Catalytic Mechanism Revealed by the Crystal Structure of Undecaprenyl Pyrophosphate Synthase in Complex with Sulfate, Magnesium, and Triton*

Received for publication, March 17, 2003, and in revised form, May 16, 2003
Published, JBC Papers in Press, May 19, 2003, DOI 10.1074/jbc.M302687200

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Undecaprenyl pyrophosphate synthase (UPPs) catalyzes chain elongation of farnesyl pyrophosphate (FPP) to undecaprenyl pyrophosphate (UPP) via condensation with eight isopentenyl pyrophosphates (IPP). UPPs from *Escherichia coli* is a dimer, and each subunit consists of 253 amino acid residues. The chain length of the product is modulated by a hydrophobic active site tunnel. In this paper, the crystal structure of *E. coli* UPPs was refined to 1.73 Å resolution, which showed bound sulfate and magnesium ions as well as Triton X-100 molecules. The amino acid residues 72–82, which encompass an essential catalytic loop not seen in the previous apoenzyme structure (Ko, T.-P., Chen, Y. K., Robinson, H., Tsai, P. C., Gao, Y.-G., Chen, A. P.-C., Wang, A. H.-J., and Liang, P.-H. (2001) *J. Biol. Chem.* 276, 47474–47482), also became visible in one subunit. The sulfate ions suggest locations of the pyrophosphate groups of FPP and IPP in the active site. The Mg"⁺ is chelated by His-199 and Glu-213 from different subunits and possibly plays a structural rather than catalytic role. However, the metal ion is near the IPP-binding site, and double mutation of His-199 and Glu-213 to alanines showed a remarkable increase of *Kₘ* value for IPP. Inside the tunnel, one Triton surrounds the top portion of the tunnel, and the other occupies the bottom part. These two Triton molecules may mimic the hydrocarbon moiety of the UPP product in the active site. Kinetic analysis indicated that a high concentration (>1%) of Triton inhibits the enzyme activity.

Undecaprenyl pyrophosphate synthase (UPPs) catalyzes the consecutive condensation reactions of eight molecules of isopentenyl pyrophosphate (IPP) with farnesyl pyrophosphate (FPP) to form a lipid carrier to mediate bacterial peptidoglycan synthesis (1–3). This enzyme belongs to a family of prenyltransferases that make linear IPP condensation products with designate chain lengths (4). These enzymes are divided into trans-type and cis-type, which catalyze the trans- and cis-double bond formation during each IPP condensation, respectively (5, 6). Unlike trans-prenyltransferases, which tend to make short and medium chain-length products ranging from C₁₅ to C₅₀, UPPs and other cis-prenyltransferases mostly generate C₇₅₆ long-chain products. Significant sequence homology has been found within the cis- and trans-prenyltransferases, but the two groups of enzymes do not share sequence similarity (7, 8). Among the trans-prenyltransferases, the crystal structure of avian farnesyl pyrophosphate synthase has been solved almost a decade ago, and the mechanism has been elucidated (9). However, only recently have the first crystal structures of cis-prenyltransferase (UPPs) become available (10, 11), and they provide a template for modeling other cis-enzymes such as dehydrodolichyl pyrophosphate synthase from yeast and human and a polyprenyl pyrophosphate synthase discovered in *Arabidopsis thaliana* (12, 13).

UPPs from *Escherichia coli* is a dimer of identical subunits of 253 amino acids. The three-dimensional structure of the apoenzyme reveals an elongated tunnel-shaped active site crevice surrounded by two α-helices and four β-strands (11). Previous site-directed mutagenesis studies suggested that the substrates FPP and IPP are bound on top of the tunnel, and the farnesyl moiety of FPP migrates toward its bottom during product chain elongation (14–16). The tunnel is sealed at the bottom by the side chain of Leu-137 (11). In the crystal structure of UPPs from *Micrococcus luteus*, a sulfate ion bound to a conserved structural P-loop represents the location of the pyrophosphate moiety of FPP (10).

The previous *E. coli* UPPs crystal was grown using polyethylene glycol (PEG), and no sulfate ion was observed (11). Metal ion was not found in both apo-UPPs structures, although the enzyme requires Mg²⁺ for activity. Two protein conformers of the *E. coli* UPPs were observed, i.e. one with a bound PEG fragment that adopts a narrower conformation than the other one with water molecules in the active site, indicating the possible open and close mechanism for substrate binding and product release. These two conformers have the most striking difference in the position of the α₃ helix, which is connected to a loop containing amino acids 72–82. In the proposed catalytic model, the loop may play an essential role in pulling the α₃ helix toward the active site (17). Perhaps because of the requirement of its flexibility in enzyme catalysis, this loop was invisible in the previous crystal structure of the apoenzyme. Neither was this loop observed in the crystal structure of UPPs from *M. luteus*, in which both subunits had the closed conformation (10).

To answer the questions regarding the location of substrate...
binding, the role of the metal ion, and the function of the flexible loop in enzyme catalysis, we report here the structure of UPPs with sulfates, Mg$^{2+}$ ions, and two molecules of Triton X-100 occupying the tunnel in conjunction with the kinetic results of a few relevant mutants. Triton at low concentration has been shown to increase the UPPs steady-state reaction rate (18). However, a high concentration of Triton in the crystallization condition resulted in the occupancy of Triton in the active site. Therefore, the dose dependence of Triton in altering the reaction velocity was also examined.

**EXPERIMENTAL PROCEDURES**

**Materials**—Radiolabeled [14C]IPP (55 mCi/mmol) was purchased from Amersham Biosciences, and FPP was obtained from Sigma. Reverse-phase thin layer chromatography (TLC) plates were purchased from Merck. Toy DNA polymerase was obtained from Invitrogen. The plasmid mini-prep kit, DNA gel extraction kit, and a nickel-nitrilotriacetic acid resin were purchased from Qiagen. Potato acid phosphatase plasmid mini-prep kit, DNA gel extraction kit, and a nickel-nitrilotriacetic acid resin were purchased from Qiagen. Potato acid phosphatase from Merck. Reversed-phase thin layer chromatography (TLC) plates were purchased from Amersham Biosciences, and FPP was obtained from Sigma. Reverse-phase thin layer chromatography (TLC) plates were purchased from Amersham Biosciences, and FPP was obtained from Sigma.

**Crystallographic Analysis**—For purification of enzymes, we previously reported protocol of using nickel-nitrilotriacetic acid column was followed (18). The purified wild-type UPPs was crystallized using the hanging drop set-up from Hampton Research (Laguna Niguel, CA), while attempts were tried to incorporate FPP into the crystal. In the end, 2 μl of mother liquid containing 0.01 M cobalt chloride, 0.1 M MES, and 1.8 M ammonium sulfate at pH 6.5 was mixed with 2 μl of protein solution consisting of 10 mg/ml UPPs, 2% Triton X-100, 5 mM MgCl$_2$, and 660 μM FPP. The mixture was equilibrated against 200 μl of the mother liquid at 25 °C. Crystals started to appear within 10 days. This condition was different from the previous one for the Se-Met enzyme (11), but the crystals turned out to be isomorphous. Diffraction experiments on the UPPs crystal was carried out at ~150 °C on beam line 17B2 of the National Synchrotron Radiation Research Center in Hsinchu, Taiwan. Data were processed and scaled by employing the program HKL2000 (19). For computational refinement, manual modification, and analysis of the crystal structure, the programs CNS (20), O (21), and CCP4 (22) were used.

Using 2.0-Å resolution data and the apoenzyme model that we solved previously, but with all solvent and cofactor molecules removed, an initial R-value of 0.52 was calculated, and it was reduced immediately to 0.39 after rigid body refinement. The Se-Met residues in the original model were replaced with methionines, and subsequent energy minimization yielded an R-value of 0.29 before calculation of the first Fourier map. According to the density level of 1.5 σ, 327 water molecules were added to the model, and the amino acid side chains were adjusted. Strong densities in the active site tunnel of one subunit (monomer B) clearly showed two Triton molecules, but they were modeled as a series of solvent atoms. Possible densities for sulfate and metal ions were also modeled as water molecules.

Prior to subsequent refinement, 5% of randomly selected reflections were set aside to calculate R$_{free}$ values for monitoring progress of refinement (23). The model yielded R and R$_{free}$ values of 0.227 and 0.256 after simulated annealing and temperature factor refinement when the resolution was increased to 1.8 Å. Explicit models for the two Triton molecules, the sulfate, and the magnesium ions were constructed, and the polypeptide termini were also modified according to the Fourier maps. With the bound cofactors and 393 water molecules, the R and R$_{free}$ values were reduced to 0.200 and 0.231, respectively. At this point, densities for residues 72–82 of monomer B became interpretable in the map, and the corresponding fragment was modeled. Finally, the resolution was increased to 1.73 Å, and more water molecules were added according to 1.0 σ density level in the 2F$_{o}$–F$_{c}$ map. Statistical numbers for the diffraction data set and the refined model are listed in Table I.

**Site-directed Mutagenesis of UPPs—**UPPs mutants were prepared by using PCR techniques in conjunction with the E. coli Bos-12 UPPs gene template in the pET32Xa/LIC vector. The mutagenic primers used were prepared by MdBio, Inc. The mutagenic oligonucleotides for performing site-directed mutagenesis are as follows: 5’-GCTTGGGCGATTGAAAC-3’ for H48A; 5’-GCGGAGGTCCGATTGAT-3’ for H199A; and 5’-GGAGGAGATGCACT-3’ and 5’-GCCATGCGACTT-3’ for the H199A/E213A double mutant. Subsequently, the forward primer 5’-GTATTAGGGGTAGCACTGCTTCTG-3’ and the reverse primer 5’-AGAGGAAGGTAGAGCCTACCCGT-3’ were used in combination with the PCR products obtained using the above mutagenic oligonucleotides to create the full-length mutant UPPs genes. The F50 cleavage site (IEGR) and the complementary sequences to the sticky ends of the linear vector pET32Xa/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 Tris-acetate-EDTA (TAE) buffer, and the gel was then stained with ethidium 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 gene 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 

\[ A_{	ext{max}} = 0.6 \]

and induced with 1 mM isopropyl-1-thiogalactopyranoside. After 4–5 h, the cells were harvested by centrifugation at 7,000 \( \times g \) for 15 min.

### Measurements of \( K_m \) and \( k_{cat} \) Values for Mutant UPPs—For the measurement of kinetic parameters, mutant UPPs (0.01 µM H199A, 0.5 µM H199A/E213A, or 0.5 µM H434A) was utilized to initiate the reaction of FPP and [\(^{14}C\)]IPP in 200-µl solutions. For IPP \( K_m \) and \( k_{cat} \) determinations, 5 µM FPP was utilized to saturate the enzyme, and IPP concentrations of 0.5–5-fold \( K_m \) were employed. For FPP \( K_m \) measurements, 0.2–20 µM FPP were used along with 20 µM [\(^{14}C\)]IPP. All reactions were carried out in 100 mM KOH-Hepes buffer, pH 7.5, 50 mM KC1, 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 µM 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 using a Beckman LS6500 scintillation counter. Initial velocity data were fitted to Equation 1 to obtain \( K_m \) and \( k_{cat} \) values by non-linear regression using KaleidaGraph computer program. The \( k_{cat} \) was calculated from \( \frac{V_{max}}{[E]} \), as shown in Equation 1,

\[
\frac{V}{V_{max}} = \frac{[S]}{K_m + [S]} \quad \text{(Eq. 1)}
\]

where \( V_{max} \) is the initial velocity, \([E]\) is the enzyme concentration, \([S]\) is the substrate concentration, \( V \) is the maximum velocity; and \( K_m \) is the Michaelis constant.

### Enzyme Activity Measurement Under Different Concentrations of Triton

X-100—To measure the kinetic constant of *E. coli* UPPs in the presence of Triton X-100, the enzyme reaction was initiated by adding 0.1 µM UPPs to a reaction mixture containing 0.01% Triton X-100, whereas to reaction mixtures containing 0.01, 0.05, 0.07, 0.1, 0.5, or 1.5% of Triton X-100 (v/v), 0.01 µM enzyme was added. All reactions were carried out in 5 µM FPP, and 50 µM [\(^{14}C\)]IPP along with 100 mM Hepes-KOH buffer (pH 7.5), 50 mM KC1, and 0.5 mM MgCl2. Portions of the reaction mixture were periodically withdrawn within 10% substrate depletion and mixed with 10 µM EDTA for reaction termination. The radiolabeled products were then extracted with 1-butanol, and the radioactivities associated with aqueous and organic phases were separately quantitated by using a Beckman LS6500 scintillation counter. Under the saturating concentration of FPP and IPP (5-fold \( K_m \) value), the rate of IPP condensation was determined as \( k_{cat} \) (s\(^{-1}\)).

### RESULTS AND DISCUSSION

#### Overall Structure—The refined model of UPPs in complex with sulfate, Mg\(^{2+}\), and Triton contains amino acid residues 13-71 and 86-240 in one subunit (monomer A) and 17-241 in another (monomer B). Fig. 1, A and B show ribbon diagrams of the dimeric protein structure. There are two sulfate ions in the active site of each monomer and two Triton X-100 molecules bound to the active site tunnel of monomer B. Two magnesium ions are bound to equivalent positions at the dimer interface. Not included in these figures are a fifth sulfate ion and an unidentified compound with a five-member ring, both observed at crystal contact regions, plus 688 water molecules. The two UPPs subunits have very similar structures as those observed in the apoenzyme crystal (11), with monomers A and B assuming the closed and open conformations, respectively. The entire dimers of UPPs can be superimposed with a root mean square deviation of 1.59 Å between bound and apo forms for all 3,383 equivalent non-hydrogen atoms. Excluding the terminal residues that have large deviations, the root mean square deviation is 1.18 Å for 3,300 atoms and 0.594 Å if only 1,656 backbone atoms are compared.

The most significant difference in the protein model of apo-UPPs and the current UPPs model is the presence of the additional loop 72–82 in monomer B, as shown in Fig. 1C. In the final difference Fourier map, although some of the side chains still lack clear densities, the polypeptide backbone is well defined. The loop was not in contact with neighboring molecules, and it became visible probably because the bound Triton molecules stabilized this particular conformation of UPPs in the crystal. The residues of 79–82 extended the helix \( \alpha \) 3 by more than one turn at the N terminus, which is capped with Pro-78. The succeeding residue, Ser-83, was moved by more than 2 Å away from the active site. Indeed, the entire \( \alpha \) 3 helix was displaced by 1.1 Å before the kink at Glu-96 and −1.0 Å for residues 97–103. It became more straightened than in the original model. The adjacent helix \( \alpha \) 4 was also shifted by about 1 Å. Thus, the structure of monomer B in the present UPPs crystal has an even more open conformation than that of apo-UPPs.

In contrast, monomer A remained largely unchanged as in the apo-UPPs crystal. Only slight shifts were observed in the helices \( \alpha \) 3 and \( \alpha \) 4, with maximal displacements of 1 Å, although the kink of helix \( \alpha \) 3 seems to occur at Glu-96 also rather than at Ala-92, as in apo-UPPs. Apparently, monomer A assumes a closed conformation, as in the apoenzyme. The \( B\)-values of residues 88–96 are between 40 and 60 Å\(^2\), whereas those of 86–87 are over 60 Å\(^2\), indicating some disorder in this region. Other regions with \( B\)-values higher than 40 Å\(^2\) are located in residues 114–115 of monomer A and 74–81 of monomer B, corresponding to the loops of \( \beta \)-c- and \( \beta \)-b, respectively. Movement of these two helices, which constitute the forewing in the butterfly-shaped UPPs molecule (11), accounts for the conversion between open and closed conformations and, thus, modulates binding and release of the substrate and product from the tunnel-shaped active site.

Adjacent to the flexible loop, residues 69–71 at the C terminus of strand \( \beta \) 8 show maximal displacements (Fig. 1C). Specifically, the entire residues of Ala-69, Phe-70, and Ser-71 in monomer B were moved by 3.3, 5.8, and 7.5 Å, respectively. The phenyl group of Phe-70 was moved by 14.0 Å from the original position in apo-UPPs, where it was only 2 Å away from a bound sulfate ion in the current structure. Such structural changes presumably were a result of the bound sulfate ion and Triton molecules in the active site. It now interacts directly with the tert-ocetyl phenyl head group of Triton (see below). The neighboring side chains of Leu-85, Leu-88, Phe-89, Trp-91, and Ser-95 in the \( \alpha \) 3 helix were also rotated to accommodate the Triton molecule. Similar rearrangements for residues 69–71 were also observed in monomer A, but the side chains in helix \( \alpha \) 3 remained in their original positions, perhaps because of the absence of the Triton molecule. The N-terminal part of helix \( \alpha \) 3 that encompasses residues 79–85 and the connecting loop of 72–78 to strand \( \beta \) 8 were not observed in monomer A. Presumably, these residues were flexible, as in the apoenzyme.

### The Sulfate Ions—Both FPP and IPP substrates, as well as the reaction intermediates and the final product UPP, have a highly negatively charged pyrophosphate moiety in the molecule. In the previously solved crystal structure of farnesyl pyrophosphate synthase, the allylic and homoallylic substrates are bound via Mg\(^{2+}\) ions, which are coordinated with the active site Asp residues in the conserved DDXXD motifs (9). However, a similar DDXXD motif was not found in cis-prenyltransferases. In contrast, the crystal structure of UPPs from *M. luteus* (10) showed a sulfate ion bound to a highly conserved structural P-loop that contains the positively charged Arg-32 (equivalent to Arg-30 in the *E. coli* enzyme). This structural P-loop is supposed to interact with the pyrophosphate of the...
allylic substrate FPP. A second binding site for pyrophosphate of the homoallylic substrate IPP was also proposed (10, 16). It involves two positively charged arginine residues, 197 and 203 (Arg-194 and Arg-200 in *E. coli*). In both cases, Mg\(^{2+}\) ions were also supposed to participate in substrate binding by bridging the pyrophosphates of FPP and IPP with the acidic side chains of Asp-29 and Glu-216* (Asp-26 and Glu-213* in *E. coli*). In our refined UPPs structure there are five sulfate ions, and both monomers have two sulfates bound in the active site, as residues in the counter subunit are designated by an asterisk after the position number (e.g. Glu-216*).

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shown in Figs. 1 and 2. The first sulfate ion (S1) forms five well-defined hydrogen bonds with the backbone nitrogen atoms of Gly-27 (G27), Gly-29 (G29), and Arg-30 (R30) and the side chain of Arg-30. B, the second sulfate (S2) makes hydrogen bonds with the side chains of Arg-194 (R194), Arg-200 (R200), and Ser-202 (S202) as well as the backbone N of Arg-242* (R242*). Other nearby residues of Asp-26 (D26), Arg-39 (R39), and Arg241* (R241*) are also shown. For comparison, the model of apo-UPPs is superimposed and shown with pink bonds. Panels A and B were drawn using monomers B and A, respectively. Two Mg²⁺ ions are bound to equivalent sites at the dimer interface, and one is shown in panel C. C, the octahedral architecture of each cation is constituted by the side chains of His-199 (H199) and Glu213* (E213*) from different subunits and four water molecules. The water molecules are further hydrogen bonded to protein atoms, forming a network that maintains an ordered structure in the nearby region. Bonds colored in pink and blue are for monomers A and B, respectively, whereas the superimposed apo-UPPs model is shown using thin sticks.
molecules was also hydrogen-bonded to the backbone nitrogen atom of Ser-99 and a third water molecule, which was bound to the backbone oxygen and nitrogen atoms of Asp-94 and Lys-98, respectively. Insertion of these two water molecules between the normally hydrogen-bonded backbone atoms actually produced the kink at Gly-96 in the α3 helix. Similar intercalation of waters in the α3 helix was also observed for monomer A, but the densities were weaker.

Compared with the original model of apoenzyme, quite a few rearrangements in the UPPs structure occurred upon sulfate binding. In monomer A, the side chain of Arg-30 remains in original position, but the guanidium group was flipped over to bind the S1 sulfate ion. Originally, it was hydrogen bonded to Asp-26. The side chain of Asp-26 was rotated 120° for the χ1 angle upon binding sulfate, and the carbonyl group became bonded to the side chain of Arg-194 (Fig. 2A). The side chain of Arg-200 formed a salt bridge with Glu-213 in apo-UPPs. In the present structure, the guanidium group was moved 4 Å upon swinging the side chain by 120° for the χ1 angle to interact with the S2 ion. The side chain of Arg-39 also underwent slight reorientation. In monomer B of the apo-UPPs, the side chain of Arg-30 had a different conformation, which did not interact with Asp-26. Upon sulfate binding, it moved 5 Å toward the S1 ion by rotating almost 180° about the χ3 angle and made identical interactions with the sulfate as in monomer A (Fig. 2A). The side chain of Asp-26 in monomer B was also rotated 120° and made hydrogen bonds with Arg-194. The side chain of Arg-200, originally bound to Glu-213 and Glu-240*, was moved 5 Å toward the S2 ion by 120° rotation about the χ3 angle. Arg-39 remained almost unchanged, as in monomer A.

Consistent with the model proposed for the UPPs from M. luteus, the S1 and S2 sulfate ions observed in our crystal structure may represent the locations of the pyrophosphate groups of the allylic and homoallylic substrates FPP and IPP, respectively, in E. coli UPPs. However, no magnesium ion was observed to make direct interactions with the bound sulfate ions. As shown below, the magnesium ions are bound in other places, and they function in a different way than those in FPPs or other enzymes with pyrophosphate substrates.

The Magnesium Binding Sites—The role of the metal ion has often been argued in metal-requiring enzymes. In prenyltransferases, the common mechanism may be that Mg$^{2+}$ chelated by Asp residues coordinates with the pyrophosphate moiety of substrate FPP and facilitates the nucleophilic attack by making the pyrophosphate a better leaving group. The trans-type prenyltransferases all have two DDXXD motifs responsible for allylic substrate (FPP) and homoallylic substrate (IPP) binding (24). The Asp residues in the motif play essential role in Mg$^{2+}$ binding, and the substitution of these Asp residues with Ala led to the remarkable decrease of substrate affinity and turnover number (25, 26). However, none of these motifs is found in the cis-type enzymes. In the UPPs from E. coli, a possible candidate for binding Mg$^{2+}$ in the active site is Asp-26 (or Asp-29 in the M. luteus enzyme). As shown above, this residue did have significant conformation change of the side chain upon binding sulfate, but no Mg$^{2+}$ ion seemed to be involved. Nevertheless, it remains unanswered whether Mg$^{2+}$ will participate in binding if the anions are actually pyrophosphates.

In the refined structure of UPPs crystallized in the presence of 5 mM MgCl$_2$, there are two Mg$^{2+}$ ions bound to the enzyme (Fig. 1). The two Mg$^{2+}$ binding sites are equivalent. They are located in the dimer interface and related by the molecular dyad axis. As shown in Fig. 2C, each ion is octahedrally coordinated with six ligands; one of them is the ND1 atom in the side chain of His199, another is the OE1 atom of Glu213, and the other four are water molecules. The water molecules are directly hydrogen bonded with the backbone O of Gly197, the side chain OE1 of Glu198 and the backbone N of His199 in one subunit, as well as the side chain OE2 of Glu213 and the backbone O of Ala235 in another subunit. The hydrogen bond network is further extended with the involvement of ordered water molecules in this region. The Mg$^{2+}$ binding site is 11 Å from the S2 site for sulfate ion in both monomers, and there is no direct interaction between the bound cation and anion.

In the previous structure of apo-UPPs, the C termini of both subunits were disordered, wherein no densities were observed beyond residue 240 of monomer A and residue 238 of monomer B. In the current structure, the C termini are also disordered. However, some notable rearrangements were observed. In both subunits, the imidazole ring of His-199 was rotated 90° into a proper orientation for coordinating the Mg$^{2+}$ ion (Fig. 2C). Without the cation, the Mg$^{2+}$ binding site was occupied by the positively charged side chain of Arg-239*. It was moved 11 Å to the other side of peptide backbone in the presence of Mg$^{2+}$ ion and redirected toward the S2 sulfate ion in the active site. The side chain of Glu-240* was salt bridged with that of Arg-200 in the apo-UPPs, but in the current model the backbone atoms were displaced by 4.5 Å, and the side chain also moved 10 Å away from the active site, facing the solvent. Similar conformations of Arg-239 and Glu-240 are seen in both monomers, whereas the additional Arg-241 of monomer B is 3.4 Å from the S2 ion in monomer A. As shown above, Arg-200 is directly bonded to the S2 sulfate ion, while Arg-239* and Arg-241* may also be involved. Consequently, the binding of Mg$^{2+}$ is likely to generate a more ordered structure of the C terminus for interactions with the pyrophosphate substrate.

### Effects of Mutants on the UPPs Activity—As shown in Table II, our results from a previous mutagenesis study showed that substitution of Asp-26 in E. coli UPPs by alanine decreased the $k_{cat}$ to only one-thousandth (10$^{-3}$) of that for the wild-type enzyme without significant change of the $K_m$ values for FPP and IPP (14). Therefore, Asp-26 is important for catalysis but not for substrate binding. The IPP condensation mechanism of the enzymes for polyprenyl pyrophosphate synthesis has been well established (5, 6). A carboxylation is first generated by eliminating the pyrophosphate in the allylic substrate, with the assistance of charge neutralization by Mg$^{2+}$ or protonation of the leaving group. A proton on the second carbon of the ho-

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| UPPs | $k_{cat}$ | $K_m$ (FPP) | $K_m$ (IPP) | Relative $k_{cat}$ |
|------|----------|-------------|-------------|-------------------|
| Wild type | 2.5 ± 0.1 | 0.4 ± 0.1 | 4.1 ± 0.3 | 1 |
| D26A | (3.30 ± 0.03) × 10$^{-3}$ | 0.5 ± 0.1 | 1.41 ± 0.3 | 1 × 10$^{-3}$ |
| H45A | (2.6 ± 0.2) × 10$^{-3}$ | 3 ± 0.3 | 63 ± 3.6 | 1 × 10$^{-3}$ |
| H199A | 2.2 ± 0.2 | 0.28 ± 0.08 | 16 ± 5 | 1 |
| E213A | (2.6 ± 0.2) × 10$^{-2}$ | 0.7 ± 0.1 | 280 ± 20 | 1 × 10$^{-2}$ |
| H199A/E213A | (2.5 ± 0.2) × 10$^{-3}$ | 0.66 ± 0.1 | 279 ± 83 | 1 × 10$^{-3}$ |

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

* Kinetic constants obtained from Ref. 14.
moiety of Triton consisting of a phenyl ring and a tert-octyl group is entirely hydrophobic. In the T1 molecule, it is buried near its bottom. The head group of the second Triton molecule, T2, also makes direct hydrophobic interactions with that of T1—prenyl portion of the product. The Triton molecules together may mimic the entire product in the tunnel.

Fig. 3. The structures of Triton X-100 molecules and the occupied tunnel of UPPs. A, in the UPPs active site, two molecules of Triton X-100 were found: one appeared linear (left, T1) and the other curved (right, T2). The models are superimposed on the final 2Fₐ – Fₜ map contoured at the 1.0 σ level. Densities in the initial Fourier maps were comparable with the clear densities shown here, especially for the tert-octyl phenyl head groups. During refinement, the PEG tails were extended, yielding final numbers of 24 and 30 atoms in the T1 and T2 molecules, respectively. This figure was produced using BobScript (30) and Raster3D. B, the monomer B is viewed with the active site tunnel in the front. This subunit is in complex with two sulfates (S1 and S2), a magnesium ion (Mg), and two molecules of Triton X-100 (T1 and T2). C, a schematic diagram for the cis- and trans-prenyl moieties of product UPP. The cis-trans C₁₅ portion of the product, which is located in the bottom of the active site cleft, can be represented by the linear Triton molecule, T1. The other circular Triton T2 resembles the cis-prenyl portion of the product. The Triton molecules together may mimic the entire product in the tunnel.

Regarding the role of Mg²⁺ in substrate binding, our fluorescence studies showed that the FPP substrate still binds to UPPs and quenches its intrinsic fluorescence even in the absence of Mg²⁺ (17). However, the IPP binding absolutely requires Mg²⁺. As discussed above, binding of Mg²⁺ resulted in significant structural changes in the C-terminal regions of the enzyme, which allowed side chain rotations of Arg-200 and Arg-239 into a proper orientation for binding the S2 sulfate ion. Previous studies showed that Gu-213 is involved in IPP binding, because replacement of Glu-213 with Ala resulted in a significant 70-fold increase of IPP Kₐ value and a 100-fold reduction of kₐcat (14, 16) (Table II). In the current structure, His-199 and Glu-213⁰ constitute two ligands for binding Mg²⁺, and we further examined the importance of His-199 by mutating it to Ala. As listed in Table II, the mutant H199A shows a four times larger Kₐ value for IPP. However, its role seems not as important as that of Glu-213. The His-199/Glu-213 double mutant also displays 70-fold larger IPP Kₐ and 1,000-fold smaller kₐcat values. Therefore, Glu-213 is essential in binding Mg²⁺, but His-199 is optional. These results are consistent with the previous observation that both carboxyl oxygen atoms in the side chain of Glu-213 contribute to Mg²⁺ binding. On the other hand, in all three mutants the Kₐ values for FPP remained nearly unchanged, providing further evidence for the
this hydrophobic cluster, two adjacent side chains of Trp-75 and Leu-85 tend to form a lid to cover the active site cleft. The PEG tail makes a U-turn at the second ethylene glycol unit, which is opposed by Met-25 and Trp-221. The third unit is in contact with Gly-46 and Val-50, and it is close to the side chain of His-43, with a distance of 4.5 Å. The 4th and 5th ethylene glycol units fold back on the phenyl head group, and they also interact with the tert-octyl group of the T1 molecule as well as the side chains of Phe-89 and Ala-92. The remaining 4–5 units of the PEG tail protruded out of the active site and were not observed because of disorder.

The hydrophobic nature of the Triton molecules and their propensity to replace the natural substrate FPP in binding to the active site cleft suggest that the Triton structure should somehow resemble the reaction intermediates or product of the active site cleft. Because of the different stereochemistry (cis) UPPs catalyze from that (all-trans) of substrate FPP, the product UPP likely adopts a folded structure. The length of the observed PEG tail of the T1 molecule is comparable with that of a straightened all-trans farnesyl group of the substrate, whereas the remainder of T1 together with the entire T2 molecule has about the same size of the cis-polyprenyl moiety of the product. Thus, the linear part of T1 and the other parts of Triton molecules mimics the trans- and cis-prenyl parts of the product, respectively. In addition, the shortest distance between the Triton molecules and the sulfate ions S1 and S2 are 7.6 and 12.4 Å, respectively. Consequently, it is more likely for the S1 site to serve for binding to the pyrophosphate moiety of the allylic substrate FPP.

\textbf{High Concentration of Triton Inhibits the Enzyme Activity—}
Our previous studies have shown that Triton X-100 at a low concentration of 0.1% increased the steady-state \( k_{cat} \) of \( E. \ coli \) UPPs reaction by 190-fold (18). The rationale for this activity stimulation by Triton can be attributed to its ability to provide the preferred interaction with the product, which is highly hydrophobic, and thus facilitate product release from the active site, which is the rate-limiting step of steady-state catalysis. In the present structure, two Triton molecules were found to occupy the UPPs active site and prohibit binding of the natural substrate FPP. Therefore, we determined the UPPs activity in the presence of high concentration of Triton X-100 as used in the protein crystallization experiments. As shown in Fig. 4, the enzyme activity is increased with the addition of a low concentration of Triton, which is similar to the previous results. However, when the concentration is higher than 1%, the UPPs activity is decreased with the addition of Triton X-100. The reaction rate at each Triton concentration was determined by counting the radioactivity in the butanol layer. The plot of kinetic constant versus the percentage of Triton X-100 illustrates the effect of Triton on the IPP condensation rate of steady-state reaction.

\begin{table}[h]
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
Residue 1 & Atom 1 & Residue 2 & Atom 2 & Distance (Å) \\
\hline
T1 head & C8 & T2 head & C8 & 4.02 \\
T1 head & C4 & T2 tail & C26 & 3.54 \\
T2 head & C12 & T2 tail & C25 & 3.56 \\
T1 head & C2 & Ala-47 (α2) & CB & 4.22 \\
T1 head & C3 & Ala-92 (α3) & CB & 4.41 \\
T1 head & C8 & Leu-107 (βC) & CD1 & 4.43 \\
T1 head & C8 & Ile-141 (βD) & CD1 & 4.46 \\
T1 head & C12 & Leu-100 (α3) & CD2 & 3.70 \\
T1 head & C13 & Val-54 (α2) & CG2 & 3.68 \\
T1 head & C14 & Val-50 (α2) & CG1 & 3.79 \\
T1 head & C14 & Leu-139 (βD) & CD2 & 3.98 \\
T1 tail & C19 & Val-54 (α2) & CG1 & 3.61 \\
T1 tail & C19 & Leu-100 (α3) & CD2 & 4.03 \\
T1 tail & C20 & Val-105 (βC) & CG2 & 4.31 \\
T2 tail & C22 & Ala-58 (α2) & CB & 3.90 \\
T2 head & C2 & Ala-143 (βD) & CB & 3.62 \\
T2 head & C2 & Ala-143 (βD) & CZ & 3.80 \\
T2 head & C3 & Leu-120 (α4) & CD2 & 4.08 \\
T2 head & C3 & Ile-124 (α4) & CD1 & 4.07 \\
T2 head & C4 & Ile-109 (βC) & CG1 & 3.49 \\
T2 head & C7 & Ala-92 (α3) & CB & 3.72 \\
T2 head & C8 & Leu-93 (α3) & CD2 & 4.03 \\
T2 head & C10 & Phe-89 (α3) & CE1 & 3.76 \\
T2 head & C11 & Phe-70 (βB) & CE2 & 4.05 \\
T2 head & C12 & Ala-69 (βB) & CB & 3.44 \\
T2 head & C14 & Ile-141 (βD) & CG2 & 3.84 \\
T2 tail & C20 & Met-25 (βA) & CB & 3.65 \\
T2 tail & C20 & Trp-221 (βP-α7) & CD1 & 3.40 \\
T2 tail & C22 & Val-50 (α2) & CG2 & 3.90 \\
\hline
\end{tabular}
\caption{Hydrophobic interactions between Triton molecules and UPPs. Atom pairs in the interacting Triton moieties or amino acid residues with minimal contact distances (<4.5 Å) are listed. The corresponding secondary structure elements are given in parentheses for amino acids.}
\end{table}
activity drops. Presumably a high concentration of Triton increases the chance for Triton to occupy the active site, converts most UPPs molecules into the full-open conformation, and thus inhibits the enzyme reaction.

**Active Site Structure and Catalytic Mechanism**—In the current crystal structure of UPPs, the sulfate ions show the locations of pyrophosphate binding sites for substrate. The Mg$^{2+}$ ions induce conformational changes in the C-terminal region to facilitate binding of IPP. The two Triton molecules in the active site cleft may represent the cis and trans -prenyl moieties of the product. In a plausible mechanism of UPPs, the deprotonated IPP acts as a nucleophile to attack the carbocation formed from FPP by His-43-assisted dissociation of the pyrophosphate group, similar to other prenyltransferase reactions (27). As suggested by our structural analysis, Asp-26 and His-43 play key roles in the enzyme reaction in which Asp-26 acts as general base to deprotonate IPP and His-43 provides a proton to FPP for the dissection of its pyrophosphate group. Both of their mutations to Ala resulted in a significant decrease of $k_{\text{cat}}$ (Table II). The loop of $\beta\beta\alpha3$ containing Glu-73, Trp-75, and Arg-77 may also be involved in the catalysis. Our previous site-directed mutagenesis studies have shown that the mutation of these three residues to Ala led to lower substrate affinity and catalytic activity (11). An overview of the active site structure with the proposed reaction mechanism for UPPs catalysis is shown in Fig. 5.

The subunit conformations with the primary substrate analogue of sulfate (monomer A) and the additional product analogue of Triton (monomer B) are different, particularly in the $\alpha3$ helix. The interconversion between two conformations of open and closed forms is implicated in substrate binding and product release. The conformational change during substrate binding and catalysis of UPPs has been probed previously using steady-state and stopped-flow fluorometers (17). It was shown that FPP binding quenches the fluorescence of Trp-91 in the $\alpha3$ helix, which moves toward the active site during substrate binding and thereby results in a closed conformation to provide better interaction of UPPs with the substrate. After the reaction, the crowding prenyl chain of the product shifts the UPPs structure to an open conformer for product release.
As a summary, the UPPs turnover is shown in Fig. 5D. The binding of FPP followed by an incoming IPP initiates the condensation reaction. The condensation occurs with the release of pyrophosphate from FPP, leading to addition of five carbon atoms to the growing hydrocarbon chain. A similar reaction is repeated by incorporating another IPP molecule, and it proceeds until the FPP chain elongation yields the C55 final product.

The all-trans C15 portion of the product reaches the bottom trans-prenyl portion of the product. cis- Specifically, although the loop of 72–82 can be seen in the present unpublished data.

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Acknowledgment—We thank National Synchrotron Radiation Research Center for beam time allocation.

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Catalytic Mechanism Revealed by the Crystal Structure of Undecaprenyl Pyrophosphate Synthase in Complex with Sulfate, Magnesium, and Triton
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J. Biol. Chem. 2003, 278:29298-29307.
doi: 10.1074/jbc.M302687200 originally published online May 19, 2003

Access the most updated version of this article at doi: 10.1074/jbc.M302687200

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