used are: IPP, isopentenyl pyrophosphate; FPPs, farnesyl-pyrophosphate synthase; FPP, farnesyl pyrophosphate; UPPs, undecaprenyl-pyrophosphate synthase; UPP, undecaprenyl pyrophosphate; NNTA, nickel-nitrioltriacetic acid; PEG, polyethylene glycol; r.m.s.d., root mean square deviation; PCR, polymerase chain reaction.
and we show that some aspartate and glutamate residues may play a structural and catalytic role and that their mutations affect the enzyme activity and/or substrate binding (24). Recently, the three-dimensional structure of UPPs from Micrococcus luteus solved by Fujihashi et al. (25), and it is completely different from those of E-type prenyltransferases. We report the structure of E. coli UPPs, which is similar to the structure of M. luteus UPPs except that two protein conformers were discovered for E. coli UPPs. The large amino acid residues Ile-62, Leu-105, Val-106, and His-103, occupying the bottom portion of an elongated hydrophobic tunnel from the structure of E. coli UPPs were replaced with Ala using site-directed mutagenesis to study the mechanism of product chain length determination. In addition, two amino acid residues, Ala-69 and Ala-143, located near the top of the hydrophobic tunnel in E. coli UPPs, were substituted with Leu and Val, respectively, as seen in the Mycobacterium tuberculosis Z-type short chain FPPs (26) to examine the possible formation of a short chain product. Furthermore, a flexible loop without observable electron densities of residues 72–83 was found in both UPPs structures. Mutagenesis studies of the M. luteus enzyme suggest that loop residues Asn-77 and Trp-78 are important for catalysis and FPP binding (27). To address the role of this loop in forming the FPP product, we generated the loop mutants S71A, E73A, N74A, and H57A, R77A, and E81A and measured the impact of loop residues Asn-77 and Trp-78 are important for catalysis and FPP binding (27). To address the role of this loop in forming the FPP product, we generated the loop mutants S71A, E73A, N74A, and H57A, R77A, and E81A and measured the impact of loop residues Asn-77 and Trp-78.

### EXPERIMENTAL PROCEDURES

#### Materials—Radiolabeled [14C]IPP (55 mCi/mmol) was purchased from Amersham Pharmacia Biotech, and FPP was obtained from Sigma. Reversed-phase thin layer chromatography (TLC) plates were purchased from Merck. Taq DNA polymerase was obtained from Life Technologies, Inc. The plasmid mini-prep kit, DNA gel extraction kit, and NTA resin was purchased from Qiagen. Potato acid phosphatase (2 units/mg) was purchased from Roche Molecular Biochemicals. FXa and the protein expression kit (including the pET32Xa/LIC vector and competent JM109 and BL21 cells) were obtained from Novagen. The B834 (DE3) competent cells for the production of seleno-Met UPPs was also from Novagen. Seleno-methionine was purchased from Sigma. All commercial buffers and reagents were of the highest grade.

#### Site-directed Mutagenesis of UPPs—UPPs mutants were prepared by using PCR techniques in conjunction with the E. coli BstI22 UPPs gene template in the pET32Xa/Lic vector (23). The mutagenic primers used were purchased by MDBio Inc. The mutagenic oligonucleotides for performing site-directed mutagenesis are as follows: 5′-AACGAGTGCT-GAGGCGTTA-3′ for 162A, 5′-CTGACCCGAGCTAACTG-3′ for H103A, 5′-CAACGGCGGCTGCGCT-3′ for V105A, 5′-ATCCGCTGACTTCGA-3′ for A143V, 5′-GGTATTGAGGGTCGCATGTTG-3′ for I62A, and 5′-GGTATTGAGGGTCGCATGTTG-3′ for L137A. Thirty cycles of PCR were performed using the above mutagenic oligonucleotides to create the full-length mutant UPPs genes. The FXa cleavage site (IEGR) and the complimentary sequences to the sticky ends of the linear vector pET-32Xa/Lic were included in these primers. Thirty cycles of PCR were performed using a thermocycler (Applied Biosystems) with the melting temperature at 95 °C for 2 min, annealing temperature at 42 °C for 2 min, and polymerization temperature at 68 °C for 40 s. The PCR product was subjected to electrophoresis on 0.8% agarose gel in TAE buffer, and then the gel was stained with etidium bromide. The part of the gel containing the band of the correct size was excised, and the DNA was recovered using a DNA elution kit. The constructed DNA of a mutant enzyme was ligated to the vector by incubation for 1 h at 22 °C. The recombinant UPPs plasmid was then used to transform E. coli JM109 competent cells that were streaked on a Luria-Bertani (LB) agar plate containing 100 μg/ml ampicillin. Ampicillin-resistant colonies were selected from the agar plate and grown in 5 ml of LB culture containing 100 μg/ml ampicillin overnight at 37 °C. The mutation was confirmed by sequencing the entire UPPs mutant gene of the plasmid obtained from the overnight culture. The correct construct was subsequently transformed to E. coli BL21 for protein expression. The 5-ml overnight culture of a single transformant was used to inoculate 500 ml of fresh LB medium containing 100 μg/ml ampicillin. The cells were grown to A600 = 0.6 and induced with 1 mM isopropyl-β-thiogalactopyranoside. After 4–5 h, the cells were harvested by centrifugation at 7,000 × g for 15 min.

#### Enzyme Purification—Approximately 3 g of cell paste, collected from LB broth of E. coli expressing mutant UPPs by centrifugation, was suspended in 25 ml of lysis buffer containing 25 mM Tris-HCl, pH 7.5,
Structure and Mechanism of E. coli UPPs

Measurements of kinetic parameters, mutant UPPs (0.1–10 mM E81A and W75A) was utilized to initiate

Preparation of Seleno-Met Modified UPPs—To produce seleno-Met modified UPPs, the plasmid containing UPPs gene was transformed into B834 (DE3)-competent cell. The single transformant was first grown overnight at 37 °C in LB containing 100 μg/ml ampicillin. The cells were then used to inoculate 2 liters of Lemaart Medium (28) with the supplement of 50 mg/liter seleno-methionine. Isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 0.75 mM for induction after the cell density reached A600 = 0.4. The cells were grown for another 10 h at 37 °C and then harvested by centrifugation. The subsequent purification procedure for seleno-Met UPPs was the same as for UPPs. The NiNTA-purified seleno-Met UPPs was in buffer containing 25 mM Tris-HCl, pH 7.5, and 150 mM NaCl. Triton X-100 was added into protein solution to 0.1%, and the protein was concentrated to ~5 mg/ml.

Crystallographic Analysis—UPPs was crystallized using the hanging drop set-up from Hampton Research (Laguna Niguel, CA) by mixing 2 μl of the UPPs solution (5 mg/ml in 0.1% Triton X-100) with 2 μl of the mother liquor (25% PEG1000 in 100 mM sodium cacodylate, pH 5.5), equilibrating with 500 μl of the mother liquor. Crystallization of the seleno-Met enzyme was achieved under a slightly different condition by mixing 2 μl of the UPPs solution (5 mg/ml in 0.1% Triton X-100) with 2 μl of the mother liquor (10% PEG8000, 8% ethylene glycol, in 100 mM KOH-Hepes, pH 7.5), equilibrating with 500 μl of the mother liquor. In both cases, long rods of crystals appeared in 1 week.

Diffraction data were collected at the National Synchrotron Light Source beamline X4A (Brookhaven National Laboratory) at four wavelengths (low remote, inflection point, peak, and high remote) at and near the selenium absorption edge. We attempted to determine the phases using data from all four wavelengths. However, the resulting MAD-phasing electron density map could not be interpreted. Subsequently, phases were determined with the program suite SOLVE (29; also at website www.solve.lanl.gov) in the single wavelength anomalous diffraction mode, using only the inflection wavelength data in the resolution range of 20 to 1.8 Å. Six selenium sites (of 10) were found. Density modification with RESOLVE (30) yielded an overall figure of merit of 0.53 (with figure of merit = 0.86 in the 20 to 3.0-Å range).

kcat and kcat' Values for Mutant UPPs—For the measurements of kinetic parameters, mutant UPPs (0.1 μM S71A, N74A, and R77A and 0.01 μM E81A and W75A) was utilized to initiate the reaction of FPP and [14C]IPP in 200-μl solutions. For IPP Ks and kcat determinations, 10 μM FPP was utilized to saturate the enzyme, and IPP concentrations of 0.5-5 Ks were employed. For FPP Ks measurements, 0.2–10 μM FPP were used along with 20 μM [14C]IPP. All reactions were carried out in 100 mM KOH-Hepes buffer, pH 7.5, 50 mM KCl, and 0.5 mM MgCl2 at 25 °C in the presence of 0.1% Triton X-100. To measure the initial rate, 40-μl portions of the reaction mixture were periodically withdrawn within 10% substrate depletion and mixed with 10 mM EDTA for reaction termination. The radiolabeled products were then extracted with 1-butanol, and the radioactivities associated with aqueous and butanol phases were separately quantitated by a Beckman LS6500 scintillation counter. Initial velocity data were fitted to Equation 1 to obtain kcat and kcat' values by non-linear regression (KaleidaGraph computer program). The kcat was calculated from

\[ \frac{v_0}{v = v_{max} [E]/(K_{m} + [S])} \] (Eq. 1)

where v0 is the initial velocity; [E] is the enzyme concentration; [S] is the substrate concentration; vmax is the maximum velocity; and Km is the Michaelis constant.

Product Formation and Analysis—The reaction rates of the wild-type and mutant enzymes I62A, H103A, L137A, and V105A were measured under the same conditions with 0.1 μM enzyme, 5 μM FPP, 50 μM [14C]IPP, 0.5 mM MgCl2, 50 mM KCl, and 0.1% Triton X-100 in 100 mM KOH-Hepes buffer, pH 7.5. Based on the reaction rate, the reaction mixture containing 0.5 μM mutant UPPs under the above reaction condition was incubated at 25 °C for 2 and 96 h in the presence and

FIG. 2. A, GRASP representation (39) of the UPPs dimer with one subunit shown as “worm” tracing and another subunit as molecular surface with charge potential. The range is approximately ~10 to 10 kBT and coded red to blue. The unobserved loop is drawn as orange spheres in the left subunit. The red arrow indicates the location of the hydrophobic tunnel, which is also seen in the right subunit as a hole in the surface. Several conserved residues in the vicinity of Asp-26, including Arg-30, His-43, Phe-70, Glu-198, Arg-200, and Ser-202, are labeled, as well as three additional positively charged residues Lys-33, Lys-34, and Arg-39. B, possible active site residues of UPPs. The ribbon diagram of the protein is viewed close-up in a direction similar to that of A, centered at the substrate-binding site. The side chains of nearby conserved residues, including the previous nine and Asn-28, are shown as ball-and-stick models. The figure is produced using MolScript and Raster3D (40).
absence of 0.1% Triton, respectively, to obtain the products. The reaction was then terminated with 10 mM EDTA, and the radiolabeled products were extracted with 1-butanol (IPP was in aqueous phase). After the removal of 1-butanol by evaporation, the 20% propanol solution containing 4.4 units/ml acid phosphatase, 0.1% Triton X-100, and 50 mM sodium acetate, pH 4.7, was used to convert polypropenyl pyrophosphate products to the corresponding polypropenols (35). The polypropenols were extracted with n-hexane, and the n-hexane volume was reduced by evaporation. The residual n-hexane solution was spotted on a reversed-phase TLC plate, which was then developed using acetone/water (19:1) as the mobile phase. The plate with radiolabeled products was analyzed by autoradiography using a bioimaging analyzer (Fuji film BAS-1500). The products were identified by the $R_f$ values reported previously (23). The percentages of the intermediates and product formed in the UPPs reaction were calculated from the measured intensities normalized by the numbers of $[^{14}C]$IPP incorporated.

Single Turnover Experiments of A69L and A143V—A rapid-quench apparatus (Kintec, Austin, TX) was employed to terminate the reactions catalyzed by A69L and A143V after a specified reaction time. For each reaction, 15 μl of enzyme solution, preincubated with FPP, were mixed with an equal volume of $[^{14}C]$IPP solution to start the reaction. The reaction solution contained 10 μM mutant enzyme, 2 μM FPP, 50 μM $[^{14}C]$IPP, 0.5 mM MgCl$_2$, 50 mM KCl, and 0.1% Triton in the buffer of 100 mM KOH-Hepes, pH 7.5, and 25 °C. The reaction was terminated by the addition of 67 μl of 0.6 M NaOH. It was demonstrated previously that 0.4 μl (after mixing) base was able to stop the reaction immediately (23). Quenched reaction solutions were analyzed for intermediates and product by using the TLC method described above.

RESULTS AND DISCUSSION

Overall Structure—Each asymmetric unit of the crystal unit cell contains one UPPs dimer, which is the active form of the enzyme. The dimer has a peanut shape of 35 × 70 Å when viewing along the dyad axis direction. When the dimer is viewed perpendicular to the dyad axis, it has the shape of a butterfly (Fig. 1). Each subunit contains two domains, a catalytic domain (the butterfly wings) and a pairing domain (the butterfly body). The dimer has a novel global folding structure in which the two monomers are tightly associated via an extensive interface between the edges of the central β-sheet and between a pair of long α-helices (α5 and α6).

The catalytic domain adopts a parallel α/β topology that resembles the Rossmann fold. There are six parallel β-strands with the arrangement of a β3-β3-β2-β3-β2-β3 topology. The six-stranded β-sheet adopts an incomplete cone shape with about 40% of the surface open. The C terminus lies on the pointed side with a hole of about 10 Å in diameter, where the putative active site is located (see below). The N-terminal end of the cone has a diameter of about 25 Å.

There are 7 α-helices, with α1 (Gly-27 to Gly-36), α2 (Arg-39 to Asn-60), α3 (Ser-83 to His-103), α4 (Asn-117 to Gly-133), and α7 (Asp-225 to Glu-240) on the outer surface of the β-cone, and α5 and α6 fill the opening of the β-cone. Three 310 helices connect between α5 and α6 (Glu-168 to Ile-172), β3 and β2 (Leu-206 to Ala-210), and β2 and α7 (Leu-220 to Phe-224), respectively. All helices are mostly straight, except for α3, which is kinked. It is interesting to note that the location of the kink in α3 is different in the two subunits that may be relevant to the product release (see below). The conformations of the two subunits are nearly identical, except for the conformation and the relative disposition of helix α3.

Helices α5 and α6 and their associated turns are involved in the dimer formation with the major stabilization coming from the stacked sheet structure of the α5-α5 hydrophobic interactions. It is further stabilized by 24 hydrogen bonds involving 11 residues of one subunit, and their equivalents of the other subunit, plus 6 water molecules. The side chain of Arg-148 is stacked with that of Thr-207 from the other subunit.

The second region of the interface, constituted by strand β6, its proceeding turns, and the polypeptide C terminus, also involves hydrophobic interactions. The patch formed by the side chains of Ile-201, Leu-206, Ile-209, Ala-210, Leu-214, and Phe-216 extends the hydrophobic core of one subunit (on the concave side of the β-cone) across the molecular dyad to another subunit. There are 42 hydrogen bonds between the subunits.

Product and Substrate Binding Site—An intriguing question regarding the mechanism of UPPs is the exquisite ability of the enzyme to synthesize precisely a product containing 11 prenyl units (55-carbon). We have examined the three-dimensional structure of the enzyme to seek a clue to this question. We have found that there is an elongated tunnel within which the hydrophobic amino acids side chains cover the entire surface. Interestingly, this hydrophobic tunnel, surrounded by 4 β-strands (β2-β3-β2-β3) and 2 α-helices (α2 and α3), is not the same for the two subunits. In one subunit, the tunnel is narrow within which an elongated electron density envelope, suggestive of an aliphatic fragment, is clearly visible. In the other subunit, the tunnel is more open where only water molecules are found. Fig. 2A shows the surface drawing of the enzyme in which the open subunit is oriented in such a way that the tunnel is clearly visible. On the top of tunnel, there are several conserved hydrophilic amino acids in the vicinity of Asp-26, including Asn-28, Arg-30, His-43, Phe-70, Ser-71, Arg-194, and Glu-198. Fig. 2B shows the detailed arrangement of these amino acids. This region has been suggested as the FPP- and IPP-binding site based on site-directed mutagenesis studies (24, 36).

We compared the two subunits by least squares superposition shown in Fig. 3A. In this diagram, we are looking into the tunnel from the side. Most of the backbones are superimposed very well, except for the two helices α3 and α4, with a r.m.s.d. of 2.7 and 1.5 Å, respectively. Here the magenta and red helices are associated with the “open tunnel” and “closed tunnel” forms of the two subunits, respectively. It is interesting to note that both α3 helices are kinked, but at different locations. The magenta helix is kinked by ~30° at Glu-96, and the red helix is kinked by ~45° at Ala-92.

The red helix is translocated upward by about one-half turn of α-helix (2.7 Å) and laterally toward α2 helix by 2.5 Å, making the inside space of the tunnel tighter and shorter. The resulting tight space allows a better binding of the hydrophobic aliphatic fragment such that it becomes visible in the electron density (Fig. 3A). This aliphatic fragment may be coming from either the Triton X-100 or PEG8000.

The detailed structure of the tunnel may be schematically illustrated using a cylindrical projection diagram in Fig. 3B. It can be seen that the interior of the tunnel is completely hydrophobic, except near Asp-26 of the upper left corner where the putative substrate-binding site is. The depth of the hydrophobic tunnel is ~30 Å, which is sufficient to accommodate the C55-prenyl chain.

Comparison of UPPs Structures from E. coli and M. luteus—The structures of UPPs from E. coli and M. luteus are globally similar to each other, as evident in the root mean square deviation (r.m.s.d.) of 1.38 Å for 377 matched pairs of α-carbon atoms. The r.m.s.d. is 0.79 Å for 274 atom pairs when a matching criterion of 1.5 Å is used. The largest deviations are located in helices α3 and α4, which are relevant to the catalytic function of UPPs. As mentioned above, two conformations for the two subunits of E. coli UPPs dimer are observed. In contrast, the two subunits in M. luteus UPPs have identical structure that is similar to the closed form of E. coli UPPs. Compared with the closed form of E. coli UPPs, the α4 helix in one subunit of the M. luteus enzyme significantly differs by rotation of about 20° anchored on its C terminus, and the maximal displacement is ~6.5 Å at its N terminus, whereas α3 differs by
only about 2 Å. On the other hand, both of the entire helices α3 and α4 are moved by 5–7 Å in the other subunit, which show a manifestation of contrasting open and closed forms. In addition, helix α6 and the associated loops also show deviation of 1.5–3 Å in the two UPPs structures, possibly correlated with the open-closed conformational change. Because only the closed form was observed for M. luteus UPPs, Fujihashi et al. (25) reported a large cleft surrounded by the S2- and S4-strands and the H2 and H3 helices (equivalent to α2 and α3 in the terminology used in this paper). We observe the open form in which two additional β-strands (βA and βC) may also constitute the hydrophobic tunnel. These structural deviations might result from the different crystal packings or different conditions utilized in protein crystallization. Triton and PEG were included in crystallization of E. coli UPPs, whereas the heavy atom derivatives were contained in the soaking buffer of M. luteus enzyme.

**Products Generated by I62A, H103A, V105A, and L137A UPPs**—The structure of E. coli UPPs reveals a hydrophobic tunnel formed by 4 β-strands (βA-βB-βD-βC) and 2 helices (α2 and α3), sufficiently large to accommodate the entire UPP. Large amino acid residues, which include Ile-62, Leu-137, Val-105, and His-103, occupy the bottom portion of the tunnel (Fig. 3B). We proposed that one or more of these residues might play a role in determining product chain length, analogous to the above-mentioned amino acid residue located upstream from the first DDXXD motif in E-type prenyltransferases. To test this proposal, site-directed mutagenesis was performed to replace these large residues with Ala, and the chain lengths of the products synthesized by the mutant enzymes were examined by TLC analysis. The activities of H103A, V105A, L137A, and I62A mutants, assayed with 5 mM FPP and 50 μM [14C]IPP, are within a 5-fold range of the wild-type UPPs activity. Reactions of 0.5 mM wild-type UPPs and 0.5 mM mutant UPPs, with 5 mM FPP and 50 μM [14C]IPP for 2 h in the presence of Triton and 96 h in the absence of Triton, lead to the formation of product mixtures shown in Fig. 4, A and B. Previous studies (23) have shown that Triton X-100 facilitates product release, and the UPPs reaction is fast in the presence of Triton. The
major product for the wild-type UPPs-catalyzed reaction carried out in the presence of Triton is C_{55} (C_{55}:C_{60} = 96:4, 1st lane of Fig. 4A). This is also the case for the H103A, V105A, and I62A UPPs-catalyzed reactions (2nd, 3rd, and 5th lanes in Fig. 4A). L137A UPPs, on the other hand, promote the synthesis of C_{55} and C_{60} polymers mainly and C_{65} to a lesser extent (C_{55}:C_{60}:C_{65} = 35:25:12, 1st lane, 4th lane) of Fig. 4A). Because of the slow product release that occurs in the absence of Triton (Fig. 4B), wild-type, H103A, V105A, and I62A UPPs cause the formation of C_{55}-type products with C_{55} as the major component. As calculated from the imaging intensity divided by the number of IPP incorporated in each product, wild-type and H103A show similar products (C_{55}:C_{60}:C_{65}:C_{70}:C_{75} = 10:9:8:7:6, Fig. 4A, 1st lane). V105A and I62A mutations result in higher ratios of C_{60} and C_{65} compared with that of the wild-type UPPs, and V105A synthesizes significant amount of C_{55} (C_{55}:C_{60}:C_{65}:C_{70}:C_{75} = 35:33:21:9:2, 5th lane for I62A enzyme). In contrast, L137A UPPs produces C_{70} and C_{75} as the major products (C_{55}:C_{60}:C_{65}:C_{70}:C_{75}:C_{80} = 3:5:17:40:29:5, Fig. 4B, 4th lane).

Based on these findings, we hypothesized that the substitution of the large side chain in Leu-137 with a smaller side chain in Ala removes the floor of the tunnel, thereby allowing for the formation of longer chain length products. Based on the crystal structure, the Ile-62 and His-103 side chains are directed away from the tunnel, and the side chains of Leu-137 and Val-105 are pointed toward the tunnel interior. Leu-137 is positioned on the same side of the a1 helix used to bind FPP, whereas Val-105 is not. Apparently, Leu-137 is positioned to prevent further elongation of C_{55}. However, Val-105 may also play a role in determining product chain length because its mutations increase the ratios of C_{56}, C_{60}, and C_{65}, but not as critical as Leu-137. This conclusion is supported by the observation that dehydrodolichyl-pyrophosphate synthase from Saccharomyces cerevisiae, which catalyzes formation of longer chain length C_{70}-C_{90} products (37), has an Ala (Ala-154) at the position corresponding to Leu-137 in E. coli UPPs (see Fig. 5). Because of the presence of the small Ala residue, the chain elongation in the yeast enzyme does not stop at C_{55} but continues to generate longer chain products.

**Intermediates of A69L and A143V UPPs-catalyzed Reactions Monitored under Single Turnover Conditions**—An unusual Z-type FPPs, recently found in *M. tuberculosis* and shown to be homologous to UPPs in primary sequence, synthesizes a short chain rather than typical long chain polyisoprenyl pyrophosphate product (26). Two amino acid residues, Ala-69 and Ala-143, located near the top of the hydrophobic tunnel in *E. coli* UPPs, are replaced by Leu and Val, respectively, in *M. tuberculosis* and *M. luteus* UPPs (Swiss-Prot P35196, M. tuberculosis, Rv1086 from *M. tuberculosis*, the dehydrodolichyl-pyrophosphate synthase RER2 from *E. coli* UPPs, and M. luteus UPPs correspond to Ala-154 in yeast RER2 protein.

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**Fig. 5. Alignment of amino acid sequences of the E. coli UPPs (Swiss-Prot Q47675), the *M. luteus* UPPs (GenBank™ AB004319), the Z-type farnesyl-pyrophosphate synthase Rv1086 from *M. tuberculosis*, and the yeast dehydrodolichyl-pyrophosphate synthase RER2 (Swiss-Prot P35196) (Mlt, *M. luteus*; Mtub, *M. tuberculosis*). Black and gray outlines indicate identical and similar amino acid residues, respectively. The asterisks indicate the amino acid residues mutated in this study. The amino acid residues corresponding to Ala-69 and Ala-143 of *E. coli* UPPs are Leu-84 and Val-156 in the *M. luteus* UPPs and Ala-72 and Leu-137 in *M. tuberculosis* UPPs, respectively. The amino acid residues corresponding to Ala-154 in yeast RER2 and Ala-143 of *E. coli* UPPs are Leu-84 and Val-156 in the *M. luteus* UPPs and Ala-72 and Leu-137 in *M. tuberculosis* UPPs, respectively.**

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**Table 1: Amino Acid Residues Corresponding to Ala-69 and Ala-143 of *E. coli* UPPs**

| Ecoli: | -------- | | MSLATAPLSEQLPAHGC | ALKOCHQKAKQGKIRA-FHEKA | 45 |
| Mtub: | MFPIKEAIHHIRBHAQIGERFPRGAPQSNPRIP | - | 40 |
| Mlt: | MEIIIPRLKEEPLYELYLRRLCGLGSDKPSILAVQGQG | SAGYDVCYFEM | 69 |
| yeast: | METDGPIQHSFVLEIWTTRILSNLRKCPH | PEVKQGHCHEFRELDI | 59 |

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**Table 2: Amino Acid Residues Corresponding to Ala-154 of Yeast RER2**

| Ecoli: | -------- | | MSLATAPLSEQLPAHGC | ALKOCHQKAKQGKIRA-FHEKA | 45 |
| Mtub: | MFPIKEAIHHIRBHAQIGERFPRGAPQSNPRIP | - | 40 |
| Mlt: | MEIIIPRLKEEPLYELYLRRLCGLGSDKPSILAVQGQG | SAGYDVCYFEM | 69 |
| yeast: | METDGPIQHSFVLEIWTTRILSNLRKCPH | PEVKQGHCHEFRELDI | 59 |
the next intermediate is gradually formed (23). In contrast, the C<sub>30</sub> intermediate of A69L reaction accumulates to a greater extent (e.g., C<sub>30</sub>C<sub>35</sub>C<sub>40</sub>C<sub>45</sub>C<sub>50</sub>):0.14:2.5:1.5:27:55 at the end of 15 s) than the other intermediates. The quantitative time course data of A69L reaction are shown on the bottom of Fig. 6A. The substitution of Ala-69 with Leu might also force a slight change of the direction of chain elongation leading to shorter product C<sub>50</sub>. In contrast, the A143V-catalyzed reaction displays only the transiently formed intermediates similar to the wild-type UPPs but with 3-fold smaller rate constant for IPP condensation (Fig. 6B, quantitative data not shown). Based on the data, the Ala-69 but not Ala-143 is positioned in UPPs such that it does not affect the binding of intermediates shorter than C<sub>30</sub> but interferes with proper binding of C<sub>35</sub> and longer polymers.

It was proposed that the FPP site is located in the area of Asp-26, the upper-left corner of the tunnel shown in Fig. 3B. It is apparent from the crystal structure that Ala-69 is closer to the binding site of FPP, and Ala-143 is more distant from this site. In addition, Ala-69 in E. coli UPPs is conserved in M. luteus enzyme, but the corresponding residue of Ala-143 is a larger residue Leu in M. luteus UPPs (see Fig. 5). Our experimental data indicate that a small residue like Ala at position 69 but not 143 is required for continued chain elongation to C<sub>35</sub> catalyzed by UPPs. The accumulation of C<sub>30</sub> in the single turnover of A69L indicates that the space between Ala-69 and the FPP-binding site allows the incorporation of three IPP molecules. However, the mutation of Ala-69 to Leu does not completely block the chain elongation at this point (probably due to the large room of the hydrophobic tunnel), and the reaction continues to produce C<sub>45</sub> and finally C<sub>50</sub>. However, reversible release and temporary accumulation of the C<sub>30</sub> intermediate occur with this mutant.

The Roles of Amino Acid Residues in the Disordered Loop of 72–83—The 72–83 loop is not visible in both x-ray crystal structures of UPPs (see Fig. 3B), and thus it is not possible to know what role, if any, these residues play in catalysis. Previous studies indicated that Asn-77 and Trp-78 in M. luteus UPPs (corresponding to Asn-74 and Trp-75 in E. coli enzyme) are important in catalysis and FPP binding. Substitution of Asn-77 with Ala, Asp, or Glu causes a 10<sup>2</sup>–10<sup>3</sup>-fold reduction in <i>k<sub>catal</sub></i>. Replacement of Trp-78 with Ile, Arg, or Asp increases the FPP <i>K<sub>m</sub></i> by ∼5–20-fold (27). In the current study, we sequentially replaced the polar amino acid residues and Trp-75 of the loop of the E. coli enzyme with Ala and measured the kinetic properties of the mutant enzymes (Table II). S71A, N74A, and R77A UPPs show markedly decreased <i>k<sub>catal</sub></i> values. W75A and E81A UPPs as well as the previously characterized E73A UPPs (24) display smaller reductions in <i>k<sub>catal</sub></i>. The <i>k<sub>catal</sub></i> values for S71A, E73A, N74A, W75A, R77A, and E81A are 0.11, 0.3, 2.2 × 10<sup>−2</sup>, 1.1, 1.4 × 10<sup>−4</sup>, and 0.4 s<sup>−1</sup>, respectively, whereas that of the wild-type enzyme is 2.5 s<sup>−1</sup>. Consistent with the behavior of the corresponding M. luteus UPPs mutants (27), the N74A mutation in E. coli UPPs results in a 100-fold decrease of <i>k<sub>catal</sub></i> value with no increase in FPP and IPP <i>K<sub>m</sub></i> values. The substitution of Trp-75 with Ala increases the <i>K<sub>m</sub></i> values for FPP and IPP 8- and 11-fold, respectively, in E. coli UPPs reaction. E81A and S71A have significantly lower IPP affinities as indicated by their increased (22–33-fold) IPP <i>K<sub>m</sub></i> values.

These results indicate that the loop is responsible mainly for IPP binding, and Trp-75 is involved in FPP binding. As shown in Fig. 2B, Ser-71 (positioned at the end of the loop) together with Phe-70 are located in the substrate-binding site and may interact with IPP and place it in a correct position relative to FPP for the condensation reaction to occur. This hypothesis is supported by a recent report suggesting that Phe-73 and Ser-74 (equivalent to Phe-70 and Ser-71 in E. coli enzyme) are important for IPP binding and catalysis in M. luteus UPPs (36). In addition, our studies demonstrate that Glu-81 and some other amino acid residues in the loop, which are invisible in the crystal structure, are also critical in substrate binding and/or catalysis in E. coli UPPs.

Two protein conformers are observed in the E. coli UPPs structure. The major difference is that the α3 helix is kinked to a greater degree in the conformer that contains Triton or PEG (used in the crystallization solution). This conformer (closed form) has a tighter space, which allows for better binding of the hydrophobic aliphatic fragment. The other conformer (open
form) only contains water in the substrate binding region. As shown in Fig. 3B, the α3 helix is linked to the flexible loop 71–83. IPP binding might trigger the movement of the loop toward the substrate-binding region of Asp-26, and this might induce a conformational change from the open form to the closed form that results in enhanced interactions of enzyme with substrate. After UPP is formed, the closed form can switch back to the open form to release the product. The loop might be utilized as a hinge for the interconversion of two conformers and as a control mechanism for substrate binding and product release.

Conclusions—The E. coli UPPs structure reported here is consistent with that of the M. luteus UPPs. However, compared with the M. luteus UPPs structure, our E. coli enzyme structure has a better resolution (1.8 versus 2.2 Å) and provides more detail in structural information. Furthermore, two enzyme conformers of E. coli UPPs are identified, which may be related to a gate opening/closing mechanism for the release of the product. The site-directed mutagenesis studies reported in this paper provide further insight into the catalytic mechanism of UPPs by identifying the following: (i) important amino acid residues that determine the product chain length, and (ii) the functions of amino acid residues located on the flexible active site loop. We conclude that the bulky side chain of Leu-137 serves to block further elongation of UPP based on the finding that substitution of Leu-137 with Ala results in the synthesis of longer polypropenyl products. The Ala-69 amino acid is located at a distance corresponding to three isoprene units away from FPP bound in E. coli UPPs. The substitution of Ala-69 with Leu results in long lived accumulation of a short chain intermediate C30. Thus, the elongation process needed for formation of the correct C55 product in wild-type UPPs is regulated by the small size of the Ala residue located at position 69 and the large size of the Leu resided at position 137.

The amino acid residues of the flexible loop are important for catalysis as well as substrate binding, particularly IPP binding. The flexibility of the UPPs structure and the involvement of several residues in IPP binding are required because the enzyme catalyzes multiple IPP condensations leading to a large and highly hydrophobic product which must exit the active site.

The site-directed mutagenesis studies presented here along with the previously reported data provide a model (see Fig. 7) for UPPs catalysis and substrate binding that can be used to design studies, while the determination of the crystal structure of the enzyme substrate complexes is in progress. In addition to the hypothetical FPP and IPP binding mode reported by Fujihashi et al. (25), we provide information on the amino acid residues involved in the product chain length determination and the role of a flexible loop in the model. According to this model, FPP is bound with its PPi group near the Asp-26, Arg-30, and Ghu-198 area, likely with the help of Mg2+ ion(s). The C15-farnesyl chain is bound with its tail pointing toward the bottom of the tunnel. The tunnel at this stage is probably in the closed form, and the 71–83 loop is open and flexible. An incoming IPP (possibly assisted by the loop of residues 71–83) attacks the carbocation formed from the bound FPP. After the reaction, the free PPi leaves and the pyrophosphate moiety of the C20 compound is translocated to the Asp-26 site, ready for the next cycle. The extended C30 oligoprenyl chain passes by Ala-69 and condenses with five additional IPP units until the final product reaches Leu-137 located on the bottom of the tunnel. When the chain length reaches C55, the dimethyl end is stopped at the end of the tunnel where the opening is small. At this point, the crowding between the prenyl chain and the 71–83 loop at the top of the tunnel forces the α3 helix to move away from the closed position into the open position. The fully synthesized C55-PP product can exit easily from the open gate, especially in the presence of Triton or membrane lipid bilayer.

![FIG. 7. A model of UPPs catalysis and substrate binding. As described in the text, FPP and IPP are bound near Asp-26 and Ghu-213 in the region located on the top of the hydrophobic tunnel. FPP undergoes chain elongation by condensation with IPP and reaches the chain length of C55. The Leu-137 is at the most critical position to block the further elongation of the product.](http://www.jbc.org/)

**Table II**

| UPPs  | $k_{cat}$ | $K_m$ (FPP) | $K_m$ (IPP) | rel$e_{cat}^a$ |
|-------|---------|-------------|-------------|--------------|
| Wild type | 2.5 ± 0.1 | 0.4 ± 0.1 | 4.1 ± 0.3 | 1 |
| S71A  | 0.11 ± 0.01 | 1.0 ± 0.2 | 133 ± 14 | 4 × 10$^{-2}$ |
| E73A  | 0.30 ± 0.01 | 0.4 ± 0.1 | 16.2 ± 2.2 | 0.1 |
| N74A  | (2.20 ± 0.03) × 10$^{-2}$ | 0.4 ± 0.1 | 8 ± 0.6 | 1 × 10$^{-2}$ |
| W75A  | 1.1 ± 0.1 | 3.2 ± 0.3 | 46 ± 4 | 0.5 |
| R77A  | (1.4 ± 0.1) × 10$^{-4}$ | 1.6 ± 0.3 | 15.7 ± 2.5 | 5 × 10$^{-3}$ |
| E81A  | 0.4 ± 0.1 | 0.8 ± 0.1 | 88 ± 10 | 0.2 |

*a $k_{cat}$ relative to that of wild type.

*b Data taken from Ref. 24.
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Mechanism of Product Chain Length Determination and the Role of a Flexible Loop in *Escherichia coli* Undecaprenyl-pyrophosphate Synthase Catalysis

Tzu-Ping Ko, Yi-Kai Chen, Howard Robinson, Pei-Chun Tsai, Yi-Gui Gao, Annie P.-C. Chen, Andrew H.-J. Wang and Po-Huang Liang

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