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Significance of Highly Conserved Aromatic Residues in *Micrococcus luteus* B-P 26 Undecaprenyl Diphosphate Synthase

Yugesh Khare[1], Yuan-Wei Zhang[*], Masahiro Fujihashi[2], Kunio Miki[2,3] and Tanetoshi Koyama[†,1]

[1]Institute of Multidisciplinary Research for Advanced Materials, Tohoku University, 2-1-1 Katahira, Aoba-ku, Sendai 980-8577; [2]Department of Chemistry, Graduate School of Science, Kyoto University, Sakyu-ku, Kyoto 606-8502; and [3]RIKEN Harima Institute/SPring-8, 1-1-1 Koto, Mikazukicho, Sayo-gun, Hyogo 679-5148

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Undecaprenyl diphosphate synthase catalyzes the sequential condensation of eight molecules of isopentenyl diphosphate (IPP) in the *cis*-configuration into farnesyl diphosphate (FPP) to produce undecaprenyl diphosphate (UPP), which is indispensable for the biosynthesis of the bacterial cell wall. This *cis*-type prenyltransferase exhibits a quite different mode of binding of homoallylic substrate IPP from that of *trans*-type prenyltransferase [Kharel Y. *et al*. (2001) *J. Biol. Chem.* 276, 28459–28464]. In order to know the IPP binding mode in more detail, we selected six highly conserved residues in Regions III, IV, and V among nine conserved aromatic residues in *Micrococcus luteus* B-P 26 UPP synthase for substitution by site-directed mutagenesis. The mutant enzymes were expressed and purified to homogeneity, and then their effects on substrate binding and the catalytic function were examined. All of the mutant enzymes showed moderately similar far-UV CD spectra to that of the wild-type, indicating that none of the replacement of conserved aromatic residues affected the secondary structure of the enzyme. Kinetic analysis showed that the replacement of Tyr-71 with Ser in Region III, Tyr-148 with Phe in Region IV, and Trp-210 with Ala in Region V brought about 10–1,600-fold decreases in the k_{cat}/K_m values compared to that of the wild-type but the K_m values for both substrates IPP and FPP resulted in only moderate changes. Substitution of Phe-207 with Ser in Region V resulted in a 13-fold increase in the K_m value for IPP and a 1,000–2,000-fold lower k_{cat}/K_m value than those of the wild-type, although the K_m values for FPP showed about no significant changes. In addition, the W224A mutant as to Region V showed 6-fold and 14-fold increased K_m values for IPP and FPP, respectively, and 100–250-fold decreased k_{cat}/K_m values as compared to those of the wild-type. These results suggested that these conserved aromatic residues play important roles in the binding with both substrates, IPP and FPP, as well as the catalytic function of undecaprenyl diphosphate synthase.

Key words: circular dichroism, farneyl diphosphate, isoprenoid, prenyltransferase, site-directed mutagenesis, undecaprenyl diphosphate.

Abbreviations: IPP, isopentenyl diphosphate; UPP, undecaprenyl diphosphate; FPP, (all-*E*)-farnesyl diphosphate; DedolPP, dehydrodolichyl diphosphate; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; TLC, thin layer chromatography; CD, circular dichroism; Tris, tris(hydroxymethyl)-aminomethane.

Isoprenoids are some of the most structurally and functionally diverse compounds ubiquitously occurring in nature. They are widely distributed in organisms, serving as steroids, carotenoids, respiratory quinones, prenylated proteins, and glycosyl carrier lipids. More than 23,000 structurally diverse isoprenoid compounds have been identified and they all share a common biosynthetic mechanism in that all of their carbon skeletons are constructed from linear prenyl diphosphates through the action of prenyltransferases (prenyl diphosphate synthases). Prenyltransferases catalyze the consecutive (1′-4)-condensation of isopentenyl diphosphate (IPP) starting with the elimination of a diphosphate group from an allylic diphosphate. By repeating the stereospecific condensation between IPP and an allylic diphosphate, prenyltransferase can synthesize prenyl diphosphate with a certain chain length and stereochemistry fixed by the specificities of individual enzymes. According to the stereochemistry of the products, prenyltransferases are classified into two sub-groups, *trans*- and *cis*-types (*1*, *2*).

In bacteria, undecaprenyl diphosphate (UPP) synthase catalyzes the sequential *cis*-condensation of eight molecules of IPP into farnesyl diphosphate (FPP) as an allylic primer to produce UPP. UPP has been identified as the direct precursor of the glycosyl carrier lipid that transports intracellular carbohydrates outside of the cell membrane during the biosynthesis of bacterial cell wall polysaccharide components such as peptidoglycan and lipopolysaccharide. In eukaryotic cells, dehydrodolichyl diphosphate (DedolPP) synthase catalyzes the *cis*-prenyl

[*]Present address: Department of Pharmacology, School of Medicine, Yale University, New Haven, CT 06520, USA.
[†]To whom correspondence should be addressed. Tel: +81-22-217-5621, Fax: +81-22-217-5620, E-mail: koyama@tagen.tohoku.ac.jp

chain elongation to form DedolPP, which has a much longer *cis*-polyisoprenoid chain than the bacterial equivalent, UPP (*3*). DedolPP is an essential precursor of the carbohydrate carrier lipid in the biosynthesis of *N*-linked glycoproteins or glycosylphosphatidylinositol-anchored proteins (*4*).

During the past 16 years, *trans*-type prenyltransferases have been widely studied at the molecular level, and structural genes for many enzymes have been cloned and characterized. Multiple alignment of the deduced amino acid sequences of many *trans*-type prenyltransferases from a wide range of organisms revealed the presence of seven conserved regions including two characteristic aspartate-rich motifs (DDXXD) (*5*, *6*). These DDXXD motifs have been shown to be essential for the catalytic function as well as substrate binding by means of site-directed mutagenesis (*7–10*). X-ray crystallographic and intensive site-directed mutagenesis studies (*11–16*) have revealed the mechanisms of substrate binding, catalysis and product chain length regulation for short chain *trans*-type prenyltransferases.

On the other hand, no information had been available on the structure of a *cis*-type prenyltransferase until our recent isolation of the gene for UPP synthase from *Micrococcus luteus* B-P 26 (*17*). Shortly afterwards, homologous genes for the bacterial enzymes from *Escherichia coli*, *Haemeophilus influenzae*, and *Streptococcus pneumoniae* (*18*), and eukaryotic enzymes from *Saccharomyces cerevisiae* (*19*) and *Arabidopsis thaliana* (*20*) were identified and isolated. Multiple alignment of the deduced amino acid sequences of these *cis*-type prenyltransferases has revealed five conserved regions, which are completely different to those of *trans*-type prenyltransferases (*3*, *18*). The DDXXD motifs, which have been shown to be essential for *trans*-type prenyltransferases, are completely absent in *cis*-type prenyltransferases. Fujihashi *et al.* (*21*) determined the crystal structure of *M. luteus* B-P 26 UPP synthase, the first three-dimensional structure of a *cis*-type prenyl chain elongating enzyme. To our surprise, this enzyme shows a novel protein fold, which is completely different from the common "isoprenoid synthase fold" of the enzymes related to isoprenoid biosynthesis (*22*). Then, Ko *et al*. (*23*) reported the structure of *E. coli* UPP synthase, which is essentially the same as that of *M. luteus* B-P 26 UPP synthase (*21*). The crystal structures of these *cis*-type prenyltransferases have a large hydrophobic cleft around a structural P-loop, which is believed to be the binding site for the diphosphate moiety of an allylic substrate. Mutational analysis of UPP synthase has revealed the amino acid residues (D29, R33 and R80) in the structural P-loop and RX_5RX_nE motif in Region V along with the FS-motif in Region III of *M. luteus* B-P 26 UPP synthase that are essential for the binding of the diphosphate moieties of allylic and homoallylic substrates, respectively (*23–27*). These results indicated that *cis*-prenyl chain elongating enzymes exhibit a different mode of binding of both substrates, FPP and IPP, from that of *trans*-type enzymes. It would be of particular interest to further elucidate the differences and similarities at the molecular level of these *cis*-and *trans*-type enzymes, which have evolved from different ancestors.

In *M. luteus* B-P 26 UPP synthase, there are six highly conserved aromatic residues in conserved Regions III, IV, and V, as shown in Fig. 1 (*3*). These aromatic residues are located near the large cleft or the interface of the homodimeric structure of the protein, and are thought to be important for the UPP synthase reaction (*21*). To investigate the role of the aromatic residues during catalysis, these residues (Tyr-71, Tyr-148, Phe-207, Trp-210, Trp-224, and Phe-227) were selected for substitution by site-directed mutagenesis. This paper describes elucidation of the roles of aromatic residues in the UPP synthase in substrate binding and catalysis.

EXPERIMENTAL PROCEDURES

Materials and General Procedures—Nonlabeled IPP and FPP were synthesized according to the procedure of Davisson *et al*. (*28*). [1-^{14}C]IPP (1.95 TBq/mol) was purchased from Amersham Pharmacia Biotech. Restriction enzymes and other DNA modifying enzymes were from Takara Shuzo and Toyobo. Potato acid phosphatase was a product of Sigma. Precoated reversed phase thin layer chromatography (TLC) plates, LKC-18, were purchased from Whatman. *E. coli* B strain BL21(DE3) was used as the host for expression of the target gene. Restriction enzyme digestions, transformations, and other standard molecular biological techniques were carried out as described by Sambrook *et al*. (*29*). Bacteria were cultured in Luria-Bertani (LB) or M9YG medium (*5*). All other chemicals were of analytical grade.

Protein Sequence Alignment—Protein sequence database SWISS-PROT was searched for amino acid sequences similar to that of *M. luteus* B-P 26 UPP synthase. Multiple alignment of amino acid sequences was performed using GENETYX genetic information processing software (Software Development).

Expression Vector System and Site-Directed Mutagenesis—The expression plasmid for *M. luteus* B-P 26 UPP synthase (pMluUEX) was employed as described in the previous report (*26*). Site-directed mutagenesis was performed according to the protocol for the GeneEditor *in vitro* site-directed mutagenesis system (Promega). The single-stranded wild-type UPP synthase gene, used as a template in the mutagenesis reaction, was prepared by R408 helper phage infection of *E. coli* JM109 cells (Takara) that contain pMluUEX. The mutagenic oligonucleotides designed to produce the desired point mutations were 5′-AGTTGAAAACGCGGACAGCGTTAAGTAC-3′ (for Y71S, the mismatched pair is underlined); 5′-ACGCCCACCAGAATTCAGTGCAAACAC-3′ (for Y148S); 5′-ACGCCCACCAAAATTCAGTGCAAACAC-3′ (for Y148F); 5′-CCAAATTAAAGAGTTACTTAAGCGTTCTTC-3′ (for F207S); 5′-ATATGAACATTGCGCAATTAAAAAGTTAC-3′ (for W210A); 5′-AAAATCCGGCGCGAATTCATCTATAAATAC-3′ (for W224A); and 5′-TTCTTCATTAGAATCTGGCCAGAATTC-3′ (for F227S). Introduction of the mutations was confirmed by sequencing the whole nucleotide sequences using the dideoxy chain–termination method with a DNA sequencer (LI-COR, model 4200).

Overproduction and Purification of UPP Synthase Mutants—The procedures employed for the overproduction and purification of UPP synthase mutants, Y71S, Y148S, Y148F, F207S, W210A, W224A, and F227S, as

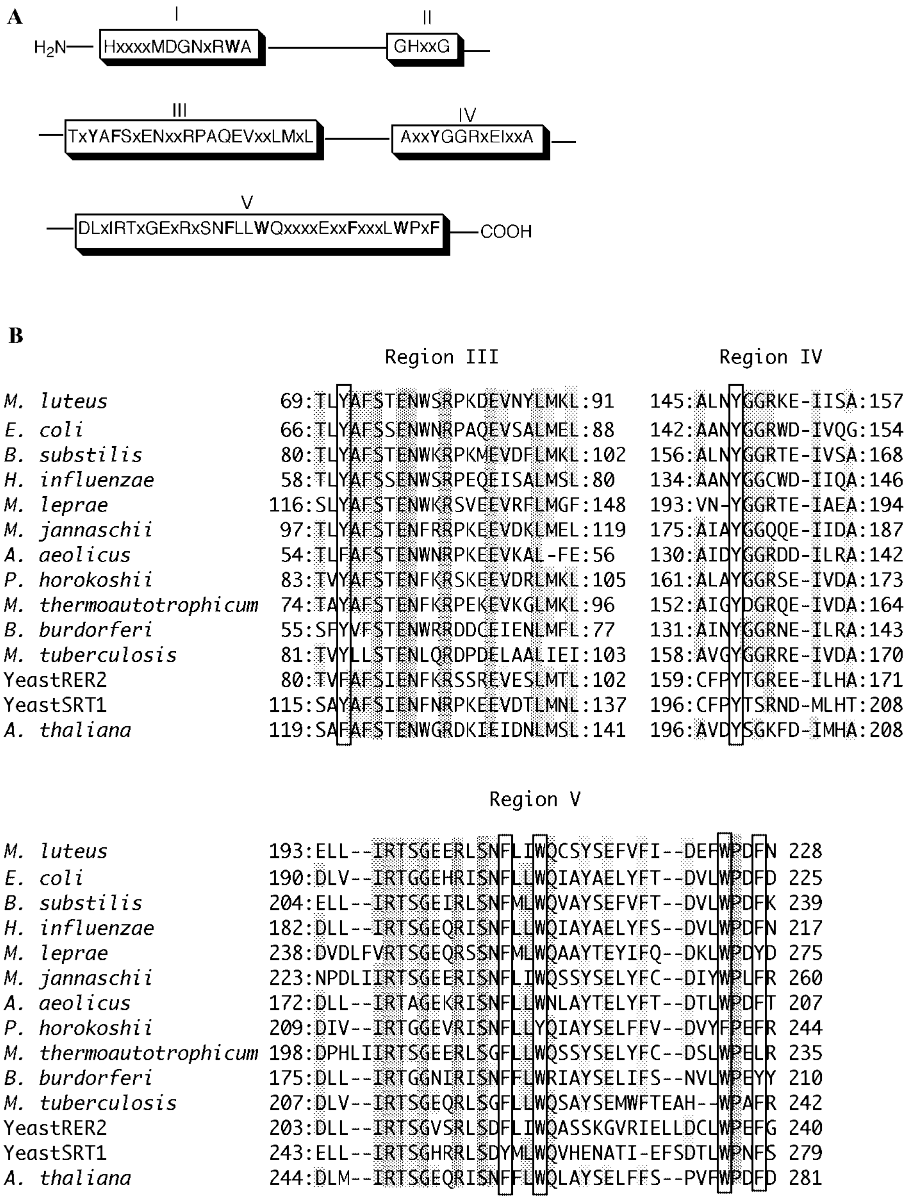

Fig. 1. **(A) Schematic drawing of the five highly conserved regions of *cis*-type prenyl chain-elongating enzymes.** Nine conserved residues are shown in bold. **(B) Multiple alignment of the amino acid sequences of *cis*-prenyl chain-elongating enzymes.** Only the residues corresponding to the conserved Regions III, IV, and V are shown. Black and gray outlines indicate identical and similar amino acids residues, respectively. The conserved aromatic amino acid residues of *M. luteus* B-P 26 UPP synthase mutated in this study are boxed. Sequences: *M. luteus* B-P 26 (GeneBank™ accession number AB004319), *E. coli* (Swiss-Prot Q47675), *B. substilis* (Swiss-Prot Q31751), *H. influenzae* (Swiss-Prot P44938), *Mycobacterium leprae* (Swiss-Prot P38119), *Methanococcus jannaschii* (Swiss-Prot Q58767), *Aquifex aeolicus* (Swiss-Prot O67291), *Pyrococcus horikoshii* (Swiss-Prot O59258), *Methanobacterium thermoauotrophicum* (Swiss-Prot O26334), *Borrelia burgdorferi* (Swiss-Prot O51146), *Mycobacterium tuberculosis* (Swiss-Prot O53434), yeast RER2 (Swiss-Prot P35196), yeast SRT1 (Swiss-Prot Q03175), and *Arabidopsis thaliana* (GeneBank™ accession number AF162441).

well as the wild-type enzyme, were essentially similar to those previously reported by us (*26*). Mutant enzymes were analyzed for purity by SDS-PAGE with Coomassie Brilliant Blue staining, and the fractions that showed more than 90% purity were used for further characterization. Protein concentrations were measured by the method of Bradford with bovine serum albumin as the standard (*30*).

UPP Synthase Assay and Product Analysis—Enzyme activity was measured by determining the amounts of [1-^{14}C]IPP incorporated into butanol-extractable polyprenyl diphosphates. The standard assay mixture contained, in a final volume of 0.2 ml, 100 mM Tris-HCl buffer, pH 7.5, 0.5 mM $MgCl_2$, 10 μM FPP, 10 μM [1-^{14}C]IPP (37 MBq/mol), 0.05% (w/v) Triton X-100, and a suitable amount of enzyme solution. After incubation at 30°C for 30 min, the reaction products were extracted with 1-butanol saturated with water, and then the radioactivity in the butanol extract was measured with an Aloka LSC-1000 liquid scintillation counter.

For kinetic studies, the concentration of allylic substrate FPP or homoallylic substrate [1-^{14}C]IPP was varied, while the other substrate, [1-^{14}C]IPP or FPP, was kept constant at 200 or 500 μM, respectively. Calculation of kinetic parameters was performed using EnzymesKinetics software version 1.5 (Trinity Software).

For product analysis, the radioactive prenyl diphosphate products in the reaction mixture were hydrolyzed to the corresponding alcohols with potato acid phosphatase according to the method we previously reported (*31*). The alcohols were extracted with pentane and then analyzed by reversed phase TLC with a solvent system of acetone/water (19:1). The positions of authentic standards were visualized with iodine vapor, and the distribution of radioactivity was examined. The TLC plates were exposed to Fuji imaging plates at room temperature for 1

Table 1. **Kinetic Constants of the wild-type and mutated UPP synthases.**

Enzyme	IPP			FPP		
	K_m (μM)	k_{cat} ($\times 10^3$) (s^{-1})	k_{cat}/K_m ($s^{-1}/\mu M$)	K_m (μM)	k_{cat} ($\times 10^3$) (s^{-1})	k_{cat}/K_m ($s^{-1}/\mu M$)
WT[a]	7.8± 2.8	1,550±90	198	8.3± 1.3	1,220±64	147
Y71S	25.4± 3.1	110±12	4.3	7.7± 2.3	111±10	14
Y148S	–	–	–	–	–	–
Y148F	22.9± 4.2	115±10	5	2.8± 0.6	15± 2	5
F207S	103.1± 9.4	10± 1	0.1	22.2± 3.1	3± 0.26	0.14
W210A	17.5± 1.1	2± 0.2	0.12	1.5± 0.01	2± 0.08	1.3
W224A	35.6±10.1	71±3	2	121.3±36.3	71± 9	0.6
F227S	ND	ND	ND	ND	ND	ND

[a]Data taken from our previous report (*26*). ND, not determined. –, no activity.

day, and then analyzed with a Fuji BAS 1000 Mac bioimage analyzer.

Circular Dichroism (CD) Spectroscopy—CD spectra of the wild-type and mutant enzymes were recorded at 22°C with a JASCO J-720 spectropolarimeter. The results are expressed as the mean residue ellipticity, $[\Theta]_M = (\Theta_{obs}/10)(M/lc)$, where Θ_{obs} is the observed ellipticity at a given wavelength, M the mean residue mass, l the cuvette path-length in centimeters, and c the protein concentration expressed as g/ml. The unit for $[\Theta]_M$ is deg·cm^2·dmol^{-1}. Far-ultraviolet CD spectra were recorded between 190 and 250 nm in a 0.01 cm path-length quartz cell with protein concentrations of 0.1–0.5 mg/ml. The signals were digitized at 10 nm intervals and averaged with a 4 s step time constant using AVIV 60DS V4 software. All CD spectra were corrected by subtracting the corresponding base line spectra obtained under identical conditions. All measurements were performed in 100 mM Tris-HCl buffer, pH 7.5, containing 0.5 mM EDTA, and 15 mM NaCl. The helical contents were estimated at a single wavelength (208 nm) according to the equation reported by Greenfield and Fasman (*32*).

RESULTS

Introduction of Site-Directed Mutagenesis of M. luteus B-P 26 UPP Synthase—Figure 1 shows the amino acid sequence alignment of Regions III, IV, and V of several *cis*-type prenyltransferases as well as a schematic drawing of the highly conserved Regions of *cis*-type prenyltransferases. Six aromatic amino acid residues [Tyr-71 in Region III (at S2), Tyr-148 in Region IV (at the loop joining S4 and H6), two Trp residues at positions 210 (at the loop joining S5 and S6) and 224 (at H9), and two Phe residues at 207 (at the loop joining S5 and S6) and 227 (at the loop joining H9 and H10) in Region V in *M. luteus* B-P 26 UPP synthase (*21*)] are highly conserved among these *cis*-type prenyltransferases. To determine whether or not these aromatic residues play any role in substrate binding and catalysis, they were replaced with other amino acids by site-directed mutagenesis to produce Y71A, Y148S, Y148F, F207S, W210A, W224A, and F227S. To construct expression systems for these mutated enzymes, oligonucleotide-mediated mutagenesis was performed using the expression plasmid pMluUEx as a template, which carries the wild-type UPP synthase gene (*26*). *E. coli* BL21(DE3) was transformed with each of the expression plasmids, and the UPP synthase mutant was overproduced in *E. coli* cells and purified by three chromatographic steps according to the procedure that we previously reported (*26*). However, one mutant, F227S, could not be purified because of its insolubility in the Tris-HCl buffer. All other mutant enzymes showed chromatographic properties similar to those of the wild-type UPP synthase during the purification procedures. All mutants gave a protein band of identical size on SDS-PAGE analyses with Coomassie brilliant blue staining, and the purities of all the mutant enzymes were more than 90% (data not shown).

Kinetic Analysis of the UPP Synthase Mutants—Steady-state kinetic data for the mutant enzymes as well as the wild-type were determined with [1-^{14}C]IPP and FPP as substrates, and the results are listed in Table 1. F207S, W210A, and W224A showed approximately 100–

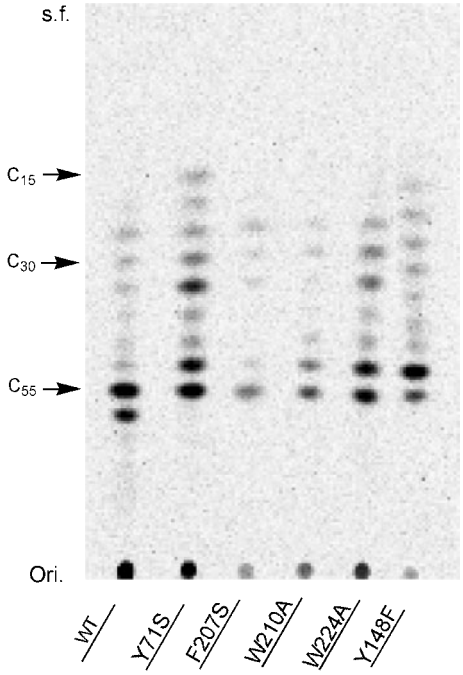

Fig. 2. **Reversed phase TLC radiochromatograms of the product alcohols derived on the reaction of the wild-type UPP synthase and its mutants with FPP and [1-^{14}C]IPP as substrates.** After enzymatic hydrolysis the product alcohols were analyzed as described under EXPERIMENTAL PROCEDURES. Arrows indicate the positions of authentic alcohols: C_{15}, (*E,E*)-farnesol; C_{20}, (all-*E*)-geranylgeraniol; C_{30}, (all-*E*)-hexaprenol; C_{55}, all-*E*-farnesyl-all-*Z*-octaprenol.

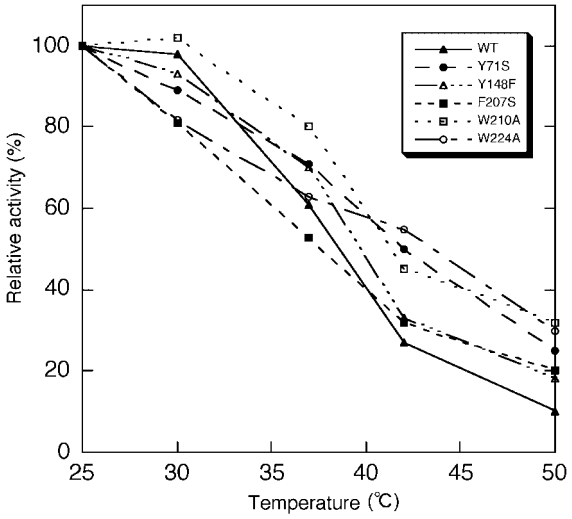

Fig. 3. **Thermostability of the mutant enzymes and wild-type.**

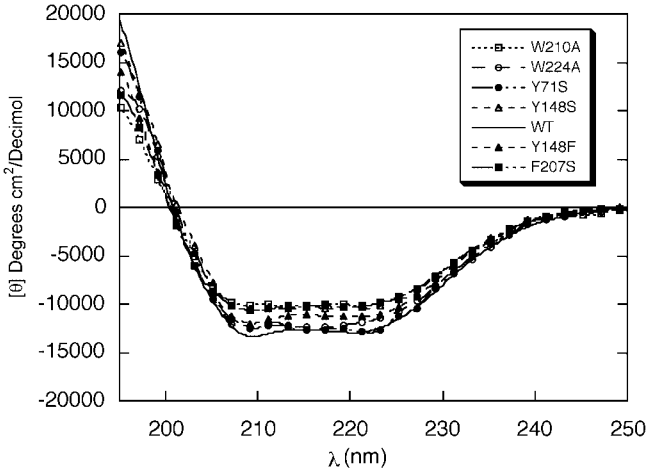

Fig. 4. **Far-UV CD spectra of *M. luteus* B-P 26 UPP synthase and its mutants.** CD spectra were measured under the conditions given under "EXPERIMENTAL PROCEDURES."

2,000-fold decreases in catalytic efficiency (k_{cat}/K_m) compared to the wild-type. Hence, it was necessary to substantially increase both the enzyme concentrations and incubation times to obtain reliable reaction rate values for these less reactive mutant enzymes. Three mutant enzymes, Y71S, F207S, and W210A, showed comparable Michaelis constants for allylic substrate FPP to that of the wild-type UPP synthase. Y71S and W210A showed 2–3-fold increased K_m values for homoallylic substrate IPP. Moreover, Y71S showed 10–45-fold decreased k_{cat}/K_m values while W210A showed 100–1,600-fold decreased k_{cat}/K_m values compared to that of the wild type. On the other hand, F207S showed a 13-fold increased K_m value for IPP and 1,000–2,000-fold lower k_{cat}/K_m values compare to those of the wild-type. However, W224A showed increased K_m values for both substrates, FPP and IPP (14- and 6-fold, respectively), and 100–250-fold decreased k_{cat}/K_m values compared to those of the wild-type. Y148S was found to be inactive, although it could be purified to homogeneity and showed chromatographic properties similar to those of the wild-type during purification. Interestingly, Y148F exhibited similar K_m values for IPP and FPP, but 30–40-fold lower k_{cat}/K_m values as compared to the wild-type.

Product Analysis—An appropriate amount of each mutant enzymes as well as the wild-type was used for the enzymatic reaction to analyze their reaction products. After enzymatic reaction at 37°C for 3 h using FPP and [1-^{14}C]IPP as substrates, the radioactive prenyl diphosphate products were hydrolyzed enzymatically to the corresponding alcohols. The results of TLC analyses of the alcohols are shown in Fig. 2. Most of the mutants showed product distribution patterns similar to that of the wild-type enzyme, which produced UPP (C_{55}) as the major product as well as minor amounts of some intermediates having shorter prenyl chains and a small amount of a C_{60} product. The major products migrated in a similar manner to that of the authentic C_{55}-OH, all-*E*-farnesyl-all-*Z*-octaprenol, indicating that the enzymatic reactions proceeded through *cis*-prenyl chain elongation. On the other hand, the production of only a little UPP was detected in the reaction with mutant F207S even if the amount of the mutant enzyme was increased to 5-fold in the reaction mixture and the incubation time was prolonged to overnight.

Heat Stability of the UPP Synthase Mutants—The heat stabilities of the mutant enzymes were examined by analyzing the remaining activity after heat treatment at various temperatures (25, 30, 37, 42, and 50°C) for 30 min. All of the mutants exhibited comparable stability to that of the wild-type (Fig. 3).

CD Analysis of the Secondary Structure of the Wild-type and Mutant Enzymes—Far-UV CD spectrum analysis was performed to determine whether or not these aromatic mutations affect the secondary structure of the enzyme. All of the mutants showed similar spectral profiles to those of the wild-type, with minima at 209 and 223 nm and a maximum near 195 nm. The α-helix contents of all mutant proteins (43% for Y71S, 39% for Y148S, 42% for Y148F, 39% for F207S, 38% for W210A, and 44% for W224A) are found to be essentially the same as that of the wild-type (45%) indicating that the overall secondary structure was not appreciably affected by the point mutations (Fig. 4). The helical content of the wild-type estimated from the far-UV CD spectra is consistent with that determined on X-ray crystallography (40.6%). However, the possibility that the aromatic substitutions have some effect on the local conformation of small segments of the protein cannot be excluded.

DISCUSSION

M. luteus B-P 26 UPP synthase has a novel protein fold, which is totally different from that of *trans*-type prenyl chain-elongating enzymes. This fact raised the possibility that *cis*-type prenyltransferases may have quite different modes of substrate binding and catalysis from those of *trans*-type enzymes. Our recent finding of homoallylic substrate recognition sites in *M. luteus* B-P 26 UPP synthase has confirmed that there is a significant difference in substrate binding between *trans*- and *cis*-type prenyl chain-elongating enzymes (*26*).

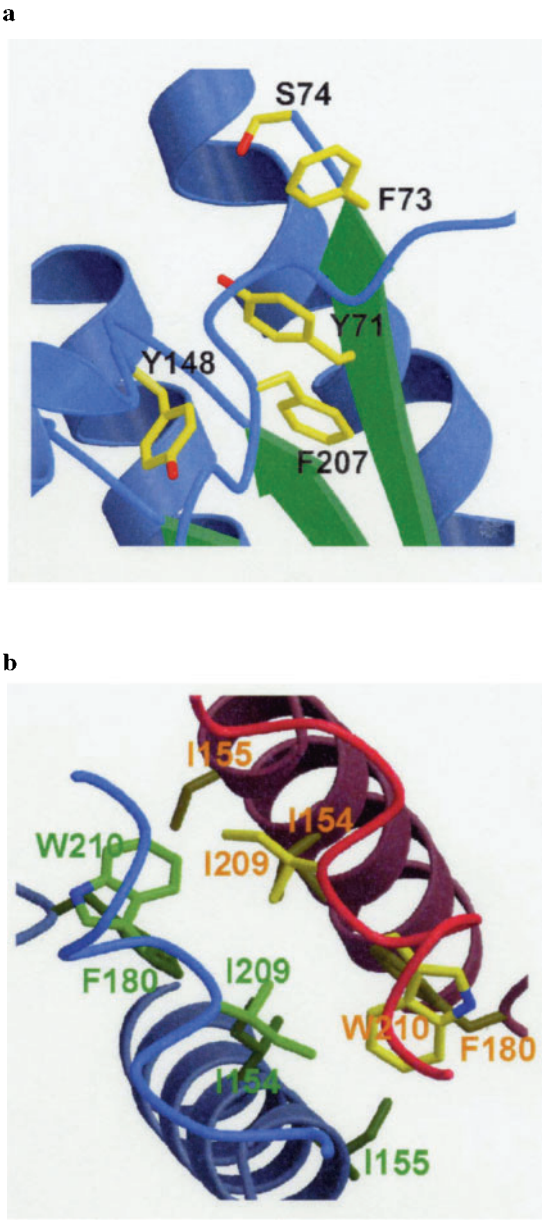

Fig. 5. **Three-dimensional structure of the *M. luteus* B-P 26 UPP synthase (PDB ID 1F75) around the locations of the side chains of the conserved aromatic amino acid residues investigated in this study.** (a) Y71, Y148, and F207, which reside in close vicinity to the FS-motif. (b) W210 along with the hydrophobic residues, I209, I154, and I155, at the interface of the homodimeric enzyme.

There are six highly conserved aromatic amino acid residues (Tyr-71 in Region III, Tyr-148 in Region IV, and Phe-207, Trp-210, Trp-224, and Phe-227 in Region V in *M. luteus* B-P 26 UPP synthase) among *cis*-type prenyltransferases, which are located in the interior of a hydrophobic cleft or at the interface between two monomers in the homodimeric enzyme structure (*21*). We replaced these aromatic residues with Ala and Ser, respectively, which have a smaller side chain and are less hydrophobic, and examined their roles during catalysis. Although Y148S could be purified to homogeneity, it showed little enzyme activity. F227S could not be purified after disruption of the cells due to its insolubility.

Our previous site-directed mutagenesis analyses of *M. luteus* UPP synthase indicated that the FS-motif in Region III plays an important role in homoallylic substrate binding as well as catalysis (*26*). The FS-motif is located adjacent to the charged triangle formed by Arg-197, Arg-203, and Glu-216, and which may recognize the diphosphate moiety of IPP. In the crystal structure of *M. luteus* B-P 26 UPP synthase, Tyr-71, Phe-207, and Tyr-148 are located in close proximity to the homoallylic substrate binding site (*21*). The F207S mutant gave a 13-fold increase in the K_m value for IPP and 1,000–2,000-fold decreased catalytic efficiency (k_{cat}/K_m), suggesting that F207 is indispensable for substrate binding as well as catalysis. Y71S showed a 3-fold increase in the K_m value for IPP and 11–14-fold decreased catalytic activity, which may be explained by that Y71 is less important for substrate binding but rather important for catalysis. However, we could not determine the kinetic parameters for Y148S as it appeared to be an almost inactive enzyme. Interestingly, the substitution of Tyr-148 with Phe led to an enzyme, which resulted in similar K_m values for IPP and FPP, and 30–40-fold lower k_{cat}/K_m values as compared to the wild-type. This suggests that the tyrosine residue at position 148 is very critical for keeping the enzyme in an active form. In the three-dimensional structure of the UPP synthase, the Y71, F207, and Y148 residues are stacked through a triangular interaction manner, and their aromatic side chains face onward the FS-motif, indicating their implication to support the IPP binding site (Fig. 5a). Substitution of Phe-207 with a small and less hydrophobic residue, Ser, may affect the intervention of the proper orientation of the FS-motif, which may lead to reduced binding affinity for IPP and decreased catalytic activity. In fact, Phe-207 and Tyr-71 are located in close proximity and thus can interact with each other, as shown in Fig. 5a. Replacement of Phe-207 with a smaller and less hydrophobic residue may cause some movement of Tyr-71, which may result in a change in the position of Phe-73. Moreover, Tyr-148, which is completely conserved in all species, seems to be located at the critical position to support the entire conformation around Tyr-71. Substitution of Tyr-148 with a smaller residue Ser, Y148S, must greatly affect the binding and catalytic function, and thereby change the entire protein structure into an inactive form. Two Gly residues at positions 149 and 150, which are located immediately adjacent to Tyr-148, make the loop between S4 and H6 structurally restricted. This may suggest that Tyr-148 is located at a key position, supporting the IPP binding site to keep an enzymatically active form. This hypothesis is supported by a report on dehydrodolichyl diphosphate synthase showing that the *rer2* mutant, which has mutation at the residue equivalent to Gly-150 of *M. luteus* B-P 26 UPP synthase, is deficient in enzymatic activity (*19*). This was further supported by replacement of Tyr-148 with Phe, which is active but has highly reduced catalytic activity, indicating that not only the aromatic ring, but also the hydroxyl-group of Tyr-148 is important for the enzymatic activity of UPP synthase.

The W224A mutant showed a 15-fold increased K_m value for FPP and a moderate change in the K_m value for

IPP. The k_{cat}/K_m value was decreased by 100–250-fold compared to that of the wild-type. W224 is located at the backside of the structural P-loop motif composed of residues 30–33 in the crystal structure of *M. luteus* B-P 26 UPP synthase. The aromatic side chain of W224 points towards the structural P-loop. The fact that W224A showed a 15-fold larger K_m value for FPP also coincides with the probable role of the structural P-loop motif in recognition of the diphosphate moiety of FPP (*21*). Because of the difference in size between tryptophan and alanine, the substitution which alters the hydrophobic interaction may lead to the movement of a number of catalytic residues around the P-loop, resulting in 15-fold lower allylic substrate binding affinity and 100–250-fold decreased catalytic efficiency. These results indicated that W224 plays a very important role in substrate binding as well as catalysis.

On the other hand, W210A showed no significant change in the K_m value for either allylic or homoallylic substrates, while the k_{cat}/K_m values decreased by 100–1,600-fold compared to that of the wild-type. Trp-210 is located at the interface between two monomers in the homodimeric enzyme, and is stacked with Phe-180 of the same monomer, and Ile-209, Ile-154 and Ile-155 of the other monomer, as shown in Fig. 5b. The dramatic reduction of the catalytic efficiency of the W210A mutant can be explained by that substitution of Trp-210 with Ala may alter the hydrophobic interaction of these amino acid residues on the interface between two monomers of the UPP synthase, resulting in alteration of the proper conformation of the active sites for substrate binding and catalysis. Determination of the UPP synthase structure with substrate(s) bound would confirm the hypothetical mode of substrate binding of the *cis*-type prenyl chain-elongating enzymes, which is totally different to that of the *trans*-type enzymes.

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