Effect of Site-Directed Mutagenesis of the Conserved Aspartate and Glutamate on *E. coli* Undecaprenyl Pyrophosphate Synthase Catalysis†

Jian-Jung Pan, Lee-Wei Yang, and Po-Huang Liang*

Institute of Biological Chemistry, Academia Sinica, Nankang, Taipei 11529, Taiwan

Received May 30, 2000; Revised Manuscript Received August 4, 2000

ABSTRACT: Undecaprenyl pyrophosphate synthase (UPPs) catalyzes condensation of eight molecules of isopentenyl pyrophosphate with farnesyl pyrophosphate to yield C_{55}-undecaprenyl pyrophosphate. We have mutated the aspartates and glutamates in the five conserved regions (I to V) of UPPs protein sequence to evaluate their effects on substrate binding and catalysis. The mutant enzymes including D26A, E73A, D150A, D190A, E198A, E213A, D218A, and D223A were expressed and purified to great homogeneity. Kinetic analyses of these mutant enzymes indicated that the substitution of D26 in region I with alanine resulted in a 10^3-fold decrease of k_{cat} value compared to wild-type UPPs. Its IPP K_m value has only minor change. The mutagenesis of D150A has caused a much lower IPP affinity with IPP K_m value 50-fold larger than that of wild-type UPPs but did not affect the FPP K_m and the k_{cat}. The E213A mutant UPPs has a 70-fold increased IPP K_m value and has a 100-fold decreased k_{cat} value compared to wild-type. These results suggest that D26 of region I is critical for catalysis and D150 in region IV plays a significant role of IPP binding. The E213 residue in region V is also important in IPP binding as well as catalysis. Other mutant UPPs enzymes in this study have shown no significant change ($<$5-fold) of k_{cat} with exception of E73A and D218A. Both enzymes have 10-fold lower k_{cat} value relative to wild-type UPPs.

Prenyltransferases, a group of enzymes involved in the isoprenoid biosynthesis, catalyze the condensation of isopentenyl pyrophosphate (IPP)[1] with allylic pyrophosphate to generate polyprenyl pyrophosphate (*1*). The enzymes were classified as Z- and E-type that catalyze cis and trans double bond formation from the IPP condensation, respectively (*2*). The E-type enzymes such as farnesyl pyrophosphate synthase (FPPs) which utilizes IPP and C_{10} geranyl pyrophosphate (GPP) to produce C_{15} farnesyl pyrophosphate (FPP) have been shown to share amino acid sequence homology and possess two aspartate-rich DDXXD motifs (*3*−*5*). Several site-directed mutagenesis studies of FPPs have suggested that the aspartates in these regions were critical for enzyme catalysis and some were important for substrate binding (*6*−*15*). The respective substitution of charged side chain of the three Asp residues with Ala in the first conserved motif was reported to have a decrease of k_{cat} by 4−5 orders of magnitude for yeast FPPs (*9*). Each of the mutations of the first and second Asp to Ala in the second DDXXD motif of yeast FPPs has also resulted in 4−5 orders of magnitude lower k_{cat}. Mutation of the first and second Asp to Ala and Ile, respectively, in the second conserved motif also caused the significant reduction of k_{cat} by 4−5 orders of magnitude for FPPs from *Bacillus stearothermophilus* (*10*). However, the third Asp mutation to Ala only yielded 6−16-fold of lower activity. The avian liver FPPs crystal structure has shown that these two aspartate-rich characteristic motifs are located in the deep cleft that forms the substrate binding pocket (*16*). The carboxyl groups of Asp in these motifs all point into the cleft and might bind the pyrophosphate moieties of the substrates through Mg^{2+} bridges (*7*, *8*, *16*).

Z-Prenyl chain-elongation prenyltransferases represent unique members of the family (*1*, *2*). Unlike the E-type enzymes which mostly synthesize short- and medium-chain polyprenyl pyrophosphates, the Z-type enzymes catalyze long-chain polyprenyl pyrophosphate synthesis with cis double bonds formed. The bacterial undecaprenyl pyrophosphate synthase (UPPs) is a Z-type prenyltransferase that catalyzes the condensation of eight molecules of IPP with *trans*,*trans*-FPP to produce C_{55} undecaprenyl pyrophosphate (UPP) as shown in Figure 1 (*17*). The UPP hydrolyzes to undecaprenyl phosphate that serves as carbohydrate carrier in bacterial cell wall glycoconjugate biosynthesis (*18*). While the 3-D structure and site-directed mutagenesis studies have been available for E-type farnesyl pyrophosphate synthase, the information regarding structure of *Z*-prenyl chain-elongation enzyme is lacking. Recently, the gene encoding undecaprenyl pyrophosphate synthase was cloned from bacterium *Micrococcus luteus* B-P 26 (*19*). Later, the UPPs genes have been identified from 25 bacterial strains including Gram-positive and Gram-negative bacteria (*20*). These

† This work was supported in part by a grant from Academia Sinica.

* To whom correspondence should be addressed. Phone: 886-2-2785-5696, ext. 6070. Fax: 886-2-2788-9759. E-mail: phliang@gate.sinica.edu.tw.

[1] Abbreviations: IPP, isopentenyl pyrophosphate; FPPs, farnesyl pyrophosphate synthase; FPP, farnesyl pyrophosphate; GPP, geranyl pyrophosphate; UPPs, undecaprenyl pyrophosphate synthase; UPP, undecaprenyl pyrophosphate; PCR, polymerase chain reaction; IPTG, isopropyl-β-D-thiogalactopyrano-side; NiNTA, nickel nitrilo-triacetic acid; Tris, tris(hydroxymethyl)aminomethane; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EDTA, ethylenediaminetetraacetic acid; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; K_m, Michaelis constant; k_{cat}, turnover number.

10.1021/bi001226h CCC: $19.00
Published on Web 10/21/2000

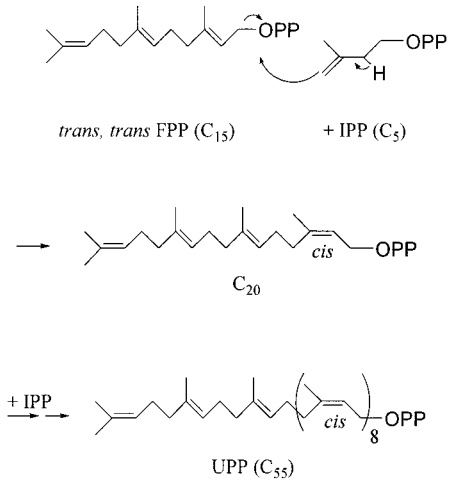

FIGURE 1: IPP condensation reactions catalyzed by undecaprenyl pyrophosphate synthase. The eight molecules of IPP were added to *trans*,*trans*-FPP to generate C_{55} undecaprenyl pyrophosphate as final product. The formed double bonds are in cis configuration.

Region I

20-**H**VAII**MD**$_{26}$**G**N**GR**W**A**

Region II

42-**GH**K**AG**

Region III

66-**T**LY**AFSSE**$_{73}$NWN**R**PAQ**EV**SAL**M**E**L**

Region IV

142-**AAN**Y**GGRW**D$_{150}$**I**VQG

Region V

190-**D**$_{190}$**L**VI**RTGGE**$_{198}$H**RI**S**NFLLWQ**IAYAE$_{213}$LY**FTD**$_{218}$**VLWPD**$_{223}$**FD**

FIGURE 2: Five regions in *E. coli* UPPs amino acid sequence homologous to other UPPs enzymes. The highly conserved amino acid residues in these regions are highlighted. The Asp and Glu mutated in this study are numbered. The sequence alignment was published by ref *20*.

provide the opportunity for site-directed mutagenesis approach to identify the essential amino acid residues for substrate binding and catalysis.

The Z-type UPPs has no sequence homology to the E-type prenyltransferases. Among the UPPs enzymes, five conserved regions in amino acid sequence as shown in Figure 2 were identified (*20*). Since aspartates in the two characteristic DDXXD motifs have been shown to be critical for FPPs activity, we mutated the Asp and Glu to Ala in the five conserved regions and performed the kinetic parameter measurements on the purified UPPs mutant enzymes. Aim of the study is to identify the conserved Asp or Glu, which might play a role in substrate binding and catalysis. Through the identification of the critical Asp or Glu, the active site pocket can possibly be distinguished among the conserved regions of the UPPs. To our best knowledge, this study represents the first mutagenesis examination on the Z-type polyprenyl pyrophosphate synthase.

EXPERIMENTAL PROCEDURES

Chemicals. Radiolabeled [^{14}C]IPP (55 mCi/mmol) was obtained from Amersham Pharmacia Biotech. and unlabeled FPP substrate was product of Sigma Co. Reversed-phase TLC was purchased from Merck chemical Co. NiNTA, plasmid mini-prep kit and DNA elution kit were obtained from QIAGEN. Taq DNA polymerase was purchased from Gibco Life Technologies. Potato acidic phosphatase (2 units/mg) was product of Boehringer Mannheimer. FXa cleavage kit containing FXa and Xarrest Agarose was obtained from Novagen. Vector pET32Xa/Lic was also the product of Novagen. All other reagents and buffer were of the highest commercial purity. The Millipore ultrapure water was exclusively used in our studies.

Site-Directed Mutagenesis of UPPs. Our previously constructed plasmid which contains the pET32Xa/Lic vector with insertion of *Escherichia coli Bos-12* UPPs encoding gene was employed as template for site-directed mutagenesis using polymerase chain reaction (PCR) technique (*21*). The 30 cycles of PCR reactions were performed using a thermocycler (Biometra, Germany) with 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 mutagenic oligonucleotides for performing site-directed mutagenesis were 5′-ATCATTATGGCCGGCAAT-3′ for D26A (mismatched base is underlined); 5′-TTTAGTAGTGCAAACTGG-3′ for E73A; 5′-GGACGTTGGGCTATAGTC-3′ for D150A; 5′-GCCCCTGTAGCTTTAGTA-3′ for D190A; 5′-GGCG-CATCGCATTAGTAA-3′ for E198A; 5′-GCCTATGCCG-CACTTTAC-3′ for E213A; 5′-CTTTACAGCTGTTCTCTG-3′ for D218A; and 5′-CGCTTTCGATGAACAAGA-3′ for D223A. The forward and reverse primers used with the above mutagenic oilgonucleotides to create full-length mutant UPPs gene were 5′-GGTATTGAGGGTCGCATGTTGTCTGCT-3′ and 5′-AGAGGAGAGTTAGAGCCATCAGGCTGT-3′, respectively. These primers contain the nucleotides encoding FXa cleavage site (IEGR) and the complementary sequences of the sticky ends of the linear vector pET-32Xa/LIC for ligation. The PCR products were purified by 0.8% agarose gel containing trace of ethidium bromide for DNA detection under UV light. The DNA was sliced from gel and purified using DNA elution kit. After the amplified and purified UPPs mutant gene was ligated into pET-32Xa/LIC, the resulting UPPs expression vector was transformed into the *E. coli* BL21 (DE-3) competent cell (Novagen). The introduction of mutation was confirmed by sequencing the entire UPPs mutant gene using dideoxy chain-termination method (*22*).

Overproduction and Purification of Mutant UPPs. The procedure reported previously for the expression and purification of wild-type UPPs was followed for each mutant enzyme preparation (*21*). In brief, the hexa-His tagged UPPs mutant enzyme was expressed under the control of T7 promoter and LacZ in BL21 *E. coli* host. The overexpression of the UPPs was induced by 1 mM of isopropyl-β-D-thiogalactopyranoside (IPTG) at 37 °C for 4 h after the OD_{600} of cell density has reached 0.6. The cell lysate was prepared by passing the cell suspension through French-Press apparatus (AIM-AMINCO spectronic Instruments) twice at 12 000 psi and the mixture was centrifuged to remove the cell debris. NiNTA column was employed for enzyme purification and FXa protease was used to remove the tag.

Since the secondary cleavage of UPPs accompanied with the tag removal during the incubation with FXa, the mixture after partial protease digestion was loaded onto NiNTA column to obtain untagged protein with great homogeneity as judged by SDS−PAGE electrophoresis. The reducing SDS−PAGE was performed according to the method of Laemmli (*23*). The gel was stained with Coomassie Brilliant Blue and destained using aqueous solution containing 5% methanol and 7% acetic acid. The protein concentration was determined using extinction coefficient at 280 nm calculated from amino acid sequence for each mutant.

Steady-State K_m and k_{cat} Measurements for UPPs Mutants. The enzyme reaction was initiated by adding mutant UPPs to a mixture containing various concentrations of FPP and [^{14}C]IPP with 0.01 μM enzyme except for D26A, D150A, and E213A mutant enzymes. In the measurements of IPP K_m and k_{cat} values, 10 μM of FPP was utilized to saturate with 0.01 μM enzyme and IPP concentrations of $1/2K_m$ to $5K_m$ were employed. In use of low concentration of FPP as substrate for FPP K_m measurements, the 10% FPP consumption gave low count of ^{14}C-labeled products in the butanol phase, and this count cannot be distinguished from the background ([^{14}C]IPP extracted by butanol). This led to the inaccuracy in the measurements of FPP K_m. To solve the problem, we utilized lower concentration of IPP (5 μM in most cases) to reduce the background. The FPP K_m was measurable under such a condition. All reactions were carried out in 100 mM Hepes buffer (pH 7.5), 50 M KCl, and 0.5 mM $MgCl_2$, at 25 °C in the presence of 0.1% Triton X-100. The reaction mixture was periodically withdrawn within 10% substrate depletion and mixed with 10 mM EDTA for reaction termination. The products were then extracted with 1-butanol and quantitated by a Beckmann LS6500 Scintillation Counter. The initial rate was calculated from the 10% consumption of radiolabeled [^{14}C]IPP with time. Data of initial rates versus substrate concentrations were analyzed by nonlinear regression of the Michaelis−Menten equation using the KaleidaGraph computer program (Synergy software) to obtain K_m and k_{cat} values.

Due to their extremely low activities, 1 μM D26A and 1 μM of E213A were used in the steady-state kinetic measurements. For determination of FPP and IPP K_m values of D26A, 1−10 μM FPP with 50 μM of [^{14}C]IPP and 5−60 μM [^{14}C]-IPP with 10 μM FPP were employed, respectively. Its k_{cat} value was determined from the kinetic data using 10 μM FPP and 5−60 μM [^{14}C]IPP as substrates. In the FPP K_m determination of E213A, 1−10 μM of FPP and 50 μM of [^{14}C]IPP were employed. The 80−500 μM [^{14}C]IPP and 20 μM FPP were used for IPP K_m and k_{cat} measurements. For D150A, the 0.02 μM enzyme with FPP (1−10 μM) and 50 μM [^{14}C]IPP was used to determine FPP K_m. The [^{14}C]IPP (80−500 μM) and 20 μM FPP were used for IPP K_m and k_{cat} measurements.

Enzyme Products Analysis. The UPPs reaction containing 0.2 μM enzyme (wild-type and mutant UPPs), 50 μM [^{14}C]-IPP, 7 μM FPP, 0.1% Triton X-100, 0.5 mM $MgCl_2$, and 50 mM KCl in 100 mM Hepes buffer (pH 7.5) was performed for 5 h at 25 °C. The 10 mM EDTA was used to terminate the reactions. The radiolabeled polyprenyl pyrophosphate products were extracted with 1-butanol. The 1-butanol was then evaporated and the 20% propanol solution containing 4.4 units/mL acidic phosphatase, 0.1% Triton, 50 mM sodium acetate (pH 4.7) was prepared to convert polyprenyl pyrophosphate products to corresponding alcohols according to the reported procedure (*24*). After the pyrophosphate hydrolysis catalyzed by acidic phosphatase was completed, the polyprenols were extracted with *n*-hexane. The hexane volume was reduced by evaporation. The polyprenols were separated on reversed-phase TLC using acetone/water (19:1) as mobile phase (*19*). The radiolabeled products were identified by autoradiography using a bioimaging analyzer FUJIFILM BAS-1500 (Japan) according to their R_f values reported (*19*).

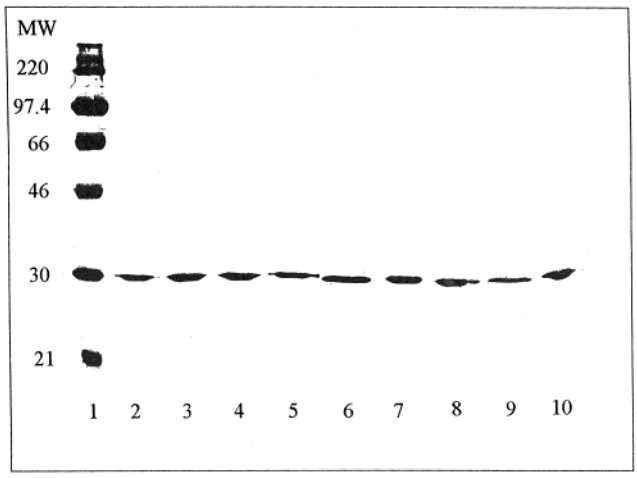

FIGURE 3: Purified UPPs mutants as shown by 12% SDS−PAGE under reducing conditions. The lanes from left to right contain the molecular weight standards (lane 1), wild-type UPPs (lane 2), D26A (lane 3), E73A (lane 4), D150A (lane 5), D190A (lane 6), E198A (lane 7), E213A (lane 8), D218A (lane 9), and D223A (lane 10). Each lane contains 2 μg of protein.

RESULTS

Overexpression and Purification of E. coli Mutant UPPs. Each UPPs mutant gene was created and amplified using PCR method and inserted into commercial vector pET-32Xa/LIC for expression of mutant enzyme with hexa-His tag and FXa protease cleavage site on the N-terminal. The engineered plasmid in *E. coli* BL21 host cell expressed high quantity of recombinant His-tagged UPPs with yield approximately 100 mg/L culture. The NiNTA column was employed for the His-tagged protein purification. The secondary cleavage of UPPs occurred during the tag removal by FXa with a slower rate. Therefore, before the tag cleavage was completed and the secondary cleavage was started by FXa protease, the mixture was loaded onto NiNTA. The flow through contained highly pure untagged UPPs mutant as shown by reducing SDS−PAGE (Figure 3). The final recovery yield for purified protein was approximately 50 mg/L culture. Approximately 2 μg of protein was included in each lane of Figure 3. The protein molecular mass for each mutant judged from the molecular weight standards on SDS−PAGE was approximately 29 kDa in agreement with prediction from amino acid sequence.

Steady-State K_m and k_{cat}. Among the UPPs enzymes identified from several species, there are five regions with highly conserved amino acid sequences (see Figure 2). In these conserved regions, we have individually mutated eight Asp and Glu residues to examine their possible roles in

Table 1: Kinetic Parameters of Wild-Type and Mutant *E. coli* UPPs

UPPs	k_{cat} (s^{-1})	K_m (FPP) (μM)	K_m (IPP) (μM)	rel k_{cat}[a]
wild-type	2.5 ± 0.1	0.4 ± 0.1	4.1 ± 0.3	1
D26A	$(3.30 \pm 0.03) \times 10^{-3}$	0.5 ± 0.1	14.1 ± 1.4	1×10^{-3}
E73A	0.30 ± 0.01	0.4 ± 0.1	16.2 ± 2.2	0.1
D150A	1.1 ± 0.1	0.4 ± 0.1	207 ± 30	0.5
E213A	$(2.60 \pm 0.02) \times 10^{-2}$	0.7 ± 0.1	280 ± 20	1×10^{-2}
D218A	0.22 ± 0.03	0.4 ± 0.1	4.4 ± 0.4	0.1

[a] k_{cat} relative to that of wild-type.

substrate binding and catalysis. These mutations were chosen based on the fact that several conserved Asp were found to play significant roles in FPPs enzyme catalysis and binding with the substrates and UPPs likely utilizes similar reaction mechanistic machinery. The kinetic parameters of these Asp → Ala and Glu → Ala UPPs mutants with significantly altered kinetic constants compared to wild-type are summarized in Table 1. The IPP and FPP K_m values of the wild-type *E. coli* UPPs were previously reported to be 4.1 ± 0.3 and 0.4 ± 0.1 μM, respectively (*21*). The k_{cat} was 2.5 ± 0.1 s^{-1} in the presence of 0.1% Triton (*21*). In contrast, D26A with a mutation in the first region showed 10^3-fold reduced activity. The k_{cat} was $(3.30 \pm 0.02) \times 10^{-3}$ s^{-1} for D26A. However, its FPP K_m (0.5 ± 0.1 μM) was not altered and the IPP K_m value was slightly changed (3-fold) to 14.1 ± 1.4 μM from 4 μM for wild-type enzyme. This D26A mutant was severely impaired in activity, suggesting that D26 plays a significant role in catalysis. When assayed under the saturated concentration of substrates, the k_{cat} was 3 orders of magnitude lower than that of wild-type. This result indicates that the activation energy required for the condensation of IPP with FPP is largely increased for D26A mutant.

Substitution of E73 with Ala in the third conserved region resulted in 10-fold reduced k_{cat} value. As shown in Table 1, the k_{cat} value of E73A was 0.30 ± 0.01 s^{-1} and its FPP and IPP K_m values were 0.4 ± 0.1 and 16.2 ± 2.2 μM, respectively. For E73A, the affinity of enzyme to IPP was slightly (4-fold) reduced relative to the wild-type enzyme.

The D150A mutation in the region IV has resulted in significantly increased IPP K_m value (207 ± 30 μM) which was 50-fold larger than the K_m value for wild-type enzyme. This indicates that the substitution of carboxyl side chain of D150 with neutral group has caused dramatic decrease in IPP affinity. However, the D150A k_{cat} value (1.1 ± 0.1 s^{-1}) was only 2-fold lower than the wild-type UPPs. The D150 in this conserved region apparently participates in the binding with IPP but is not important in enzyme activity.

Several Asp and Glu are located in the conserved region V. The D190A, E198A, E213A, D218A, and D223A were created and tested for their possible catalytic functions. These mutant enzymes have no significant change of k_{cat} values compared to the wild-type except the E213A and D218A. As shown in Table 1, the k_{cat} value for D218A was 0.22 ± 0.03 s^{-1}, 10-fold lower than the wild-type. Its FPP and IPP K_m values were 0.4 ± 0.1 and 4.4 ± 0.4 μM, respectively, which are similar to wild-type. In contrast, the E213A has k_{cat} value of $(2.60 \pm 0.02) \times 10^{-2}$ s^{-1} and FPP and IPP k_m values of 0.7 ± 0.1 and 280 ± 20 μM, respectively. The k_{cat} of E213A was approximately 100-fold reduced compared to the wild-type enzyme. Its FPP K_m was not altered from the wild-type. However, the IPP K_m value of E213A was much higher than the wild-type enzyme. Unlike D150 which is only critical for IPP binding, E213 appears to be important in IPP binding as well as enzyme catalysis.

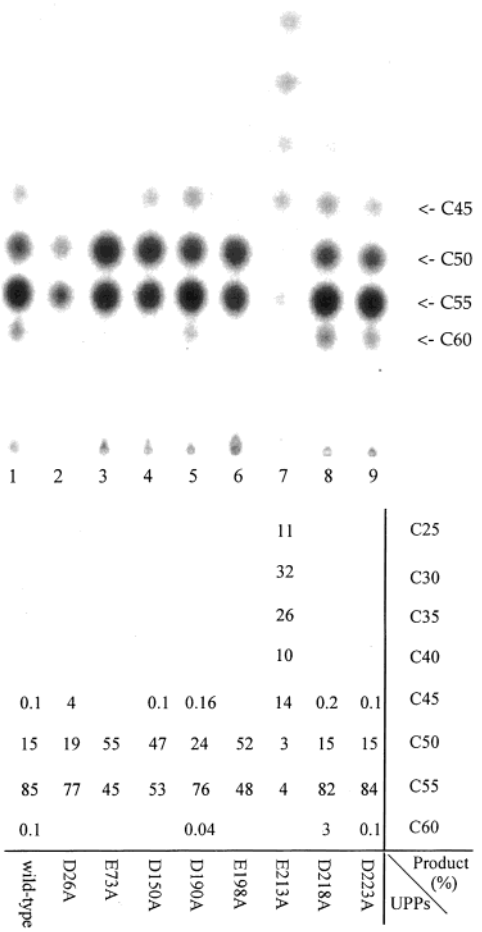

FIGURE 4: Products catalyzed by wild-type and mutant UPPs enzymes. The lanes show the products formed after 5 h reaction in 100 mM Hepes buffer (pH 7.5) containing 0.2 μM enzyme, 50 μM [^{14}C]IPP, 7 μM FPP, 0.5 mM $MgCl_2$, 50 mM KCl, and 0.1% Triton at 25 °C. The lanes from left to right represent the products formed under the catalysis of wild-type UPPs (lane 1), D26A (lane 2), E73A (lane 3), D150A (lane 4), D190A (lane 5), E198A (lane 6), E213A (lane 7), D218A (lane 8), and D223A (lane 9). The quantification of product distribution for wild-type and each mutant is shown in the bottom.

Product Formation Catalyzed by UPPs Mutants. The products catalyzed by the mutant enzymes were examined under the same reaction condition where 0.2 μM of each enzyme was incubated with 7 μM FPP and 50 μM [^{14}C]IPP in the presence of 0.5 mM $MgCl_2$, 50 mM KCl, and 0.1% Triton at pH 7.5 and 25 °C. These results are shown in Figure 4. Under such a condition, the C_{55} UPP was the major product with the minor product of C_{50} for wild-type UPPs. The most of the mutants including D26A, E73A, D150A, D190A, E198A, D218A, and D223A have produced C_{50} and C_{55} products with different ratios as summarized in the bottom of the figure. The percentages of the different products were normalized by the numbers of [^{14}C]IPP incorporated into the products. As shown in lane 2 of Figure 4, D26A has apparently lower rate in catalysis under the reaction condition and produced less quantity of products in the 5 h reaction. This is consistent with its low k_{cat} value. In contrast, the intermediate products instead of UPP were

generated by the E213A as displayed in lane 7. The extended reaction time (9 and 18 h) has resulted in C_{50} as major product and the data demonstrated the extremely slow conversion of C_{45} to C_{50} and C_{50} to C_{55} catalyzed by E213A (Figure not shown).

DISCUSSIONS

As shown in Figure 2, the UPPs enzymes from several species have displayed five conserved regions with great amino acid sequence homology. Since UPPs requires divalent ion Mg^{2+} for enzyme activity, the carboxyl groups of Asp and Glu might be involved in the binding of pyrophosphate moieties of IPP and FPP bridged by Mg^{2+}. The side chains of these residues might also participate in the enzyme catalysis such as base-assisted catalysis for abstraction of a hydrogen ion from IPP or electrostatic catalysis for making FPP a good electrophile. The several conserved Asp residues have been previously shown to be critical for the enzyme function of FPPs. By evaluating the impact on the substrate binding and enzyme activity of eight UPPs mutant enzymes with replacement of conserved Asp or Glu by Ala, we have found that some of these residues play major roles in catalytic function. The substitution of negatively charged carboxyl group with neutral side chain for D26A resulted in 10^3-fold lower k_{cat} value without significant interference on FPP and IPP binding. This result indicates that D26 plays a significant role in catalysis. In the near downstream of this Asp, there are Asn and Arg with intervention of Gly (D_{26}GNGR in conserved region I, as shown in Figure 2). In the FPPs, two Arg residues follow the first DDXXD motif and these Arg residues appear to be equally important as the Asp residues in the enzyme activity for rat FPPs (*7*) and yeast FPPs (*9*). In the UPPs, the single Arg in the first conserved region might be also an important residue. Our recent mutagenesis study has confirmed that R30A has resulted in more than 10-fold decrease in activity (Pan et al., unpublished results).

The mutation of E73 in the third conserved region has less impact on enzyme activity. The k_{cat} (0.3 s^{-1}) of E73A was almost 10-fold lower than that of the wild-type UPPs. Its FPP affinity was not changed compared to the wild-type enzyme. However, the IPP K_m was slightly increased. This might suggest that the amino acid residues in region II play less important role in enzyme catalysis compared to the D26, D150, and E213.

The D150 located in the fourth conserved region appears to play a significant role in IPP binding. The IPP K_m value (207 μM) of D150A was 50-fold larger than the wild-type K_m value. However, the k_{cat} was not significantly altered by the mutation. This indicates that D150 carboxyl side is responsible for the IPP binding. However, the change of the Asp to Ala only impacted on the IPP binding affinity but not the IPP condensation rate (k_{cat}) with FPP. This result might not be surprising because IPP acts as a nucleophile in the coupling reaction with FPP, and the electron-withdrawing ability of the enzyme-bound Mg^{2+} makes the reaction less favorable if indeed D150 binds IPP via Mg^{2+}. Therefore, the decrease of the IPP affinity for UPPs should not necessarily reduce the catalytic activity. In this case, 2-fold lower k_{cat} was observed for D150A.

Conserved region V has the largest number of amino acid residues among the five regions. We have examined D190A, E198A, E213A, D218A, and D223A in this region, and among them, E213A and D218A have significantly altered kinetic parameters. It was previously suggested that D190 might be an important residue based on the sequence comparison (*20*). However, we observed no significant change of kinetic constants for D190A compared to the wild-type. In contrast, the less conserved E213 (some UPPs enzymes have non-D or E amino acid at this position) has shown more important role in catalysis. The E213A k_{cat} was 100-fold lower than that of wild-type UPPs. Although the FPP K_m value was not changed, its IPP K_m was dramatically increased, indicating that the mutation has caused the severe decrease of IPP affinity. Unlike the D26A mutant which has 10^3 lower k_{cat} while still producing C_{55} as final product, E213A generated short intermediate products (Figure 4). The lack of UPP formation catalyzed by E213A in 5 h suggested that IPP condensations in late stage were extremely slow due to poor IPP binding of mutant enzyme and/or the participation of E213 in the binding of large intermediates. Therefore, the relatively slow late IPP condensation steps led to the accumulation of short polymers in 5 h reaction. In contrast, for D26A reaction under the same condition, the formation of C_{55} might be faster than the initial IPP coupling so C_{55} was observed as final product although it also has a low k_{cat}. The product monitored in the shorter time intervals for this mutant was still C_{55} but with less quantity (data not shown). Therefore, the initial IPP condensation for D26A might have a slow rate close to its k_{cat} value.

We have previously demonstrated that the rate-limiting step of the UPPs reaction in the presence of Triton is the chemical step and each of the IPP condensation steps has approximately the same rate (*21*). Under single-turnover condition where the product release was not involved, the rate of IPP condensation was equal to the k_{cat} measured under steady-state condition. Therefore, the reduction of the catalytic efficiency for the each of the mutant D26A, D150A, E213A, E73A, and D218 shown in this study was likely due to the decrease of the chemical step rate. This was supported by our single-turnover experiments in which the mutant enzymes (10 μM) and [^{14}C]IPP (50 μM) were in excess of FPP (1 μM). Under this condition, the reaction rate observed for each mutant was consistent with their k_{cat} (data not shown).

Our kinetic evaluations of the Asp → Ala and Glu → Ala mutant UPPs enzymes have suggested that certain Asp and Glu amino acid residues for *E. coli* UPPs are important for the enzyme catalysis and substrate binding. Five mutant enzymes including D26A, E73A, D150A, E213A, and D218 out of eight mutant UPPs created for this study were identified to be important for enzyme function. Among them, D26A and E213A have much lower enzyme activities than the wild-type and D150A and E213A have extremely lower IPP affinity. Similar to FPPs which has been intensively studied by crystal structure and site-directed mutagenesis, Asp residues (D26 and D150) of *E. coli* UPPs are important. However, Glu residue, which was not found as an important residue in FPPs reaction, is critical for UPPs catalysis. In addition to E213, most of the other UPPs enzymes have Glu residue at the position of D150 for *E. coli* UPPs (20). This unique property of Glu requirement reflects the difference of UPPs active site geometry from those of the E-type prenyltransferases. More details of the UPPs active site

structure require further mutagenesis and crystallographic studies.

ACKNOWLEDGMENT

We thank Dr. Teh-Yung Liu for helpful support.

REFERENCES

1. Ogura, K., Koyama, T., and Sagami, H. (1997) *Subcellular Biochem. 28*, Chapter 3, 1−87.
2. Ogura, K., and Koyama, T. (1998) *Chem. Rev. 98*, 1263−1276.
3. Ashby, M. N., and Edward, P. A. (1990) *J. Biol. Chem. 265*, 13157−13164.
4. Koyama, T., Obata, S., Osabe, M., Takeshita, A., Yokoyama, K., Uchita, M., Nishino, T., and Ogura, K. (1993) *J. Biochem. (Tokyo) 113*, 355−363.
5. Chen, A., Kroon, P. A., and Poulter, C. D. (1994) *Protein Sci. 3*, 600−607.
6. Marrero, P. F., Poulter, C. D., and Edwards, P. A. (1992) *J. Biol. Chem. 267*, 21873−21878.
7. Joly, A., and Edwards, P. A. (1993) *J. Biol. Chem. 268*, 26983−26989.
8. Song, L., and Poulter, C. D. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 3044−3048.
9. Koyama, T., Tajima, M., Nishino, T., and Ogura, K. (1995) *Biochem. Biophys. Res. Commun. 212*, 681−686.
10. Koyama, T., Tajima, M., Sano, H., Doi, T., Koike-Takeshita, A., Obata, S., Nishino, T., and Ogura, K. (1996) *Biochemistry 35*, 9533−9538.
11. Koyama, T., Saito, K., Ogura, K., Obata, S., and Takeshita, A. (1994) *Can. J. Chem. 72*, 75−79.
12. Koyama, T., Obata, S., Saito, K., Takeshita-Koike, A., and Ogura, K. (1994) *Biochemistry 33*, 12644−12648.
13. Ohnuma, S., Narita, K., Nakazawa, T., Hemmi, H., Hallberg, A.-M., Koyama, T., Ogura, K., and Nishito, T. (1996) *J. Biol. Chem. 271*, 10087−10095.
14. Ohnuma, S., Narita, K., Nakazawa, T., Ishida, C., Takeuchi, Y., Ohto, C., and Nishito, T. (1996) *J. Biol. Chem. 271*, 30748−30754.
15. Tashis, L. C., Proteau, P. J., Kellogg, B. A., Sacchettini, J. C., and Poulter, C. D. (1996) *Proc. Natl. Acad. Sci. U.S.A. 93*, 15018−15023.
16. Tarshis, L. C., Yan, M., Poulter, C. D., and Sacchettini, J. C. (1994) *Biochemistry 33*, 10871−10877.
17. Allen, C. M. (1985) *Methods Enzymol. 110*, 281−299.
18. Robyt, J. *Essentials of carbohydrate chemistry*, Chapter 10, pp 305−318, Springer-Verlag, New York.
19. Shimizu, N., Koyama, T., and Gura, K. (1998) *J. Biol. Chem. 273*, 19476−19481.
20. Apfel, C. M., Takacs, B., Fountoulakis, M., Stieger, M., and Keck, W. (1999) *J. Bacteriol. 181*, 483−492.
21. Pan, J. J., Chiou, S. T., and Liang, P. H. (2000) *Biochemistry 39*, 10936−10942.
22. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463−5467.
23. Laemmli, U. K. (1970) *Nature 227*, 680−685.
24. Fujii, H., Koyama, T., and Ogura, K. (1982) *Biochim. Biophys. Acta 712*, 716−718.

BI001226H