Dimer Formation of Octaprenyl-diphosphate Synthase (IspB) Is Essential for Chain Length Determination of Ubiquinone*

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Ubiquinone (Q), composed of a quinone core and an isoprenoid side chain, is a key component of the respiratory chain and is an important antioxidant. In Escherichia coli, the side chain of Q-8 is synthesized by octaprenyl-diphosphate synthase, which is encoded by an essential gene, ispB. To determine how IspB regulates the length of the isoprenoid, we constructed 15 ispB mutants and expressed them in E. coli and Saccharomyces cerevisiae. The Y38A and R321V mutants produced Q-6 and Q-7, and the Y38A/R321V double mutant produced Q-5 and Q-6, indicating that these residues are involved in the determination of chain length. E. coli cells (ispB::cat) harboring an Arg-321 mutant were temperature-sensitive for growth, which indicates that Arg-321 is important for thermostability of IspB. Intriguingly, E. coli cells harboring wild-type ispB and the A79Y mutant produced mainly Q-6, although the activity of the enzyme with the A79Y mutation was completely abolished. When a heterodimer of His-tagged wild-type IspB and glutathione S-transferase-tagged IspB(A79Y) was formed, the enzyme produced a shorter length isoprenoid. These results indicate that although the A79Y mutant is functionally inactive, it can regulate activity upon forming a heterodimer with wild-type IspB, and this dimer formation is important for the determination of the isoprenoid chain length.

Ubiquinone (Q) is an essential component of the electron transport system associated with aerobic growth and oxidative phosphorylation in many organisms. It also has been reported that ubiquinone has an important role as an antioxidant in E. coli (1), Schizosaccharomyces pombe (2, 38), Saccharomyces cerevisiae (3), and mammalian cells (4). Furthermore, it was elegantly shown that ubiquinone (or menaquinone) accepts electrons generated by the formation of protein disulfide in E. coli (5). Thus, multiple functions of ubiquinone have been proposed. The ubiquinone biosynthetic pathway, which is comprised of 10 steps including methylation, decarboxylation, hydroxylation, and isoprenoid transfer, has been studied genetically in respiratory-deficient mutants of E. coli and S. cerevisiae (6). Each of these organisms has a specific isoprenoid side chain length as part of the ubiquinone molecule, e.g. Q-6 for S. cerevisiae, Q-8 for E. coli, Q-9 for rat, and Q-10 for P. pombe and human. For this reason, ubiquinone species have been used for classification in microbial taxonomy (7). The length of the side chain of ubiquinone is precisely defined by the action of polyisoprenyl-diphosphate synthases, but not by 4-hydroxybenzoate-polyisoprenyl-diphosphate transferases, which catalyze the condensation of 4-hydroxybenzoate and polyisoprenyl diphosphate (8). When various polyisoprenyl-diphosphate synthase genes, such as the mutant GGPP synthase gene from Sulfolobus acidocaldarius, the hexaprenyl-diphosphate synthase synthase gene (COQ1) from S. cerevisiae, the heptaprenyl-diphosphate synthase gene from Haemophilus influenzae, the octaprenyl-diphosphate synthase gene (ispB) from E. coli, the solanesyl-diphosphate synthase gene (ddsA) from Rhodobacter capsulatus, and the decaprenyl-diphosphate synthase gene (ddsA) from Gluconobacter suboxydans, are expressed in an S. cerevisiae COQ1 mutant, each transformant produced mainly Q-5, -6, -7, -8, -9, and -10, respectively (9). When COQ2, which encodes 4-hydroxybenzoate-hexaprenyl-diphosphate transferase in S. cerevisiae, was expressed in an E. coli ubiA mutant cell line, the transformant produce Q-8, but not Q-6 (10). These results indicate that polyisoprenyl-diphosphate synthase determines the chain length of ubiquinone and that 4-hydroxybenzoate-polyisoprenyltransferases can accept various isoprenoid chains as a substrate.

In E. coli, ispB is an essential gene, responsible for the biosynthesis of both ubiquinone and menaquinone (11, 12). An E. coli ubiA mutant, which does not produce Q-8, is not able to grow on a non-fermentable carbon source, but can grow on glucose (10). However, an E. coli ubiA− menA− mutant, which lacks both ubiquinone and menaquinone biosynthesis genes, can grow only when a small amount of Q-8 is still produced by leakiness of the mutations. Thus, ubiA− menA− mutants with an absolute lack of production of Q-8 and menaquinone-8 cannot be isolated.

Long-chain polyisoprenyl-diphosphate synthases (C40, C45, and C50) catalyze the condensation of FPP, which acts as a primer, and IPP to produce each polyisoprenyl diphosphate with various chain lengths. These enzymes possess seven conserved regions including two DXDXXD motifs that are binding sites for the substrates in association with Mg2+ (13, 14). Short-chain polyisoprenyl-diphosphate synthases (C15 and C20), such as FPP and GGPP, geranylgeranyl diphosphate; FPP, farnesyl diphosphate; IPP, isopentenyl diphosphate; PCR, polymerase chain reaction; GST, glutathione S-transferase; NTA, nitrolotriacetic acid; HPLC, high pressure liquid chromatography.
geranylgeranyl-diphosphate synthase from *S. acidocaldarius* (16) was used to determine which amino acids are important for the determination of chain length of short-chain prenyl diphosphates. These amino acids were those at the fourth and fifth positions before an aspartate-rich motif in region II or one amino acid at the fifth position before this motif and two amino acids in region II. Recently, we reported that substitution of glycine for alanine before the first DDXXD motif in decaprenyl-diphosphate synthase allowed the enzyme to synthesize products with longer chain lengths (18). Thus, the fifth amino acid before region II of long-chain polyisoprenyl-diphosphate synthases plays an important role in the mechanism of chain length determination (18).

Generally, polyisoprenyl-diphosphate synthases are known to function as a dimer. The medium-chain polyisoprenyl-diphosphate synthases (C_{30} and C_{35}) from *Micrococcus luteus* BP26, *B. steatorrhophilus*, and *Bacillus subtilis* are composed of heterodimers (19, 20). GGPP synthase purified from bovine brain forms a homo-oligomer (150–195 kDa) (21). However, the subunit structure of long-chain polyisoprenyl-diphosphate synthases remains to be determined. Recently, geranyl-diphosphate synthase isolated from spearmint (22) was found to form a heterodimer. One subunit has similarity with known prenyltransferases, and the other has similarity with the *Arabidopsis* GGR protein (23), but the aspartate-rich motifs are not conserved.

In this study, we describe the mutational analysis of octaprenyl-diphosphate synthase (IspB) from *E. coli*. From the analysis, we found that IspB forms a homodimer that is important for the determination of isoprenoid chain length.

**EXPERIMENTAL PROCEDURES**

**Materials**—Restriction enzymes and other DNA-modifying enzymes were purchased from Takara Shuzo Co., Ltd., and New England Biolabs, Inc. IFF, (E)-farnesyl diphosphate (all-(E)-FPP), geranylgeraniol, and solanesol (all-((Z)-nonaprenol) were purchased from Sigma. [1-^{14}C]IPP (1.96 TBq/mol) was purchased from Amersham Pharmacia Biotech. Kieselgel 60 F_{254} TLC plates were purchased from Merck. Reversed-phase LKC-18 TLC plates were purchased from Whatman.

**Strains and Plasmids**—*E. coli* strains DH10B and KO229, containing the mutant *ispB*::cat (11) from *M. luteus*, were used as templates in PCRs. First, PCR was performed with the T7 and T3 primers by the method described previously (8, 28). The crude extract of ubiquinone was then used as a crude enzyme extract and then sonicated buffer. The GST-IspB protein was eluted with sonication buffer containing 10 mM reduced glutathione.

**Construction of IspB Mutants by Site-directed Mutagenesis**—Site-directed mutagenesis was performed by PCR according to the methods of Ito et al. (26). Five oligonucleotide primers (MUT, R1 (for each mutational primer), T7, and T3) (see Table II) were used in amplifications. pK056, which contains the open reading frame and downstream region of *ispB*, was used as template in PCR. First, PCR was performed with the MUT and T3 primers in one reaction and with the R1 and T7 primers in another. An aliquot of each of the reaction mixtures was mixed in a new tube to form the heteroduplex *ispB* template, and full-length mutant *ispB* was amplified with the T7 and T3 primers by PCR. This DNA fragment was digested with EcoRI and HindIII and cloned into pBluescript KS+ (11). This construct was transformed into *E. coli* DH10B and KO229 (ispB::cat) for analysis of enzyme activity and ubiquinone extraction.

**Purification of IspB**—IspB was purified from cell extracts of *E. coli* DH10B or KO229. Cells harboring pRAM-IspB, which expresses IspB containing the mutation R231A, were transformed with pSTVKKQKO56 and produced transformants that were resistant to ampicillin and kanamycin. Ampicillin-sensitive and kanamycin-resistant strains were selected following the method described above and transformed with pG79Y, and the strains harboring both plasmids were selected and named KO229/35-2.

**Ubiquinone Extraction and Measurement**—Ubiquinone extraction was performed by the method described previously (8, 28). The crude extract of ubiquinone was analyzed by normal-phase TLC with authentic standard Q-10. Normal-phase TLC was carried out on a Kieselgel 60 F_{254} plate with benzene/acetone (97:3, v/v). Samples were dried and redissolved in ethanol. The purified ubiquinone was further analyzed by HPLC with ethanol as the solvent.

**Prenyl-diphosphate Synthase Assay of Mutant IspB Proteins**—The EcoRI-HindIII fragment from pG79KO56 was recloned into pSTVK28, which had been converted from expressing chloramphenicol resistance to kanamycin resistance. GST-IspB fusion proteins from pG79KO56 were used as substrates for IspB in the *S. cerevisiae* (COQ1::URA3) (8), which is the *COQ1*-defective mutant—E. coli

**To express the mutant *ispB* genes in *S. cerevisiae* YKK6 (COQ1::URA3), a COQ1-ispB fusion gene was constructed. The S2 and A3 primers were used to amplify the *ispB* gene by PCR. The fragment was digested with EcoRI and HindIII and cloned into pSA1 (8), which harbors the amino acids of the *COQ1* mitochondrial import signal with the *COQ1* promoter. The BamHI-HindIII fragment from pSA1 was ligated into the yeast shuttle vector YEp13M4 (25). YKK6 was transformed with both plasmids by the lithium acetate method (27) and was selected on Synthetic Complete (0.67% (w/v) yeast nitrogen base, 2% (w/v) glucose and 3% (w/v) glycerol, and the appropriate amino acids)—Leu–Ura medium.

**Determination of IspB**—To overexpress and purify IspB, vectors containing the 6-His or glutathione S-transferase (GST) tag fused to IspB were constructed. The amplified BamHI-HindIII fragment containing *ispB* from pK056 was cloned into pQE31 (QiAGEN Inc.) to yield pQKO56. The amplified BamHI-HindIII fragment containing wild-type or A79T mutant *ispB* was cloned into pGEX-IX in which an XhoI linker had been inserted to yield pGKO56 or pG79Y, respectively. The plasmids were transformed into E. coli JM109. Transformants were grown to stationary phase in LB medium containing 50 μg/ml ampicillin, and 10 ml of culture was inoculated into 100 ml of the same medium. The culture was grown at 37 °C for 3 h, and recombinant protein expression was induced with 0.1 mM isopropyl-1-thio-galactoside (24, 25). From the resulting supernatants, the fusion proteins were purified by the method of Hill and co-workers (26). From the fusion protein, the His-IspB protein was eluted with 50 mM sodium phosphate, 300 mM NaCl, and 10 mM imidazole and sonicated 10 times for 10 s at 10-s intervals with an ultrasonic disintegrator in an ice bath. Ruptured cells were centrifuged at 15,000 × g for 20 min. The resulting supernatants were added to a Ni^{2+}-nitriilotriacetic acid (NTA) slurry and mixed gently at 4 °C for 60 min. This mixture was washed twice with 140 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate, and 1.8 mM potassium phosphate and then with sonication buffer. The GST-IspB protein was eluted with sonication buffer containing 10 mM reduced glutathione.

**Coexpression of Wild-type and Mutant IspB Proteins**—The EcoRI-HindIII fragment from pG79KO56 was recloned into pSTVK28, which had been converted from expressing chloramphenicol resistance to kanamycin resistance. GST-IspB fusion proteins from pG79KO56 were used as substrates for IspB in the *S. cerevisiae* (COQ1::URA3) (8), which is the *COQ1*-defective mutant—E. coli

**Ubiquinone Extraction and Measurement**—Ubiquinone extraction was performed by the method described previously (8, 28). The crude extract of ubiquinone was analyzed by normal-phase TLC with authentic standard Q-10. Normal-phase TLC was carried out on a Kieselgel 60 F_{254} plate with benzene/acetone (97:3, v/v). Samples were dried and redissolved in ethanol. The purified ubiquinone was further analyzed by HPLC with ethanol as the solvent.

**Prenyl-diphosphate Synthase Assay of Mutant IspB and Product Analysis**—Prenyl-diphosphate synthase activity was measured by the method described previously (8), in which incorporation of [1-{^{14}}C]IPP into reaction products is detected. E. coli DH10B or KO229 cells, harboring plasmids containing mutant *ispB*, were incubated to a late log phase in LB medium containing appropriate antibiotics at 37 °C. Cells were harvested by centrifugation; suspended in buffer A (100 mM potassium phosphate (pH 7.4), 5 mM EDTA, and 1 mM 2-mercaptoethanol); and ruptured by six sonication treatments, each lasting 30 s with 30-s intervals, in an ice bath. After centrifugation of the homogenate, the supernatant was used as crude enzyme extract. The crude extract was loaded onto a column of 100 mM potassium phosphate buffer (pH 7.5), 10 μM [1-{^{14}}C]IPP (specific activity of 0.92 TBq/mol), 5 mM FFP, and 200 μg of crude extract containing the enzyme in a final volume of 0.4 ml. Sample mixtures were incubated for 60 min at 30 °C. Reaction products such as prenyl diphosphates were extracted with 1-butanol-saturated water and hydrolyzed with acid phosphatase (29). The products of hydrolysis were extracted with hexane.
IspB Formation Is Essential for Chain Length Determination

Table I

| Strains or plasmids | Relevant characteristics | Source or Ref. |
|---------------------|--------------------------|----------------|
| Strain              |                          | Source or Ref. |
| E. coli DH10B       | lacZ M15                 | 24             |
| E. coli JM109       | lacZ, lacZ M15           | 24             |
| E. coli KO229       | Cm', Sp', ispB::cat; harbors pKA3 | 11             |
| E. coli KO229:35-2  | Cm', Km', Ap'; harbors pSTVKQKO56 and pG79Y | This study |
| S. cerevisiae YK6   | URA3'; COQ1: URA3;       | 8              |
| Plasmid             |                          |                |
| pBluescript KS(−)   | Ap'; lacZ                 | Stratagen       |
| pBluescript SK(+)   | Ap'; lacZ                 | Stratagen       |
| YEp13M4             | Ap'; LEU 2µm              | 25             |
| pKA3                | Sp'; 3-kb EcoRI fragment including ispB in pCL1920 | 11             |
| pQE31               | Ap'; T5 promoter, His tag, high expression vector | QIAGEN |
| pGEX-1              | Ap'; tac promoter, GST tag, high expression vector | Amersham Pharmacia |
| pSTVK28             | Km'; derived from pSTV28  | Takara Shuzo   |
| pKO56               | Ap'; 2.5-kb SspI-HindIII fragment including ispB in pBluescript | 8              |
| pSA1                | Ap'; 0.3-kb BamHI-EcoRI fragment of 5′-end COQ1 in pBluescript KS(+) | 8              |
| pGKO56              | Ap'; 1.0-kb BamHI-HindIII ispB gene in pQE31 | This study |
| pGKO56              | Ap'; 1.0-kb BamHI-XhoI ispB gene in pGEX-1X | This study |
| pG79Y               | Ap'; 1.0-kb BamHI-XhoI A79Y mutant ispB gene in pGEX-1X | This study |
| pSTVKQKO56          | Km'; 1.0-kb EcoRI-HindIII fragment from pQKO56 in pSTVK28 | This study |

Results

Site-directed Mutagenesis of IspB—It is known that the side chain length of ubiquinone is determined by the corresponding polyprenyl-diphosphate synthase (8), but it is not clear how polyprenyl-diphosphate synthase determines this length. To understand the nature of polyprenyl-diphosphate synthase, we analyzed the activity of octaprenyl-diphosphate synthase (IspB), which produces the side chain of Q-8 in E. coli. For this purpose, we constructed 15 IspB mutants by site-directed mutagenesis as shown in Fig. 1 and Table II (primers that were used). Site-directed mutagenesis was performed following the method of Ito et al. (26), and the substitutions in all mutant ispB genes were confirmed by sequence analysis.

Complementation of E. coli ispB- and S. cerevisiae COQ1-defective Mutants with Mutant ispB Genes—E. coli DH10B was transformed with the plasmids containing mutant ispB genes, and the transformants were used in ubiquinone analysis. Because DH10B harbors the wild-type ispB gene in the form of genomic DNA, the main product is expected to be Q-8. Although most DH10B cells harboring the mutant ispB gene produced Q-8, a number of mutants produced Q-8 with small amounts of Q-6 and Q-7 (data not shown); and interestingly, DH10B harboring the A79Y mutant produced mainly Q-6 (see Fig. 2A). To detect the actual ubiquinone species produced by the product of the mutant ispB gene, E. coli KO229 (ispB::cat)pKA3 (ispB), which is defective for the genomic ispB gene, but retains ispB in a plasmid, was transformed with the plasmids containing the mutant ispB genes. KO229, which harbors the mutant ispB genes and has lost the wild-type ispB plasmid (pKA3), was selected as described under “Experimental Procedures.” L31V, L32V, Y38A, Y37A/Y38A, Y38A/R321V, Y61V, F75A, K235L, R321A, R321D, and R321V mutants KO229 strains were obtained; however, Y37A, A79Y, K170G, and K170A mutant KO229 strains could not be isolated. Since ispB is essential for growth of E. coli (11), the inability to replace wild-type ispB with these mutants suggested that the Y37A, A79Y, K170G, and K170A mutants do not retain functional activity. The mutants that could complement the loss of the wild-type gene were further analyzed by ubiquinone extraction and analysis (Fig. 2). In the Y38A mutant, Q-7 was mainly produced, with lesser amounts of Q-6 and Q-8 (Fig. 2D). In the Y37A/Y38A mutant, Q-7 and Q-6 were mainly produced, with a little Q-8 (Fig. 2E). In the Y38A/R321V and R321V mutants, Q-6 was mainly produced, with a small amount of Q-5 and Q-7 (Fig. 2F, P and L, respectively); however, hardly any Q-8 was produced. In the K235L and R321A mutants, Q-8 was mainly produced; however, a minor product (Q-7) was produced at a level that was greater than that with wild-type IspB (Fig. 2, I and J, respectively). These results indicate that Tyr-38, Lys-

![Image](317x231 to 546x493)
235, and Arg-321 are involved in chain length determination.

Although ispB in E. coli is essential for growth, the chromosomal COQ1 gene (homolog of ispB) in S. cerevisiae can be deleted to produce a respiration-deficient phenotype. We took advantage of this COQ1 mutant phenotype for analysis of the function of ispB (8). To express ispB mutants and to analyze their ubiquinone production in YKK6 (COQ1::URA3), mutant ispB genes fused with 53 amino acids of the Coq1 mitochondrial import signal were constructed. YKK6 was transformed with various mutant ispB fusion plasmids, and transformants were replicated on Synthetic Complete–Leu–Ura plates containing glucose (Fig. 3A) or glycerol (Fig. 3B) as a non-fermentable carbon source. Although most of the strains grew on the glycerol plate, the Y37A, A79Y, K170G, and K170A mutant YKK6 strains did not grow, indicating that these mutants do not retain functional activity. These results are consistent with the complementation analysis of mutants in E. coli KO229 (Fig. 2).

We next analyzed the ubiquinone species produced by YKK6 harboring mutant ispB plasmids (Fig. 4). In the Y38A mutant, Q-8 was mainly produced, along with a significant amount of Q-7 (Fig. 4C). In the Y37A/Y38A mutant, Q-8 and Q-7 were mainly produced, with a lesser amount of Q-6 and Q-5 (Fig. 4D). In the R321V mutant, three ubiquinone species (Q-7, Q-6, and Q-5) were mainly produced, with a small amount of Q-8 (Fig. 4I). In the Y38A/R321V mutant, Q-5 was the main product, with lesser amounts of Q-6 to Q-8 (Fig. 4E). These results again suggest that Tyr-38 and Arg-321 are associated with chain length determination. Most species of ubiquinone produced by mutant ispB in E. coli were similar to ones produced in S. cerevisiae, but some ubiquinone species were shorter in length in S. cerevisiae compared with the ones produced in E. coli.

Arg-321 Is Important for Thermostability of IspB—We tested all ispB mutants for temperature sensitivity and found two temperature-sensitive mutants. The KO229 strain harboring the Arg-321 mutant (R321A or R321D) grew on LB medium containing chloramphenicol and ampicillin at 30 °C (Fig. 5A, a), but stopped growing or grew only slowly at 43 °C (Fig. 5A, b), whereas KO229/pKA3 grew normally at 43 °C. The growth curves of the mutants are shown in Fig. 5B. The R321A, R321D, and R321V mutant KO229 strains grew normally at 30 °C; but the R321A and R321D mutant KO229 strains did not grow at all at 43 °C, and the R321V mutant KO229 strain grew slowly and reached a plateau phase faster than KO229/pKA3 at

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**TABLE II**

| Mutant name | Primer sequence |
|-------------|-----------------|
| L31V        | 5'-CTGATTGATCCACTTGGACGT-3' |
| I32V        | 5'-ACTGATTGACCGCTGGACGGA-3' |
| Y37A        | 5'-TGACGATGTAAGCCCTAACTGA-3' |
| Y38A        | 5'-GCTGACGATCGCAGCGCCTAA-3' |
| Y37A/Y38A   | 5'-CCGCGATGCACTGGCCTAACTGA-3' |
| Y38A/R321V  | Combination of Y38A and R321V |
| Y61V        | 5'-CATTCCCTCGACGCACAGT-3' |
| F75A        | 5'-CGTTGGATAGCGCCTA-3' |
| A79Y        | 5'-CTGAGAAGCTATACGCT-3' |
| K170A       | 5'-ACGCCCGGGCTTGGGATC-3' |
| K170G       | 5'-ACGCCCGGGCTTAATC-3' |
| K235L       | 5'-CAGCGCGGGCTACAGT-3' |
| R321A       | 5'-TTAAAGATCGCTTGAAACAGG-3' |
| R321D       | 5'-TTAAAGATCGCTTGAAACAGG-3' |
| R321V       | 5'-TTAAAGATCGCTTGAAACAGG-3' |
| MUT         | 5'-AGTGGAACCTCGCGGACACGAG-3' |
| T3          | 5'-AATACGACTCACTATAG-3' |
| T7          | 5'-ATTAACCTCCTCAAAAG-3' |
| S2          | 5'-TGCAATTCTATAGTTTAAGAAAAT-3' |
| A3          | 5'-GAAATGCTGGGCGACG-3' |
| QEGST       | 5'-TCGGATCCGATGAAATTTAGAAAAAC-3' |
FIG. 3. Complementation of respiratory deficiency in the \textit{S. cerevisiae COQ1} disruptant strain by various mutant \textit{ispB} genes. The YKK6 (\textit{COQ1::URA3}) strains harboring each of the following plasmids were tested: I, YEp13M4 vector; II, IspB, III, R321V; IV, K170A; V, R321A; VI, R321D, VII, L31V, VIII, I32V, IX, Y38A; X, R235L; XI, Y788; XII, Y61V; XIII, A79Y; XIV, Y37A/Y38A; XV, Y38A/R321V; XVI, Y38A/R321V, K170G. These strains were grown on Synthetic Complete-Leu-Ura medium with glucose (A) or glycerol (B) at 30 °C for 6 days.

43 °C. These results indicate that Arg-321 is important for thermostability of IspB, as well as reconfirm that \textit{ispB} is essential for the growth of \textit{E. coli}.

Overexpression, Purification, and Characterization of IspB—As mentioned above, we constructed the 15 IspB mutants by site-directed mutagenesis and expressed them in \textit{E. coli} and \textit{S. cerevisiae}. Among these mutants, the A79Y mutant had an interesting property in that DH10B harboring the A79Y mutant produced Q-6 (Fig. 6A), whereas the A79Y mutant in \textit{S. cerevisiae} YKK6 (\textit{COQ1::URA3}) did not retain functional activity (Fig. 3). To further analyze the A79Y mutant, we purified the wild-type and A79Y mutant IspB proteins. JM109 harboring pKO56 produced His-IspB fusion proteins with a 6-His tag at the amino terminus of wild-type IspB. JM109 harboring pKO56 and pG79Y produced GST-IspB and GST-IspB(A79Y) fusion proteins, respectively, with GST at the amino terminus. His-IspB, GST-IspB, and GST-IspB(A79Y) were purified, and prenyltransferase activity was examined as described under “Experimental Procedures.” Purified His-IspB and GST-IspB retained functional activity of octaprenyl-diphosphate synthase, but GST-IspB(A79Y) had no detectable activity (data not shown). This result suggests that the substitution of tyrosine for alanine at position 79 abolishes the enzyme activity of IspB, which is consistent with the results of A79Y mutant activity in YKK6 (Fig. 3).

Wild-type IspB and IspB(A79Y) Form a Dimer Structure—We next tested, by Western blot analysis, whether wild-type IspB and IspB(A79Y) form dimers. Purified His-IspB and His-DdsA (as a negative control) were incubated with GST-IspB(A79Y) in buffer A and then subjected to Ni²⁺-NTA (Fig. 6C, lanes 2) or glutathione-Sepharose 4B (lane 5) column chromatography. The His-IspB protein was detected together with GST-IspB(A79Y) from purified products on the glutathione-Sepharose 4B column (Fig. 6C, lanes 5), but the His-DdsA protein was not detected in the GST-purified products (Fig. 6C, panel a, lane 6). Conversely, GST-IspB(A79Y) was detected with His-IspB from purified proteins separated on the Ni²⁺-NTA column (Fig. 6C, panel b, lane 2). Homodimerization of wild-type IspB and the A79Y mutant itself was also observed. When purified His-IspB was incubated with GST-IspB(A79Y) in buffer A with disuccinimidyl suberate as a protein cross-linker and subjected to electrophoresis, the band corresponding to a heterodimer with a molecular mass of 97 kDa was detected (data not shown). These results indicate that wild-type IspB and IspB(A79Y) can form a heterodimer, but these proteins cannot form dimers with the similarly structured enzyme DdsA (18). We analyzed the chain lengths of products of heterodimeric IspB consisting of wild-type and A79Y subunits in vitro. Only octaprenyl diphasphate was detected in this assay. We believe that because the homodimer of His-IspB comprises the majority of active enzyme in vitro, we could not detect the activity of the heterodimer composed of IspB and IspB(A79Y).

We then made a strain expressing both His-IspB and GST-IspB(A79Y). We constructed the pSTVKQKO56 plasmid, which expresses His-IspB, and co-transformed it with the pG79Y plasmid, which expresses GST-IspB(A79Y); and the resulting strain was named KO229/35-2. This strain produced Q-6 and Q-8 (Fig. 6B). Crude proteins were extracted from KO229/35-2...
Fig. 5. Growth of E. coli KO229 harboring Arg-321 mutant IspB. A, KO229 harboring pBRA (a) or pKA3 (b) was grown on LB plates containing chloramphenicol and ampicillin at 30 or 43 °C. B, different KO229 strains harboring each plasmid were grown on LB liquid medium containing chloramphenicol and ampicillin at 30 or 43 °C. □, pKA3; ○, pBV16 (R321V); △, pBRA (R321A); ▲, pBRD (R321D).

Fig. 6. Ubiquinone analysis and dimer formation of wild-type IspB and IspB(A79Y). A and B, shown are the results from the analysis of ubiquinones produced in DH10B harboring the A79Y mutant ispB gene and in KO229 harboring pSTVKQKO56 and pG79Y, respectively, by HPLC. C, crude proteins were extracted from KO229 harboring pSTVKQKO56 and pG79Y (KO229/35-2 strain) and incubated in buffer A containing 1.0 mM MgCl₂ and 0.1% (w/v) Triton X-100 at 30 °C for 1 h and then purified on a Ni²⁺-NTA column (lanes 1) or a glutathione-Sepharose 4B column (lanes 4). Purified His-IspB and GST-IspB(A79Y) proteins were used in incubations as described above and purified on a Ni²⁺-NTA column (lanes 2) or a glutathione-Sepharose 4B column (lanes 5). Purified His-DdsA and GST-IspB(A79Y) proteins were used for the same experiment and purified on a glutathione-Sepharose 4B column (lanes 6). Purified His-IspB (lanes 3) and GST-IspB(A79Y) (lanes 7) proteins were loaded as controls. Western blot analysis was performed using anti-6-His antibody (panel a) or anti-GST antibody (panel b). Arrowheads indicate the positions of His-IspB (panel a) or GST-IspB(A79Y) (panel b) protein. The asterisk indicates background protein recognized by the antibody.
IspB(A79Y) itself does not retain enzyme activity. With the isoprenoid product of homodimeric IspB, although erodimeric IspB to produce shorter isoprenoid chains compared IspB(A79Y) mutant can function as a component of het-

terodimeric IspB to produce shorter isoprenoid chains compared with the isoprenoid product of homodimeric IspB, although IspB(A79Y) itself does not retain enzyme activity.

DISCUSSION

In E. coli, the side chain length of Q-8 is determined by octaprenyl-diphosphate synthase (IspB) (8). To discover how IspB determines the chain length, we constructed 15 IspB mutants and expressed them in the E. coli ispB-defective mut-

tant KO229 and in the S. cerevisiae COQ1-defective mutant YKK6 and then analyzed the ubiquinone species produced. In KO229 or YKK6 expressing the IspB mutants L31V, I32V, Y61V, and F75A, the resulting ubiquinone species were almost the same as those produced by the wild-type enzyme (Figs. 2 and 4). However, in KO229 cells expressing the IspB mutant Y38A, Q-7 was the major product; and in KO229 cells expressing the Y37A/Y38A double mutant, the levels of Q-6 were increased. Although the Tyr-37 mutant of IspB did not have enzyme activity, an additional mutation at Tyr-38 restored activity. We speculate that the abnormal protein structure in the Tyr-37 mutant is compensated by an additional Tyr-38 mutation. In E. coli KO229/pKA3 cells, complementation of the lost ispB gene that resided on a plasmid with the Y37A, A79Y, K170G, and K170A mutant ispB genes was unsuccessful. Fur-

thermore, S. cerevisiae YKK6 transformed with the same genes could not grow on Synthetic Complete—Leu—Ura containing glycerol (Fig. 3). These results indicate that Tyr-37, Ala-79, and Lys-170 are important residues for activity. The pattern of ubiquinone species synthesized in E. coli or S. cerevisiae harboring the same mutant IspB was some different. This difference might be due to the difference in the intracellular conditions of two organisms because it was reported that metal ions and substrate concentration affected the produced chain length catalyzed by prenyl-diphosphate synthases (30, 31). We intentionally mutated aromatic residues located before the first as-

tPARTATE-rich motif because tyrosine or phenylalanine is important for chain length determination in FPP and GGPP synthases (30, 31). We intentionally mutated aromatic residues located before the first as-

cysteine region was different between FPP synthases and long-

chain polyprenyl-diphosphate synthases. It has been reported that the corresponding amino acid in mouse and human GGPP synthases is glutamate (15). Amino acid alignment of GGPP synthases showed that the length of the carboxyl terminus region was different between FPP synthases and long-chain polyprenyl-diphosphate synthases. It has been reported that an FPP synthase mutant from B. stearothermophilus in which the arginine was replaced by valine had catalytic activity similar to that of the wild-type enzyme, indicating that the amino acids in the carboxyl terminus are not essential for catalytic function in FPP synthase (33). These results suggest that the role of the carboxyl terminus in FPP and GGPP synthases is different from that in long-chain polyprenyl-diphos-
IspB Formation Is Essential for Chain Length Determination

IspB is an essential gene in S. cerevisiae (37), Synechocystis sp. strain PCC6803 (11), R. capsulatus (1343) and S. pombe (2). Because these synthases, except for the one from S. pombe, could be expressed in E. coli and S. cerevisiae, we believe that they form homodimeric enzymes like IspB. However, because the enzyme from the eukaryote was not able to express a functional product in the other species, eukaryotic long-chain polyprenyl-diphosphate synthase might be regulated differently. Therefore, the long-chain polyprenyl-diphosphate synthase of eukaryotic origin will be an interesting subject for further analysis.

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