Published in final edited form as:

Biochem Biophys Res Commun. 2008 December 5; 377(1): 17–22. doi:10.1016/j.bbrc.2008.09.014.

Product chain-length determination mechanism of *Z,E*-farnesyl diphosphate synthase

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Abstract

cis-Prenyltransferases catalyze the consecutive condensation of isopentenyl diphosphate (IPP) with allylic prenyl diphosphates, producing *Z,E*-mixed prenyl diphosphate. The *Mycobacterium tuberculosis Z,E*-farnesyl diphosphate synthase Rv1086 catalyzes the condensation of one molecule of IPP with geranyl diphosphate to yield *Z,E*-farnesyl diphosphate and is classified as a short-chain *cis*-prenyltransferase. To elucidate the chain-length determination mechanism of the short-chain *cis*-prenyltransferase, we introduced some substitutive mutations at the characteristic amino acid residues of Rv1086. Among the mutants constructed, L84A showed a dramatic change of catalytic function to synthesize longer prenyl chain products than that of wild type, indicating that Leu84 of Rv1086 plays an important role in product chain-length determination. Mutagenesis at the corresponding residue of a medium-chain *cis*-prenyltransferase, *Micrococcus luteus* B-P 26 undecaprenyl diphosphate synthase also resulted in the production of different prenyl chain length from the intrinsic product, suggesting that this position also plays an important role in product chain-length determination for medium-chain *cis*-prenyltransferases.

Keywords

Mycobacterium tuberculosis; Isoprenoid; Prenyltransferase; Prenyl diphosphate synthase; Undecaprenyl diphosphate synthase; Chain-length determination mechanism; Mutagenesis

cis-Prenyltransferases catalyze consecutive condensations of isopentenyl diphosphate (IPP) with allylic primer substrates to yield linear prenyl diphosphates with a definite prenyl chain length [1,2]. The products are utilized as precursors of glycosyl carrier lipids, namely, undecaprenyl monophosphate and decaprenyl monophosphate in bacteria [3,4], and dolichyl monophosphate in eukaryotes [5].

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The product chain-length determination mechanism of *cis*-prenyltransferases has not yet been elucidated, although mutational analyses of highly conserved residues and of characteristic amino acid residues in each subfamily of *cis*-prenyltransferases have enabled the understanding of the basic catalytic mechanism of those enzymes [6–9]. Based on the chain length of their products, *cis*-prenyltransferases have been classified into three subfamilies, namely, short-, medium-, and long-chain *cis*-prenyltransferases, which yield C_{15}, $C_{50–55}$, and $C_{70–120}$ products, respectively [9]. The enzymes of the three groups are thought to possess similar structures and catalytic mechanisms as their amino acid sequences show a high degree of similarity [10,11]. X-ray crystallographic analysis of the medium-chain enzyme undecaprenyl diphosphate synthase (UPS) from *Micrococcus luteus* B-P 26 and *Escherichia coli* revealed that these homodimeric enzymes have a large hydrophobic cleft on the molecular surface of each subunit [12,13]. The elongating prenyl chain of the product may stretch along this cleft during enzymatic condensation reactions.

Kharel et al. identified some of the characteristic amino acid residues that differ among various *cis*-prenyltransferases and examined the function of these residues by the introduction of mutations in *M. luteus* B-P 26 UPS [9]. In the long-chain *cis*-prenyltransferases, characteristic amino acid insertions were found at the side-wall of the cleft, and it was suggested that these insertions participate in the determination of chain length by controlling the direction of prenyl chain elongation during the catalysis. On the other hand, in the short-chain *cis*-prenyltransferase, characteristic amino acid residues found in proximity to the substrate binding site appeared to participate in the determination of chain length of the product by blocking further chain elongation.

In the present investigation, mutational studies of the *Z,E*-farnesyl diphosphate synthase, Rv1086, from *Mycobacterium tuberculosis* [11], were performed to help elucidate the mechanism of product chain-length determination of short-chain *cis*-prenyltransferases. This enzyme catalyzes the condensation of one molecule of IPP with geranyl diphosphate (GPP) to yield *Z,E*-farnesyl diphosphate (*Z,E*-FPP), and is the only short-chain *cis*-prenyltransferase identified thus far. *cis*-Prenyltransferases have five conserved regions that are thought to be involved in catalysis [14]. Therefore, mutations were generated in some of these conserved regions. The mutations affected not only the products synthesized by the enzyme but also the specificity for allylic substrates. Data from these studies and mutational studies using *M. luteus* B-P 26 UPS indicate that Leu84 plays an important role in the mechanism of product chain-length determination of short-and medium-chain *cis*-prenyltransferases.

Materials and methods

Materials

Precoated reversed-phase TLC plates, namely, LKC-18F, were purchased from Whatman, UK. Non-labeled IPP, GPP, and *E,E*-FPP were synthesized, as reported [15]. [1-^{14}C]IPP was purchased from GE Healthcare, USA. All other chemicals were of analytical grade.

General procedures

Restriction enzyme digestions, transformations, and other standard molecular biology techniques were carried out as described by Sambrook et al. [16].

Plasmid construction and site-directed mutagenesis of Rv1086

For Rv1086 expression, the corresponding gene was amplified by PCR with the following primers: Rv1086UP, 5′-GTACATATGGAGATCATC CCGCCGCGGCTC-3′; and, Rv1086RP, 5′-ACCCTCGAGTGCAACATAG GCGTCC-3′. The sequences corresponding to the NdeI and XhoI sites, which were used in subsequent experiments, are underlined in the primer sequences above in that order. The amplified fragment was cleaved with restriction enzymes and then inserted into an NdeI/XhoI-treated pET-28a vector (Novagen, USA) to construct pET-Rv1086. For construction of +EKE, a Gene Editor *in vitro* Site-Directed Mutagenesis System (Promega, USA) was used, according to the manufacturer's protocols. The single stranded *rv1086* gene used as a template in the mutagenesis reaction was prepared by infection of *E. coli* JM109 cells (TaKaRa, Japan) harboring pET-Rv1086 with R408 helper phages. For the construction of mutants L84A, L85A, L85F and L90W, a QuikChange Mutagenesis Kit (STRATAGENE, USA) was used, according to the manufacturer's protocols. The pET-Rv1086 was used as a template for the construction of mutants. Mutagenic oligonucleotides designed to produce the desired mutant enzymes were: 5′-GAGATCTGCGCAGAGA AGGAGCCGGCCAACCAC-3′ (for +EKE); 5′-CCACCGTCTACGCGTTGTC CACCGAAAACC-3′ (for L84A); 5′-CCGTCTATCTGGCGAGTACTGAAAA CCTGCAGC-3′ (for L85A); 5′-CCGTCTATCTGTTCAGTACTGAAAACCT GCAGC-3′ (for L85F); 5′-CCACCGAAAACTGGCAGCGCGATCC-3′ (for L90W). After the confirmation of the introduced mutation by a DNA sequencing, the constructed plasmids were cleaved with the restriction enzymes NdeI/XhoI, which were then inserted into NdeI/XhoI-treated pET-15b vector (Novagen, USA).

Plasmid construction and site-directed mutagenesis of M. luteus B-P 26 UPS

For the expression of N-terminus His_6-tagged *M. luteus* B-P 26 UPS, the expression plasmid pMluUEX [7] was cleaved with the restriction enzymes NdeI/BamHI. The gene fragment was inserted into an NdeI/BamHI-treated pET-15b to construct pET-HisMlUPS. For the construction of UPS mutant, a QuikChange Mutagenesis Kit was used according to the protocol of the manufacturer. The pET-HisMlUPS was used as a template for the construction of mutants. Mutagenic oligonucleotides designed to produce the desired mutant enzymes were: 5′-CTTAACGCTGTACTT ATTTTCCACGGAAAATTGGTC-3′ (for A72L); 5′-CTTAACGCTGTACT TTTTTTCCACGGAAAATTGGTC-3′ (for A72F); 5′-CTTAACGCTGTAC TGGTTTTCCACGGAAAATTGGTC-3′ (for A72W).

Expression and purification of wild type and mutant enzymes

For the expression of Rv1086 and its mutants, *E. coli* BL21(DE3) was transformed with pET-Rv1086 or with each expression plasmid. The transformants were cultivated in 50 mL M9YG broth supplemented with ampicillin (50 mg/L). When the OD_{600} of the culture reached 0.5, the transformed bacteria were induced with 0.5 mM isopropyl 1-thio-β-D-

galactoside. The cells were incubated overnight and then harvested. The cells were disrupted in lysis buffer containing 20 mM sodium phosphate buffer (pH 7.4), 100 mM imidazol, and 0.5 M NaCl. The homogenate was centrifuged at 6000g for 15 min at 4 °C. The supernatant was then applied on a HisTrap column (GE Healthcare, USA) previously equilibrated with the lysis buffer. The resin was washed with washing buffer containing 20 mM sodium phosphate buffer (pH 7.4), 150 mM imidazol, and 0.5 M NaCl. The protein bound to the resin was then eluted with elution buffer containing 20 mM sodium phosphate buffer (pH 7.4), 300 mM imidazol, and 0.5 M NaCl. Fractions with more than 90% protein purity as well as with high enzymatic activity were used for the experiments described below. For the expression and purification of *M. luteus* UPS, similar procedures as those described above were used.

Measurement of prenyltransferase activity

The assay mixture for wild type and mutant Rv1086s contained 4 μM [1-^{14}C]IPP (2 GBq/mmol), 10 μM of an allylic primer (GPP or *E*,*E*-FPP), 0.1 mM $MgCl_2$, 2.5 mM dithiothreitol, 0.3% (w/v) Triton X-100, 50 mM Tris–HCl buffer (pH 8.0), and a suitable amount of each enzyme in a final volume of 100 μL. This mixture was incubated at 30 °C for 30 min, and the reaction was stopped by chilling the mixture in an ice bath. The mixture was treated with 500 μL of 1-butanol saturated with H_2O. The butanol layer was washed with water saturated with NaCl, and the radioactivity in 50 μL of the butanol layer was measured with a LS 6500 Liquid Scintillation Counting System (BECKMAN COULTER, USA). The residual butanol layer was used for product analysis. The assay mixture for wild type and mutant *M. luteus* B-P 26 UPSs contained, 10 μM [1-^{14}C]IPP (2 GBq/nmol), 10 μM of an allylic primer (GPP or *E*,*E*-FPP), 0.5 mM $MgCl_2$, 0.05% (w/v) Triton X-100, 100 mM Tris–HCl buffer (pH 7.5), and a suitable amount of each enzyme in a final volume of 100 μL. The mixture was incubated at 30 °C for 30 min and then processed as described above.

Product analysis

Prenyl diphosphates in the residual 1-butanol layer were treated with acid phosphatase, according to the method of Fujii et al. [17]. The hydrolysates were extracted with pentane and analyzed by reversed-phase TLC using a precoated plates, LKC-18F (Whatman, UK), which were developed with the solvent mixture methanol/acetone (8/2) (for Rv1086) or acetone/H_2O (19/1) (for UPS from *M. luteus* B-P 26). Authentic standard alcohols were visualized with iodine vapor, and the distribution of radioactivity was detected with a Fuji BAS 1000 Mac Bioimage Analyzer (FUJIFILM, Japan).

Results and discussion

Mutations in conserved region III of Rv1086

The short-chain *cis*-prenyltransferase Rv1086 has three characteristic Leu residues, which differ from those in other groups of *cis*-prenyltransferases, at positions 84, 85, and 90 in conserved region III (Fig. 1A). Kharel et al. reported that *M. luteus* B-P 26 UPS mutants in which the characteristic Leu residues of Rv1086 were introduced in the corresponding positions, i.e., A72L/F73L and A72L/F73L/W78L, yielded shorter products with chain length of $C_{20–35}$. This suggested that these residues participated in the chain-length

determination mechanism [9]. To examine whether these Leu residues played a similar role in Rv1086, we replaced these residues with corresponding amino acids that are conserved in medium- and long-chain *cis*-prenyltransferases, respectively (Fig. 1B). L85F showed enzymatic activity similar to that of the wild type enzyme and produced C_{15} as the sole product (Fig. 1C, lane 5). L90W showed no activity although the amount of enzyme used was the same (data not shown). On the other hand, among the mutants constructed, only L84A showed considerable activity when *E,E*-FPP was used as an allylic substrate, while wild type Rv1086 scarcely accepted *E,E*-FPP (Fig. 1C, lane 5). L84A predominantly yielded a C_{20} product when *E,E*-FPP was used as the allylic substrate (Fig. 1C, lane 4).

In order to examine whether the second characteristic Leu residue at position 85 participated in the product chain-length determination mechanism, it was substituted with smaller amino acid, Ala (Fig. 1B). Product analysis revealed that the mutant L85A did not produce longer products than those produced by the wild type enzyme (Fig. 1D, lanes 3 and 4). These results strongly suggested that among the three characteristic Leu residues at positions 84, 85, and 90 of Rv1086, only Leu84 participated in product chain-length determination.

Recently, Wang et al. reported the crystal structure of Rv1086 (Protein Data Bank code: 2VFW) [18]. They also reported the crystal structure of Rv1086 bound with *E,E*-FPP or with citronellyl diphosphate (CITPP) (Protein Data Bank codes: 2VG1 and 2VG0, respectively) [18]. In the crystal structure bound with *E,E*-FPP, which is the geometric analog of the product of Rv1086, the prenyl chain lies in a hydrophobic cleft, and the diphosphate group of *E,E*-FPP occupies an “IPP binding site” common to *cis*-prenyltransferases [7]; this site is composed of charged amino acid residues including Arg211 and Arg217. Among the characteristic Leu residues, Leu84 is located at the edge of the hydrophobic cleft close to the center part of the prenyl chain of *E,E*-FPP bound. Based on this structure, the side chain of Leu84 appears to act as a barrier at the entrance gate of the cleft and to interfere with the elongation of the prenyl chain toward helix-3, bending it instead toward helix-2. In the structure of Rv1086 bound with CITPP, similar situations are observed; the side chain of Leu84 is located near the prenyl chain of CITPP causing it to bend toward helix-2. It is conceivable that the replacement of Leu84 with Ala would create a new chain-elongation pathway, resulting in further chain elongation. In the Rv1086 structure bound with *E,E*-FPP or with CITPP, the ω-end of the prenyl chain of *E,E*-FPP or CITPP is located near helix-2 and further prenyl chain elongation may be interfered with the amino acid residues in helix-2. Thus, wild type Rv1086, only one molecule of IPP can be added to the allylic substrate GPP during the enzymatic prenyl chain elongation because further extension of the ω-end of the prenyl chain may be blocked by the residue in helix-2.

Mutations in region III of M. luteus B-P 26 UPS

In order to examine the role of Leu84 of Rv1086, the corresponding residue, i.e., Ala72, of *M. luteus* B-P 26 UPS was replaced with other amino acids (Fig. 2A). When Ala72 was replaced with Phe or Trp, dramatic changes were observed: the prenyl chain length of the products became shorter than those of the wild type enzyme. Furthermore, A72W produced a considerable amount of a C_{15} product along with $C_{20–30}$ products when GPP was used as the substrate (Fig. 2B, lane 7). These results indicated that the residue at position 72 of *M.*

luteus B-P 26 UPS was also involved in the product chain-length determination mechanism. The amount of these intermediates seemed to increase as the size of the side chain of the substituted residue increased. Overall, these results indicated that introduction of an amino acid with a bulky side-chain interferes with the product chain elongation. Nevertheless, the prenyl chain length of the ultimate product synthesized by these mutants were equal to that of the product synthesized by the wild type enzyme. This was probably because the side chain of the substituted residue at position 72 did not completely block prenyl chain elongation. Kharel et al. reported that *M. luteus* B-P 26 UPS mutants in which characteristic Leu resides of Rv1086 were introduced in the corresponding positions, i.e., A72L/F73L and A72L/F73L/W78L, yielded products with shorter-chain than those of the products yielded by the wild type enzyme [9]. In this study, the results of *M. luteus* B-P 26 UPS mutant analysis supported the importance of Leu84 of Rv1086 in the mechanism of product chain-length determination.

Introduction of extra amino acid insertions into helix-3

Long-chain *cis*-prenyltransferases possess three to five characteristic amino acid insertions in helix-3, which is located downstream of the conserved region III (Fig. 3A). These insertions consist of charged or polar amino acids. Kharel et al. indicated that these insertion sequences of long-chain *cis*-prenyltransferase also participate in product chain-length determination, by controlling the bending direction of elongating prenyl chain. The *M. luteus* B-P 26 UPS mutants, EKE and RAKDY, which have insertions between Pro101 and Glu102 of helix-3 similar to those seen in long-chain *cis*-prenyltransferases, could produce longer prenyl chain products than those produced by the wild type enzyme [9]. On the other hand, the *Saccharomyces cerevisiae* dehydrodolichyl diphosphate synthase (DedolPS) mutant, delta Srt1p, in which the insertion sequence in helix-3 was deleted, yielded shorter products than those yielded by wild type Srt1p [9]. To examine the role of the residue at the corresponding position of Rv1086, an insertion mutant of Rv1086, named, +EKE, was constructed (Fig. 3B). This mutant contained insertions corresponding to the extra amino acid residues of human DedolPS [19] (HDS; positions 107–109) between Ala113 and Pro114 in helix-3 of Rv1086. As shown in Fig. 3C, however, mutant +EKE, produced a C_{15} compound as a final product, identical to the product generated using the wild type enzyme.

Introduction of extra amino acids into helix-3 of Rv1086 did not affect the chain length of the final product. Based on the crystal structure of Rv1086, the three amino acid insertions that were introduced in mutant +EKE were located around Ala113 and were in the vicinity of the bottom of the hydrophobic cleft (Fig. 4). In Rv1086, the region around Ala113 might not be involved in the mechanism of product chain-length determination, because Ala113 is situated away from Leu84, which is located at the edge of the hydrophobic cleft and is critical for product chain-length determination.

In conclusion, we have identified that the Leu84 is the most critical in determination of the product chain length in the short-chain *cis*-prenyltransferase Rv1086 from *M. tuberculosis*.

Acknowledgments

This work was supported in part by grants-in-aid for scientific research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (Grant 19710178 to M.N.) and from Tohoku University Exploratory Research Program for Young Scientists (TU-ERYS) and from NIH/NIAID Grants AI-065357 and AI-018357.

Abbreviations

IPP	isopentenyl diphosphate
GPP	geranyl diphosphate
CITPP	citronellyl diphosphate
***E,E*-FPP**	*E,E*-farnesyl diphosphate
***Z,E*-FPP**	*Z,E*-farnesyl diphosphate
GGPP	geranylgeranyl diphosphate
DedolPS	dehydrodolichyl diphosphate synthase
HDS	human dehydrodolichyl diphosphate synthase

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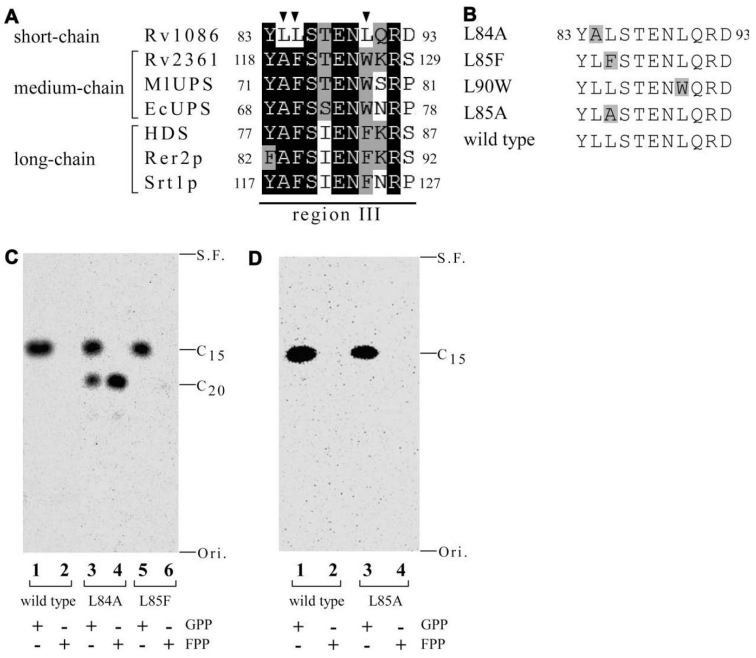

Fig. 1.

Introduction of mutations in Rv1086. (A) Alignment of amino acid sequences around conserved regions III and V of various *cis*-prenyltransferases: Rv1086, *E,Z*-FPP synthase from *M. tuberculosis* (Accession No. D70895); Rv2361c, decaprenyl diphosphate synthase from *M. tuberculosis* (H70585); MlUPS, UPS from *M. luteus* B-P 26 (BAA31993); EcUPS, UPS from *E. coli* (Q47675); Rer2p, dehydrodolichyl diphosphate (DedolPP) synthase from *S. cerevisiae* (BAA36577); Srt1p, DedolPP synthase from *S. cerevisiae* (NP_013819); and HDS, DedolPP synthases from human (BAC57588). The sequences were aligned using CLUSTAL W program [20]. Identical residues (five or more) are shown in black, and similar residues are shaded. The arrowheads indicate the characteristic amino acid residues of Rv1086. (B) Partial amino acid sequences around region III of wild type and mutant enzymes are aligned. The substituted amino acid residues are shaded. (C) TLC autoradiochromatogram of the reaction products of wild type and mutated enzymes. Lanes 1 and 2, wild type; lanes 3 and 4, L84A; lanes 5 and 6, L85F. The products were analyzed as described in Materials and methods. Each reaction mixture contained 10 μM GPP (lanes 1, 3 and 5) or *E,E*-FPP (lanes 2, 4 and 6) as an allylic substrate. Under all assay conditions, less than 30% of each substrate reacted. Ori., origin; S.F., solvent front. (D) TLC autoradiochromatogram of the reaction products of wild type and L85A. Lanes 1 and 2, wild type; lanes 3 and 4, L85A. The products were analyzed as described in Materials and methods. Each reaction mixture contained 10 μM GPP (lanes 1 and 3) or *E,E*-FPP (lanes 2 and 4) as an allylic substrate. Under all assay conditions, less than 30% of each substrate reacted. Ori., origin; S.F., solvent front.

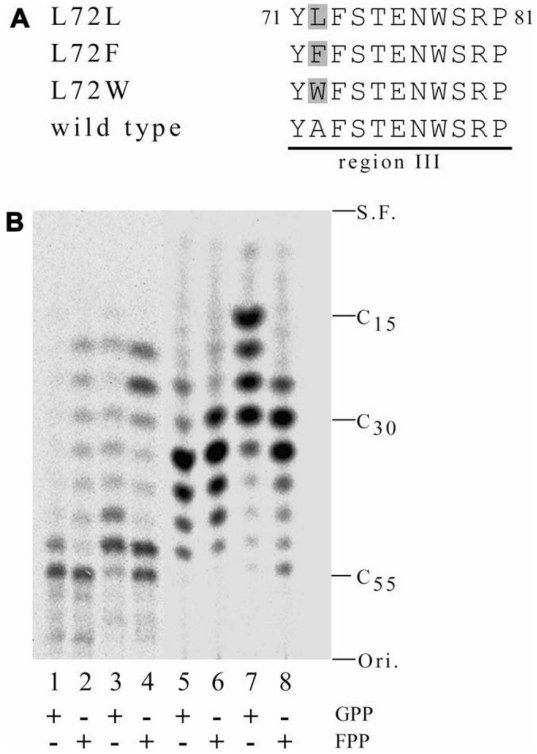

Fig. 2.

Introduction of substitution mutations into region III of UPS from *M. luteus* B-P 26. (A) Partial amino acid sequences around region III of wild type and mutant enzymes are aligned. The substituted amino acid residues are shaded. (B) TLC autoradiochromatogram of the reaction products of wild type and mutant enzymes. Lanes 1 and 2, wild type; lanes 3 and 4, A72L; lanes 5 and 6, A72F; and lanes 7 and 8, A72W. The products were analyzed as described in Materials and methods. Each reaction mixture contained 10 μM GPP (lanes 1, 3, 5, and 7) or *E*,*E*-FPP (lanes 2, 4, 6, and 8) as an allylic substrate. Under all assay conditions, less than 30% of each substrate reacted. Ori., origin; S.F., solvent front.

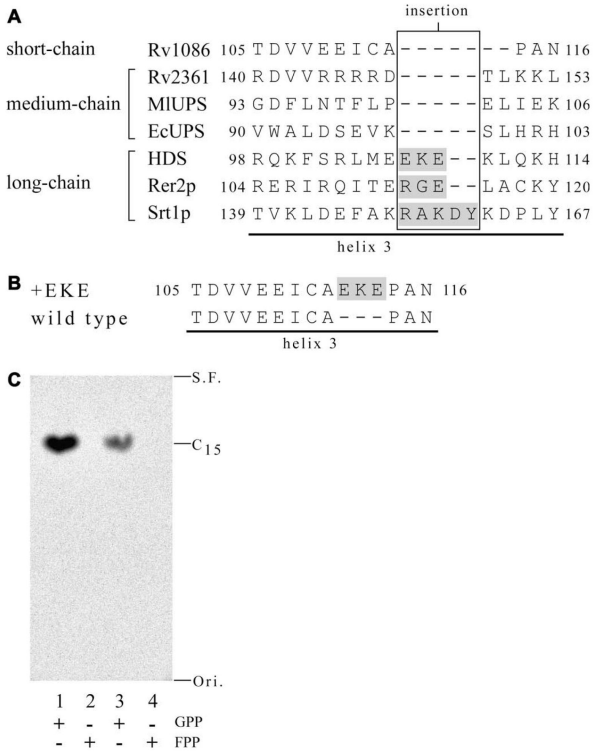

Fig. 3.

Introduction of extra amino acid insertions into helix-3 of Rv1086. (A) Alignment of amino acid sequences around helix-3 of various *cis*-prenyltransferases. Similar notations as those in Fig. 1 are used for sequences. The extra amino acid insertions that are characteristic for long-chain *cis*-prenyltransferase are shaded. (B) Partial amino acid sequences around helix-3 of Rv1086 and mutant enzymes are aligned. The introduced extra amino acid insertions are shaded. (C) TLC autoradiochromatogram of the reaction products of wild type and mutant enzymes. Lanes 1 and 2, wild type and lanes 3 and 4, +EKE. The products were analyzed as described in Materials and methods. Each reaction mixture contained 10 μM GPP (lanes 1 and 3) or *E*,*E*-FPP (lanes 2 and 4) as an allylic substrate. Under all assay conditions, less than 30% of each substrate reacted. Ori., origin; S.F., solvent front.

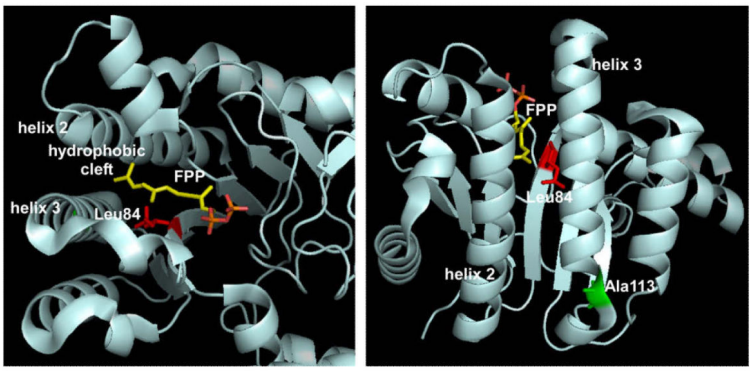

Fig. 4.

Structural information of the important amino acid residue for product chain-length determination in the crystal structure of Rv1086. Front view (left) and side view (right) are shown. Leu84 and Ala113 are shown with red and green sticks, respectively. The prenyl chain of *E*,*E*-FPP is shown with yellow sticks. This figure was prepared using the software PyMOL [21].