Title

Heteromer formation of a long-chain prenyl diphosphate synthase from fission yeast Dps1 and budding yeast Coq1

Author(s)

Mei Zhang, Jun Luo, Yuki Ogiyama, Ryoichi Saiki, Makoto Kawamukai

Journal

The FEBS journal　275(14):3653-68.

Published

03 July 2008

URL

https://doi.org/10.1111/j.1742-4658.2008.06510.x

Heteromer formation of a long-chain prenyl diphosphate synthase from fission yeast Dps1 and budding yeast Coq1

Mei Zhang, Jun Luo, Yuki Ogiyama, Ryoichi Saiki, Makoto Kawamukai*

Department of Applied Bioscience and Biotechnology, Faculty of Life and Environmental Science, Shimane University, Japan

***Correspondence**:

Makoto Kawamukai, Faculty of Life and Environmental Science, Shimane University, 1060

Nishikawatsu, Matsue 690-8504, Japan

Fax: +81 852 32 6092

Tel: +81 852 32 6587

E-mail: kawamuka@life.shimane-u.ac.jp

Running title: Heteromer formation of Dps1 and Coq1

Key words:

isoprenoid, ubiquinone, coenzyme Q, polyprenyl diphosphate synthase

Abbreviations: Dlp1, D-less polyprenyl diphosphate synthase; DMAPP, dimethylallyl pyrophosphate; DOH, decaprenol; Dps1, decaprenyl diphosphate synthase; FPP, farnesyl diphosphate; GGOH, geranylgeraniol; GGPP, geranylgeranyl diphosphate; HexOH, hexaprenol; IPP, isopentenyl diphosphate; OOH, octaprenol; PDS, prenyl diphosphate synthase; PHB, p-hydroxybenzoate; UQ, ubiquinone.

Subdivision: Gene expression, Enzymology

Summary

Ubiquinone (UQ) is an essential factor for the electron transfer system and is also a known lipid antioxidant. The length of the UQ isoprenoid side chain differs among living organisms, with 6 units in the budding yeast *Saccharomyces cerevisiae*, 8 units in *Escherichia coli*, and 10 units in the fission yeast *Schizosaccharomyces pombe* and in humans. The length of the UQ isoprenoid is determined by the product generated by polyprenyl diphosphate synthases (PDSs), which are classified into homodimer (i.e., octa-PDS IspB in *E. coli*) and heterotetramer types (i.e., deca-PDSs Dps1 and Dlp1 in *S. pombe* and in humans). Here we characterized the hexa-PDS Coq1 of *S. cerevisiae* to identify whether this enzyme was a homodimer (as in bacteria) or a heteromer (as in fission yeast). When *COQ1* was expressed in an *E. coli ispB* disruptant, only hexa-PDS activity and UQ-6 were detected, indicating that expression of Coq1 alone results in bacterial enzyme–like functionality. However, when expressed in fission yeast Δ*dps1* or Δ*dlp1* strains, *COQ1* restored growth on minimal medium in the Δ*dlp1* but not in the Δ*dps1* strain. Intriguingly, UQ-9 and UQ-10, but not UQ-6, were identified and deca-PDS activity was detected in the *COQ1*-expressing Δ*dlp1* strain. No enzymatic activity or UQ was detected in the *COQ1*-expressing Δ*dps1* strain. These results indicated that Coq1 partners with Dps1 but not with Dlp1 to be functional in fission yeast. Binding of Coq1 and Dps1 was demonstrated by co-immunoprecipitation, and the formation of tetramer consisting of Coq1 and Dps1 was detected in *S. pombe*. Thus, Coq1 is functional when expressed alone in *E. coli* and in budding yeast, but is only functional as a partner with Dps1 in fission yeast. This unusual observation indicates that different folding processes or protein modifications in budding yeast/*E. coli* versus those in fission yeast might affect the formation of an active enzyme. These results provide important insights into the process of how PDSs evolved from homo- to hetero-types.

Introduction

Ubiquinone (UQ, Coenzyme Q, or CoQ) is a natural compound present in almost all living organisms that primarily localizes to the plasma membrane (in prokaryotes) or the mitochondrial inner membrane (in eukaryotes). Ubiquinone is an essential component of aerobic growth and oxidative phosphorylation in the electron transport system [1]. Recent studies have suggested additional functions for this compound, such as in antioxidation [2, 3], disulfide formation in *Escherichia coli* [4], sulfide oxidation in fission yeast [5, 6], life span elongation in *Caenorhabditis elegans* [7], and pyrimidine metabolism in humans [8]. Because of its biochemical properties and ever-expanding known functions, UQ has become a compound of substantial interest to the research community. In particular, research has focused on the role of human-type UQ (UQ-10) in cardiovascular disease, and its use in clinical therapies and nutrition [9].

Ubiquinone is composed of a benzoquinone moiety and an isoprenoid side chain of varying lengths. Although the UQ biosynthetic pathway in *E. coli* has been almost entirely determined, such is not the case in eukaryotes [10, 11]. In *E. coli*, generation of the isoprenoid side chain is catalyzed by poly-prenyl diphosphate synthase (poly-PDS). The isoprenoid side chain is then condensed to *p*-hydroxybenzoate (PHB) by PHB-polyprenyl diphosphate transferase. A series of modification reactions of the benzoquinone ring, including methylations, decarboxylation, and hydroxylations, complete the processing of UQ. It is thought that eight enzymes are involved in UQ biosynthesis. All the eukaryotic UQ biosynthetic genes are thought to be similar to those found in *S. cerevisiae*, with the exception of those involved in isoprenoid side chain synthesis [10].

The side chain length of UQ is unique to the species of origin. For instance, *S. cerevisiae* has 6 units in its side chain, *Candida utilis* has 7 units, *E. coli* has 8 units, mice and *Arabidopsis thaliana* have 9 units, and *S. pombe* and humans have 10 units [10, 12-14]. The isoprenoid side chain length of UQ is defined by the product generated by poly-PDSs [15-17], but not by the substrate specificity of PHB-polyprenyl diphosphate transferases [13, 15]. We previously reported that the UQ side chain lengths could be altered by genetic engineering. *E. coli* ordinarily produce UQ-8, but exogenous expression of heptaprenyl, solanesyl, or decaprendyl diphosphate synthase genes from *Haemophilus influenzae*, *Rhodobacter capsulatus,* or *Gluconobacter suboxydans*, respectively, results in production of UQ-7,

UQ-9, or UQ-10, respectively [18-21]. Similarly, an *S. cerevisiae COQ1* disruptant that expresses various poly-PDS genes from different organisms can generate the provider-type UQs UQ-5, UQ-6, UQ-7, UQ-8, UQ-9, or UQ-10 [17]. Furthermore, when genetic engineering is used to enable deca-PDS production by rice mitochondria, the rice produced UQ-10 instead of the originally-synthesized UQ-9 [22].

Trans-type poly-PDSs can be categorized as short-chain (C10-C25) or long-chain (C30-C50) types according to the length of the produced isoprenoid chain. Short-chain poly-PDSs such as FPS and GGPS catalyze the initial condensation of IPP to DMAPP, and long-chain poly-PDSs catalyze the further condensation of IPP to FPP or GGPP to generate products longer than hexaprenyl diphosphate [23]. Amino acid sequence analyses have shown that seven conserved regions and two aspartate-rich motifs DDxxD are found in all trans-type poly-PDSs [24]. The first DDxxD motif is responsible for binding with FPP, and the second is responsible for IPP binding. The mechanisms of the short-chain poly-PDS proteins have been well characterized and the proteins have been solved with three-dimensional crystal structures [25, 26]. However, despite ongoing studies on the long-chain synthases and their solved three-dimensional crystal structures [27, 28], analysis of the heteromeric type of these long-chain compounds remains limited.

Long chain PDSs have been so far characterized in *E. coli* [29], *G. suboxydans* [20], *Agrobacterium tumefaciens* [30], *R. capsulatus* [19], *Rhodobacter sphaeroides* [31], *Micrococcus luteus* [32], *Sulfolobus solfataricus* [28], *Bacillus subtilis* [33], *Bacillus stearotherrmophilus* [34], *Mycobacterium tuberculosis* [35], *Trypanosoma cruzi* [36], *Plasmodium falciparum* [37], *S. cerevisiae* [38], *S. pombe* [3, 39], *A. thaliana* [40, 41], *Mus musculus* [14], and *Homo sapiens* [14]. The characterized enzymes are not always responsible for UQ synthesis; for instance, in *Bacillus* they mediate menaquinone synthesis. Long chain PDSs can be classified into homodimer- (i.e., IspB in *E. coli*), heterodimer- (i.e., GerC1 and GerC3 in *B. subtilis*), and heterotetramer- (i.e. Dps1 and Dlp1 in *S. pombe*) types based on the pattern of components. Solanesyl and deca-PDSs from mice and humans were established to be heterotetramer-types [14]. In any case, the primary structures of the core components of the heteromer-type enzymes are very similar to monomeric enzymes. These results raise the question of why heteromer-type enzymes have evolved in some species, including mice and humans.

In the present work, we characterized a *S. cerevisiae* PDS Coq1 in *E. coli* and *S. pombe*. We showed that in *E. coli,* Coq1 operates by itself as a hexa-PDS as it does in *S. cerevisiae*. To our surprise, Coq1 could not work alone in *S. pombe*; it formed a heteromer with *S. pombe* Dps1, which resulted in deca-PDS activity. A heterotetrameic enzyme was generated between Coq1 and Dps1 of different species/origins in *S. pombe*. This unexpected result provides an important insight into understanding the process by which long chain trans-PDSs have evolved from homo- to hetero-types.

Results

Isolation of *COQ1* cDNA

The *COQ1* gene encoding a hexa-PDS consists of 473 amino acids [38]. Similar to several other long-chain PDSs, such as *E. coli* IspB (octa-PDS) and *S. pombe* Dps1 (a component of deca-PDS) [39, 42], Coq1 also contains 7 highly-conserved regions through trans-PDSs, including the first aspartate-rich motif (FARM) and the second aspartate-rich motif (SARM), which are regarded as the substrate binding domains. However, unlike other PDSs, Coq1 has extended sequences between domains I and II and between domains IV and V (Fig. 1A). This unusual structure of Coq1 prompted us to check for the presence of introns in *COQ1*. We extracted RNAs from *S. cerevisiae* strain W3031A, and mRNAs were used as a template for RT-PCR to obtain a first-strand cDNA (Fig. 1B). The cDNA of *COQ1* was cloned into a pT7-Blue vector and then re-cloned into pBluescript II SK(+), yielding pBSSK-COQ1. We sequenced the *COQ1* cDNA with M13 and reverse primers. This cDNA obtained from *S. cerevisiae* mRNA completely matched with the genomic *COQ1*. Thus, despite its redundant sequence of *COQ1*, the genomic *COQ1* did not contain any introns. This *COQ1* cDNA was used in the following experiments.

Complementation by *COQ1* in an *E. coli ispB* mutant

To examine whether *COQ1* could complement a mutant defective in its homologous genes in *E. coli*, *COQ1* was expressed in an *E. coli ispB* disruptant (KO229). Because *ispB* is essential for growth in *E. coli* [18], KO229 harboring pKA3 was used to swap pKA3 with pBSSK-COQ1. KO229 harboring both pKA3 and pBSSK-COQ1 was grown for a few days in LB medium containing ampicillin; this allowed

us to obtain KO229 that harbored only pBSSK-COQ1 by selecting ampicillin-resistant but spectinomycin-sensitive strains. The ubiquinone species of the strains thus obtained were analyzed by HPLC (Fig. 2).

Wild-type *E. coli* synthesized only UQ-8 by endogenous IspB (Fig. 2B), and *E. coli* harboring pBSSK-COQ1 synthesized both UQ-6 and UQ-8 (Fig. 2C). However, the *E. coli ispB* disruptant KO229 harboring pBSSK-COQ1 produced only UQ-6 (Fig. 2D). Because the *ispB* gene is essential for *E. coli* growth and is responsible for the side chain length determination of UQ species [18], these results clearly indicated that alone, the Coq1 protein is active in *E. coli* and has hexa-PDS activity (see also Fig. 7).

Complementation of a fission yeast *dlp1* or *dps1* disruptant by *COQ1*

For UQ biosynthesis in *S. pombe*, deca-PDS is composed of a heterotetramer of Dps1 and Dlp1. Disruption of either of these two genes causes a severe growth delay on a minimum medium, a cysteine requirement for growth on the minimum medium, a sensitivity to hydrogen peroxide, and the generation of hydrogen sulfide [43]. These phenotypes can be recovered by introducing a complementary gene such as *ddsA* from *G. suboxydans* encoding deca-PDS on a plasmid [39].

To test the complementation ability of *S. cerevisiae COQ1* in fission yeast, *COQ1* expression in a fission yeast UQ-deficient strain was performed. We first constructed the plasmid pREP1-TP45-COQ1, in which a mitochondrial targeting signal sequence (TP45) from *S. pombe* Ppt1 [43] was added to the N-terminus of Coq1. This plasmid was introduced into RS312 (Δ*dlp1*) and KS10 (Δ*dps1*). Unexpectedly, the growth of the Δ*dlp1* strain, but not the Δ*dps1* strain, on minimal medium was rescued, and the growth of the *COQ1*-expressing Δ*dlp1* transformant was nearly the same as that of wild-type yeast (Fig. 3, A and B). Ubiquinone was extracted from the Δ*dlp1* strain harboring pREP1-TP45-COQ1 and was analyzed by HPLC. To our surprise, UQ-9 and UQ-10, but not UQ-6, were detected in the *COQ1*-expressing Δ*dlp1* strain (Fig. 4C). UQ-9 was produced to a much greater extent than UQ-10, with the ratio of UQ-9 to UQ-10 produced of ~1.2 to 1. The reason why UQ-9 was produced to a larger extent will be discussed later in this work. To investigate the functionality of *COQ1* in *S. pombe*, pREP1-TP45-COQ1 expressing *COQ1* was introduced into LA1 (Δ*dlp1*, Δ*dps1*), but the transformant did not grow

well on minimal medium (Fig. 3C). From these results, we conclude that Coq1 in fission yeast cannot work alone to synthesize UQ-9 and UQ-10, but may form a heteromer with the Dps1 enzyme that retains its decaprenyl diphosphate enzyme activity (see Fig 7).

S. cerevisiae Coq1 formed a heteromer with _S. pombe_ Dps1 in fission yeast

Complementation of a fission yeast *dlp1* disruptant by *COQ1* indicated that Coq1 might form a heteromer with Dps1 in fission yeast. To test for such an interaction, we co-expressed *COQ1* and *dps1* in LA1 (Δ*dlp1*, Δ*dps1*). The constructed plasmids pDS473-COQ1 and pHA-dps1, expressing fusion proteins of GST-Coq1 and HADPS1, respectively, were introduced into LA1. Consistent with the above data, the LA1 strain that harbored pDS473-COQ1 and pHADPS1 had restored growth on PM minimal medium. LA1 harboring pDS473-COQ1 and pHADPS1 produced UQ-10 as its major product (87.8% of total) along with UQ-9 (12.2% of total) (Fig. 4E). UQ-10 and UQ-9 productivity and its ratio were nearly the same as those of wild type PR110, for which UQ-10 and UQ-9 made up 92.7% and 7.3% of the total product, respectively (Fig. 4J). We observed a measurable difference of the UQ-10 and UQ-9 ratio between this data and that of the *dlp1* deletion strain expressing *COQ1* alone (Fig. 4C). This was likely due to the different expression levels of the *dps1* and *COQ1* genes. In Fig. 4E, both *COQ1* and *dps1* were expressed on the plasmids; however, *dps1* was endogenous in Fig. 4C, so that the expression level of *dps1* is lower than that of *COQ1*, thereby affecting the ratio of UQ-9 and UQ-10. Additionally, a mitochondrial import signal sequence from *S. pombe ppt1*, TP45, was added to the N-terminus of Coq1 for its expression in Fig. 4E; the altered production ratio might also be influenced by the localization of the proteins.

To determine whether human *dps1* could be functional with *COQ1*, human *dps1* was co-expressed with *COQ1* in LA1. No UQ was detected in the transformant (Fig. 4H). Although the human Dps1 protein (hDps1) had a high identity with *S. pombe* Dps1 (44.0%), hDps1 did not form a functional complex with Coq1 in *S. pombe*. We also confirmed that hDps1 and hDlp1 functionally complemented the LA1 strain, almost exclusively producing UQ-10 (Fig. 4I); this indicates that human deca-PDS could be reconstituted in *S. pombe*.

To demonstrate the interaction of Coq1 and Dps1, co-immunoprecipitation was performed in the LA1

strain harboring both pDS473-COQ1 and pHADPS1. Proteins from this strain were purified by Glutathione Sepharose 4B, and the eluted sample was subjected to Western blot analysis. If Coq1 binds with Dps1, GST purification would cause the HA-tagged Dps1 fusion protein to be pulled down as a complex with GST-tagged Coq1. Thus, GST-Coq1 and HA-Dps1 could be detected by the HA antibody or by GST. The fission yeast strain LA1 harboring GST-Dlp1 and HA-Dps1 was used as a positive control for the GST pull-down assay. Both Coq1 and Dps1 were clearly observed in the pulled-down sample, strongly suggesting the formation of a Coq1-Dps1 complex (Fig. 5A, lane 3). Formation of Dps1 and Dlp1 was observed as a positive control under the same conditions (Fig. 5A, lane 1). Conversely, in LA1 harboring GST-Coq1 and HA-Dlp1, Coq1 and Dlp1 did not form a complex in fission yeast (Fig. 5A, lane 4), consistent with the result of the genetic complementation experiments (Fig. 3).

Coq1 cannot bind with Dps1 as a functional enzyme in *E. coli.*

As was shown previously in reconstituted *E. coli*, a cooperative partnership exists between *S. pombe* Dps1 and Dlp1 [39]. To examine if Coq1 and Dps1 interact with each other for deca-PDS activity, co-expression of Coq1 and Dps1 in *E. coli* was carried out. Plasmids pGEX-COQ1 and pSTV28-HIS-dps1 were prepared and introduced into KO229 (*ispB::cat*) to create a strain that expressed both Coq1 and Dps1 without endogenous IspB. The UQ species of the strain was investigated, and we found that introducing *COQ1* and *dps1* did not result in the generation of UQ-10. Instead, the strain generated mostly UQ-6, similar to the expression of *COQ1* by itself (Fig. 2). This indicates that Coq1 executes its original functions even in the presence of Dps1 in *E. coli*.

To determine whether Coq1 interacts with Dps1 in *E. coli*, GST-fused Coq1 was purified from an *E. coli* KO229 strain expressing GST-Coq1 and HIS-Dps1 (as described above), followed by antibody detection. In the crude enzyme extracts, GST-Coq1 and HIS-Dps1 were reasonably detected by antibodies (Fig. 5B, lane 3). However, in the samples purified by GST pull-down, only the GST-fused Coq1 protein was detected (Fig. 5B, lane 4). This indicated that the His-tagged Dps1 protein did not complex with Coq1, and that in *E. coli,* Coq1 and Dps1 did not bind to each other to form a functional enzyme for deca-PDS.

S. pombe Dps1 cannot interact with Coq1 in *S. cerevisiae*

As shown above, we found that a heteromer of Coq1 and Dps1 formed in fission yeast to synthesize UQ-10 and UQ-9, but that this situation did not occur in *E. coli*. We next asked what result would be obtained if both Coq1 and Dps1 were produced in budding yeast. We constructed YEp13M4-COQ1-dps1, a plasmid containing the full length *dps1* gene with a 53-aa Coq1 mitochondrial import signal sequence at N-terminus. This plasmid was used for the expression of *COQ1-dps1* in wild-type budding yeast and in the mutant YKK6 (*COQ1::URA3*). The transformants obtained were grown on Synthetic Complete (SC)-Leu or SC-Leu-Ura medium with glucose, and were used to extract UQ for analysis. In the wild-type strain harboring YEp13M4-COQ1-dps1, UQ-6 was primarily produced (Fig. 6C); the *COQ1* mutant YKK6 that harbored the YEp13M4-COQ1-dps1 plasmid did not synthesize UQ (Fig. 6D). Similar to expression in *E. coli*, Dps1 did not work as a functional component with Coq1in budding yeast to produce UQ-10.

Prenyl diphosphate synthase activity of a Coq1-Dps1 complex

The results above indicated that decaprenyl diphosphate, the precursor of the UQ-10 side chain, was synthesized by expressing the Coq1 and Dps1 proteins in fission yeast. To confirm this, an *in vitro* enzymatic activity assay was carried out. The crude enzyme prepared from LA1 harboring pDS473-COQ1 and pHA-dps1 was reacted with [^{14}C]IPP and FPP as substrates, in order to detect prenyltransferase activity. The product generated in the reaction was hydrolyzed by acid phosphatase and separated by reverse-phase thin-layer chromatography (TLC). As expected, a decaprenol (DOH) was detected in this sample similar to wild-type fission yeast cells (Fig. 7A). Accordingly, Coq1 and Dps1 restored catalytic activity in LA1, supporting the conclusion that the Coq1-Dps1 complex encodes a deca-PDS in fission yeast.

We next examined the enzymatic activity of Coq1 and Dps1 in *E. coli*. As shown in Fig. 7B, wild-type *E. coli* DH5α, DH5α harboring pGEX-COQ1, and an *ispB* disruptant (KO229) harboring pGEX-COQ1 generated octaprenol alone, octaprenol and hexaprenol toegther, and hexaprenol alone, respectively, as their main products. These results support the notion that the Coq1 protein is active in *E.*

coli with hexa-PDS activity, and that *COQ1* could play a functional role in the replacement of the *ispB* gene. Conversely, the product pattern of KO229 that harbored both pGEX-COQ1 and pSTV28-HIS-dps1 was nearly the same as that of KO229 that harbored pGEX-COQ1 alone (Fig. 7B, lanes 3 and 4). This implied that the characteristics of Coq1 were not modified by the additional *dps1* gene. However, it is also important to note that a slight band of DOH, corresponding to deca-PDS, was observed in *E-coli* co-expressed with Coq1 and Dps1 (Fig. 8B, lane 3). It is possible that there may be some significant factors or conditions in *E. coli* that suppress the interaction of Coq1 and Dps1.

Heterotetramer formation of Coq1 and Dps1 in *S. pombe*.

Most of the long chain PDSs that synthesize UQ side chains are thought to be homodimeric enzymes [23], since to date PDS heterotetramers have only been identified in *S. pombe,* mice, and humans [14, 39]. As this study showed that Coq1 and Dps1 interact with each other to form a hetero complex having deca-PDS activity, we predicted that Coq1 and Dps1 form a tetramer rather than a dimer. To verify this, Blue Native-PAGE was used to analyze the size of the Coq1-Dps1 complex.

Crude protein extracted from LA1 cells harboring pDS473-COQ1 and pHADPS1 was purified by Glutathione Sepharose 4B, and crude and purified samples were employed in a Blue Native-PAGE. A single band with a molecule mass of ~210 kDa was detected from the purified COQ1-Dps1 sample under native conditions (Fig. 8, lane 4). This band was identified as a tetramer of Coq1-Dps1, with the molecular mass of GST-Coq1 calculated as 72 kDa and HA-Dps1 as 43 kDa. The Coq1-Dps1 band was seen at the same position in the crude extraction sample of LA1 harboring pDS473-COQ1 and pHADPS1 (Fig. 8, lane 3), while no corresponding band was seen in the protein extraction from LA1 (Fig. 8, lane 2). We can therefore conclude that Coq1-Dps1 forms a 210 kDa complex, consistent with the formation of a tetramer by Coq1 and Dps1 in *S. pombe*.

Discussion

In the present work, we characterized a *S. cerevisiae* hexa-PDS Coq1, which is responsible for the synthesis of the UQ side chain. Long chain poly-PDSs can be classified into homodimer (i.e., octa-PDS IspB in *E. coli* [29]), heterodimer (i.e., hepta-PDS in *B. subtilis* [33]), and heterotetramer (i.e., deca-

PDSs Dps1 and Dlp1 in *S. pombe* [39] and humans [14]) types. The Coq1 amino acid sequence is similar to those of other long-chain PDSs, such as *E. coli* IspB, and other PDS components, such as *S. pombe* Dps1 and human hDPS1, with a sequence similarity of ~38%, 46%, or 38%, respectively. Coq1 contains the seven conserved regions typically observed in trans-PDSs, including the putative substrate binding domains FARM and SARM [27]. Coq1 possesses small-sized amino acid residues (Ala-188 and Ser-189) at the fifth and fourth positions upstream of FARM, similar to *E. coli* IspB and *S. pombe* Dps1; this is an important characteristic of long-chain trans-PDSs. No remarkably distinct characteristics were observed for Coq1, other than its extended sequences between domains I and II, and domains IV and V. These observations led us to anticipate that Coq1 is an ordinary homomeric enzyme, similar to bacterial poly-PDSs; but further analysis revealed some unexpected characteristics for the enzyme.

When expressed in *E. coli*, *COQ1* functioned as a monomeric hexa-PDS for the generation of UQ-6. *COQ1* was able to functionally replace an essential *ispB* gene in *E. coli*. However, when expressed in fission yeast, *COQ1* was not functional by itself, but formed a heterotetromer with Dps1 to make deca-PDS for UQ-10 generation. Coq1 retained its hexa-PDS activity in *E. coli*, but this was not reproduced in fission yeast, where it partnered with Dps1 but not Dlp1 for generating deca-PDS. Coq1 did not complex with Dps1 in *E. coli* or in *S. cerevisiae*. These results were unexpected; we thought that this unexpected behavior of Coq1 might give us insight into why heteromeic PDSs are prevalent in nature, especially in higher animals.

Exogenous expression of PDSs is generally successful, as our group and others have shown. Monomeric long-chain PDSs from *G. suboxydans* [20], *A. tumefaciens* [30], *R. capsulatus* [19], *R. sphaeroides* [31], *M. tuberculosis* [35], *T. cruzi* [36], and *A. thaliana* [40, 41] can be functionally expressed in *E. coli* and some cases in *S. cerevisiae*. The expression of heteromeric enzymes from *B. subtilis*, *B. stearothermophilus* [33], *S. pombe* [3, 39], mice [14], and humans [14] are also successful in *E. coli* and some cases in *S. pombe* [6,24, this study]. It has also been reported that one component of *B. subtilis* and *B. stearothermophilus* PDSs is interchangeable [34]; similarly, *S. pombe* Dps1 is interchangeable with human hDPS1 to make an active enzyme [14]. Thus, heterologous expression of two PDS components in a non-host species sometimes allows the PDSs to become functional.

Currently, we do not have any solid explanation for why Coq1 behaves differently when expressed in

S. pombe, but we can propose several possibilities. First, it may be that different protein folding processes in budding yeast, *E. coli*, and fission yeast might affect active enzyme formation in some species but not in others. Coq1 folding might be slow in *S. pombe*, but the presence of a partner might support the folding of the Dps1-Coq1 complex. This possibility is likely, given that when *COQ1* and *dps1* were co-expressed in *E. coli*, we detected slight but measurable decaprenyl diphosphate synthesis in addition to hexaprenyl diphosphate synthesis. This indicates that some fraction of the protein forms a Coq1-Dps1 complex in *E. coli*, but that the intracellular conditions in *E. coli* are not as favorable as they are in *S. pombe*. Second, modification of the protein(s) (such as phosphorylation) might need to occur for complex formation. This modification might differ in *S. pombe* compared to that in *E. coli* or *S. cerevisiae*. This possibility is not as likely, since *S. pombe* Dps1 and Dlp1 can be functionally expressed in *E. coli* where no modifications are expected. Third, some other factor, perhaps a chaperon or some other protein, might be necessary for forming the functional enzyme. This possibility is also logical, because formation of a Coq1-Dps1 complex is not clear in *E. coli*. *S. pombe* may have other factors to allow formation of a functional complex. As it was previously reported that Coq1 mediates the formation of large complexes of UQ biosynthetic enzymes in *S. cerevisiae* [44], the role of other potential components needs to be considered. Fourth, it is possible that the interaction between Coq1 and Dps1 in fission yeast occurs by accident. We do not support this idea. *S. pombe* Dps1 requires a partner for formation of a functional complex, and the properties of the complex enzyme depend on Dps1, since the complex possesses deca-PDS but not hexaprenyl diphosphate activity. All heteromeric enzymes so far identified are non-functional alone, despite their similarity to monomeric PDSs. Obtaining a functional enzyme from mutation(s) of a non-functional component might give us insights into understanding the differences between homomeric and heteromeric enzymes.

The product chain length was first thought to be determined by certain amino acids of the PDS. However, as was shown by our group and others [14, 29, 45], the subunit structure is also very important for chain length determination. A mutated and therefore functionally-inactive octa-PDS molecule can form an active enzyme with different product specificity when the mutant is paired with the wild-type enzyme [29]. There is also evidence that heteromeric formation of a PDS changes the final product. The small subunit of geranyl diphosphate synthase from *Mentha* modifies the chain length

specificity of geranylgeranyl diphosphate synthase to produce geranyl diphosphate [46]. In this case, the geranyl diphosphate synthase subunit is not homologous to typical PDSs. In addition, formation of two similar PDS components with different activities has recently been reported in *S. pombe*. Fps1 forms a monomeric farnesyl diphosphate synthase, and also forms a heteromeric complex with the Fps1-like protein Spo9 to generate a geranyl geranyl diphosphate synthase [47]. These examples and our current results suggest that heteromeric PDS complex formation and the components being combined alter the final product(s). A solved three-dimensional structure of the heteromeric PDS is necessary in order to identify the molecular mechanism(s) of chain length determination in such enzymes.

Experimental procedures

Materials

DNA makers, DNA-modifying enzymes, and other restriction enzymes were from TOYOBO and Fermentas New England Biolabs. Protein makers were from Fermentas Life Sciences and Oriental Yeast. Antibodies were from Santa Cruz Biotechnology. IPP, all-E-farnesyl diphosphate (FPP), geranylgeraniol (GGOH), and solanesol (all-E-nonaprenol) were from Sigma Chemical. [1-^{14}C]IPP (1.96 TBq/mol) was from Amersham. Kiesel gel 60 F_{254} TLC plates were from Merck. Reversed-phase LKC-18 thin-layer plates were from Whatman Chemical Separation. The Blue Native-PAGE NOVEX Bis-Tris Gel System and the NativeMark Unstained Protein Standard were from Invitrogen (Japan K.K.).

Strains and Plasmids

The strains and plasmids used in this study are shown in Table 1. The *E. coli* strain DH5α was used in the general construction of plasmids. KO229 (*ispB::cat*), the *ispB* defective mutant of *E. coli,* harboring pKA3 (*ispB*) [18] was used as a host strain to express Coq1 and Dps1 for UQ synthesis. Wild type *S. cerevisiae*, SP1 [48] and the *COQ1*-deletion mutant YKK6 (*COQ1::URA3*) [17], were used for complementation and UQ extraction analysis. Wild-type fission yeast PR110 and single or double deletion mutants of *dps1* and *dlp1,* RS312 (Δ*dlp1::ura4*), and KS10 (Δ*dps1::ura4*) [3] were used to express Coq1 and Dps1, for complementation analysis and UQ extraction. A double disruptant of *dps1*

and *dlp1* in fission yeast was constructed. The *ura4* marker used to disrupt *dlp1* in RS312 was replaced by *ADE2,* yielding KMR1 (Δ*dlp1::ura4::ADE2),* followed by deleting *dps1* by the kanamycin resistant gene in KMR1. The obtained kanamycin-resistant double disruptant of *dps1* and *dlp1* was named LA1 (Δ*dlp1::ura4::ADE2,* Δ*dps1::kanMx6*). Disruption of *dlp1* and *dps1* were confirmed by colony PCR (data not shown).

Plasmids pBluescript II SK+/-, pBluescript II KS+/- (Stratagene), pT7Blue-T (Novagen), pSTV28 (Takara Shuzo), pSTV28K (Km^r marker) [18], pREPl [49], and YEpl3M4 [17] were used as vectors. Plasmids pGEX-KG [14], pQE31 (Qiagen), pDS473a [49], and pSLF173 [49] were used for expression of GST-, His-, or HA-tagged fusion proteins in *E. coli* or in *S. pombe.*

Construction of plasmids

The oligonucleotide primers used in this study are listed in Table 2. The ORF of the *COQ1* gene (1422 bp) was amplified with the oligonucleotides of COQ1-BamHI and COQ1-SmaI and cloned into the same sites of pBluescript II SK+ to generate pBSSK-COQ1. For expression of *COQ1* in fission yeast, the full length *COQ1* gene was amplified with the oligonucleotides COQ1-BamHI-TP45 and COQ1-SmaI and cloned into pREP1 to yield pREP1-TP45-COQ1. The *COQ1* gene was also amplified with the oligonucleotides of COQ1-BamHI and COQ1-EcoRI and cloned into pGEX-KG to generate pGEX-COQ1 for expression of the fusion protein GST-Coq1 in *E. coli*. Primers COQ1-BamHI and COQ1-SmaI were used to construct pDS473-COQ1 for GST-Coq1 expression in *S. pombe*. To construct pSTV28-HIS-dps1, the *dps1* gene was first cloned into the *Sph* I and *Sal* I sites of the pQE31 vector, and then the fragment containing 6xHIS-dps1 was digested with *Xho* I and *Sal* I and cloned into the *Sal* I site of pSTV28. The plasmid YEp13M4-COQ1-dps1 expressed the fission yeast Dps1 fused with 53 amino acids of the N-terminus of Coq1 for mitochondrial import. To yield this plasmid, *COQ1* and *dps1* genes were amplified with COQ1-a and COQ1-b or dps1-a and dps1-b primers respectively. A secondary PCR was carried out to get the *COQ1-dps1* fragment with COQ1-a and dps1-b primers, and this fragment was cloned into the YEp13M4 vector.

Expression of *COQ1* and *dps1* genes in *E. coli* or yeast strains

To express full-length *COQ1* and *dps1* genes in *E. coli,* the plasmids pGEX-COQ1 and pSTV28-HIS-dps1 were introduced into KO229 (*ispB::cat*) by plasmid swapping. For fission yeast, pDS473-COQ1 and pHA-dps1 were introduced into LA1 to express fusion proteins GST-Coq1 and HA-Dps1. *E. coli* cells transformed with the plasmid were grown at 37°C to an OD_{600} of 0.5. Isopropyl 1-thio-β-galactopyranoside was added to a final concentration of 1 mM, and the cells were cultured at 37°C for 4 h. Fission yeast cells were grown on PM minimal medium with appropriate supplements [50]. The concentration of the supplemented amino acids was 100 μg/ml. Transformants of budding yeast cells were grown on Synthetic Complete (SC) glucose media lacking uracil and leucine. The cultures were grown to the mid-to-late log phase.

Purification of Coq1 and Dps1

After the cells were collected by centrifugation at 3,000_g for 5 min, the pellets were suspended in lysis buffer [50 mM Tris (pH 8.0), 150 mM NaCl, 5 mM EDTA (pH 8.0), 10% glycerol] and ruptured by vigorous shaking with glass beads on ice. After centrifugation at 13,000_g, 4°C for 10 min, the supernatant was obtained as crude proteins. This soluble fraction was then incubated with Glutathione Sepharose 4B (Amersham Pharmacia Biotech) at 4°C for 60 min. The Glutathione Sepharose 4B beads were washed three times with 140 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate, and 1.8 mM potassium phosphate to obtain a purified GST-Coq1 protein.

Co-immunoprecipitation of Coq1 and Dps1 proteins

To examine whether Coq1 and Dps1 form a heterologous complex, the pS473-COQ1 plasmid that produces glutathione-S-transferase (GST) fused with the Coq1 protein, and the pHA-DPS1 plasmid that produces the HA-fused Dps1 protein, were introduced into *S. pombe* strain LA1. Crude proteins extracted from the transformants and purified proteins (described above) were both subjected to SDS-PAGE and the target polypeptides were detected by Western blot analysis using a rabbit anti-GST, mouse anti-HA, or mouse anti-His antibody. To detect a heteromer of COQ1 and Dps1 in *E. coli,* KO229 (*ispB::cat*) harboring both pGEX-COQ1 and pSTV28-HIS-dps1 was used.

Blue Native-PAGE

To analyze the native molecular size of the Coq1-Dps1 heteromer, samples for Blue native-PAGE (Invitrogen) were prepared according to the manufacturer's instructions. The NativeMark Unstained Protein Standard deviation from the NativePAGE NOVEX Bis-Tris Gel System contains eight proteins ranging in size from 20 to 1200 kDa.

Prenyl diphosphate synthase assay and product analysis

Modification of a method described previously was used to measure PDS activity [14]. Cells were grown to the mid-to-late log phase in the proper medium and then harvested by centrifugation. All subsequent steps were carried out at 4°C. The cells were then resuspended in a buffer containing 100 mM potassium phosphate (pH 7.4), 5 mM EDTA, and 1 mM 2-mercaptoethanol, and ruptured by vigorous shaking with glass beads 14 times for 30 s at 60 s intervals on ice. The homogenate was centrifuged at 1500_g for 10 min, and the resulting supernatant was used as a crude enzyme extract. The incubation mixture contained 2 mM $MgCl_2$, 0.2% (w/v) Triton X-100, 50 mM potassium phosphate buffer (pH 7.4), 5 mM KF, 10 mM iodoacetamide, 20 μM [^{14}C]IPP (specific activity 0.92 MBq mol^{-1}), 100 μM FPP, and 1.5 mg mL^{-1} of the enzyme in a final volume of 0.5 mL. The sample mixtures were incubated for 60 min at 30°C. Prenyl diphosphates were extracted with 1-butanol–saturated water and hydrolyzed with acid phosphatase. The hydrolysis products were extracted with hexane and analyzed by reverse-phase TLC with acetone/water (19:1, v/v). Radioactivity on the plate was detected with a BAS1500-Mac imaging analyzer (Fuji Film Co.). The spots of the marker prenols were visualized by exposure of the plate to iodine vapor.

Ubiquinone extraction and measurement

Recombinant *E. coli* strains were incubated in LB liquid medium with proper antibiotic to the mid-to-late log phase and centrifuged at 3,500 rpm for pellet collection. For yeast strains, minimum medium with appropriate supplements were used for incubation. Ubiquinone was extracted as described previously [20]. The crude extract of UQ was analyzed by normal-phase TLC with authentic UQ-10 (Kaneka) as the standard. Normal-phase TLC was carried out on a Kieselgel 60 F254 plate with

benzene/acetone (97:3, v/v). The UV-visualized band containing UQ was collected from the TLC plate and extracted with chloroform-methanol (1:1, vol/vol). The solution was evaporated to dryness and the residue was re-dissolved in ethanol. The purified UQ was further analyzed by HPLC using ethanol as a solvent.

Acknowledgements

This study was supported by a Grant-in-Aid from the Ministry of Education, Culture, Sports, Science and Technology of Japan.

References

1. Turunen, M., Olsson, J. & Dallner, G. (2004) Metabolism and function of coenzyme Q, *Biochim Biophys Acta.* **1660**, 171-199.
2. Do, T. Q., Schultz, J. R. & Clarke, C. F. (1996) Enhanced sensitivity of ubiquinone-deficient mutants of *Saccharomyces cerevisiae* to products of autoxidized polyunsaturated fatty acids, *Proc Natl Acad Sci USA.* **93**, 7534-7539.
3. Suzuki, K., Okada, K., Kamiya, Y., Zhu, X., Tanaka, K., Nakagawa, T., Kawamukai, M. & Matsuda, H. (1997) Analysis of the decaprenyl diphosphate synthase (*dps*) gene in fission yeast suggests a role of ubiquinone as an antioxidant, *J. Biochem. (Tokyo).* **121**, 496-505.
4. Bader, M., Muse, W., Ballou, D. P., Gassner, C. & Bardwell, J. C. A. (1999) Oxidative protein folding is driven by the electron transport system, *Cell.* **98**, 217-227.
5. Saiki, R., Ogiyama, Y., Kainou, T., Nishi, T., Matsuda, H. & Kawamukai, M. (2003) Pleiotropic phenotypes of fission yeast defective in ubiquinone-10 production. A study from the *abc1Sp* (*coq8Sp*) mutant, *Biofactors.* **18**, 229-235.
6. Vande Weghe, J. G. & Ow, D. W. (1999) A fission yeast gene for mitochondrial sulfide oxidation, *J. Biol. Chem.* **274**, 13250-13257.
7. Rodriguez-Aguilera, J. C., Gavilan, A., Asencio, C. & Navas, P. (2005) The role of ubiquinone in *Caenorhabditis elegans* longevity, *Ageing Res Rev.* **4**, 41-53.
8. Lopez-Martin, J. M., Salviati, L., Trevisson, E., Montini, G., DiMauro, S., Quinzii, C., Hirano, M., Rodriguez-Hernandez, A., Cordero, M. D., Sanchez-Alcazar, J. A., Santos-Ocana, C. & Navas, P. (2007) Missense mutation of the COQ2 gene causes defects of bioenergetics and de novo pyrimidine synthesis, *Hum Mol Genet.* **16**, 1091-7.
9. Rosenfeldt, F., Hilton, D., Pepe, S. & Krum, H. (2003) Systematic review of effect of coenzyme Q10 in physical exercise, hypertension and heart failure, *Biofactors.* **18**, 91-100.
10. Kawamukai, M. (2002) Biosynthesis, bioproduction and novel roles of ubiquinone, *J Biosci Bioeng.* **94**, 511-517.
11. Tran, U. C. & Clarke, C. F. (2007) Endogenous synthesis of coenzyme Q in eukaryotes, *Mitochondrion.* **7 Suppl 1**, S62-71.
12. Zhu, X., Yuasa, M., Okada, K., Suzuki, K., Nakagawa, T., Kawamukai, M. & Matsuda, H. (1995)

Production of ubiquinone in *Escherichia coli* by expression of various genes responsible for ubiquinone biosynthesis, *J Ferment Bioeng.* **79**, 493-495.
13. Okada, K., Ohara, K., Yazaki, K., Nozaki, K., Uchida, N., Kawamukai, M., Nojiri, H. & Yamane, H. (2004) The *AtPPT1* gene encoding 4-hydroxybenzoate polyprenyl diphosphate transferase in ubiquinone biosynthesis is required for embryo development in *Arabidopsis thaliana*, *Plant Mol Biol.* **55**, 567-577.
14. Saiki, R., Nagata, A., Kainou, T., Matsuda, H. & Kawamukai, M. (2005) Characterization of solanesyl and decaprenyl diphosphate synthases in mice and humans, *FEBS J.* **272**, 5606-5622.
15. Suzuki, K., Ueda, M., Yuasa, M., Nakagawa, T., Kawamukai, M. & Matsuda, H. (1994) Evidence that *Escherichia coli ubiA* product is a functional homolog of yeast COQ2, and the regulation of *ubiA* gene expression, *Biosci Biotech Biochem.* **58**, 1814-1819.
16. Okada, K., Suzuki, K., Kamiya, Y., Zhu, X., Fujisaki, S., Nishimura, Y., Nishino, T., Nakagawa, T., Kawamukai, M. & Matsuda, H. (1996) Polyprenyl diphosphate synthase essentially defines the length of the side chain of ubiquinone, *Biochim Biophys Acta.* **1302**, 217-223.
17. Okada, K., Kainou, T., Matsuda, H. & Kawamukai, M. (1998) Biological significance of the side chain length of ubiquinone in *Saccharomyces cerevisiae*, *FEBS Lett.* **431**, 241-244.
18. Okada, K., Minehira, M., Zhu, X., Suzuki, K., Nakagawa, T., Matsuda, H. & Kawamukai, M. (1997) The *ispB* gene encoding octaprenyl diphosphate synthase is essential for growth of *Escherichia coli*, *J Bacteriol.* **179**, 3058-3060.
19. Okada, K., Kamiya, Y., Zhu, X., Suzuki, K., Tanaka, K., Nakagawa, T., Matsuda, H. & Kawamukai, M. (1997) Cloning of the *sdsA* gene encoding solanesyl diphosphate synthase from *Rhodobacter capsulatus* and its functional expression in *Escherichia coli* and *Saccharomyces cerevisiae*, *J Bacteriol.* **179**, 5992-5998.
20. Okada, K., Kainou, T., Tanaka, K., Nakagawa, T., Matsuda, H. & Kawamukai, M. (1998) Molecular cloning and mutational analysis of the *ddsA* gene encoding decaprenyl diphosphate synthase from *Gluconobacter suboxydans*, *Eur J Biochem.* **255**, 52-59.
21. Park, Y. C., Kim, S. J., Choi, J. H., Lee, W. H., Park, K. M., Kawamukai, M., Ryu, Y. W. & Seo, J. H. (2005) Batch and fed-batch production of coenzyme Q10 in recombinant *Escherichia coli* containing the decaprenyl diphosphate synthase gene from *Gluconobacter suboxydans*, *Appl Microbiol Biotechnol.* **67**, 192-196.
22. Takahashi, S., Ogiyama, Y., Kusano, H., Shimada, H., Kawamukai, M. & Kadowaki, K. (2006) Metabolic engineering of coenzyme Q by modification of isoprenoid side chain in plant, *FEBS Lett.* **580**, 955-9.
23. Liang, P. H., Ko, T. & Wang, A. H. (2002) Structure, mechanism and function of prenyltransferases, *Eur J Biochem.* **269**, 3339-3354.
24. Koyama, T. (1999) Molecular analysis of prenyl chain elongating enzymes, *Biosci Biotechnol Biochem.* **63**, 1671-1676.
25. Chang, T. H., Guo, R. T., Ko, T. P., Wang, A. H. & Liang, P. H. (2006) Crystal structure of type-III geranylgeranyl pyrophosphate synthase from *Saccharomyces cerevisiae* and the mechanism of product chain length determination, *J Biol Chem.* **281**, 14991-5000.
26. Tarshis, L. C., Yan, M., Poulter, C. D. & Sacchettini, J. C. (1994) Crystal structure of recombinant farnesyl diphosphate synthase at 2.6-Å resolution, *Biochemistry.* **33**, 10871-10877.
27. Guo, R. T., Kuo, C. J., Chou, C. C., Ko, T. P., Shr, H. L., Liang, P. H. & Wang, A. H. (2004)

Crystal structure of octaprenyl pyrophosphate synthase from hyperthermophilic *Thermotoga maritima* and mechanism of product chain length determination, *J Biol Chem.* **279**, 4903-4912.
28. Sun, H. Y., Ko, T. P., Kuo, C. J., Guo, R. T., Chou, C. C., Liang, P. H. & Wang, A. H. (2005) Homodimeric hexaprenyl pyrophosphate synthase from the thermoacidophilic crenarchaeon *Sulfolobus solfataricus* displays asymmetric subunit structures, *J Bacteriol.* **187**, 8137-8148.
29. Kainou, T., Okada, K., Suzuki, K., Nakagawa, T., Matsuda, H. & Kawamukai, M. (2001) Dimer formation of octaprenyl diphosphate synthase (IspB) is essential for chain length determination of ubiquinone, *J Biol Chem.* **276**, 7876-7883.
30. Lee, J. K., Her, G., Kim, S. Y. & Seo, J. H. (2004) Cloning and functional expression of the dps gene encoding decaprenyl diphosphate synthase from *Agrobacterium tumefaciens*, *Biotechnol Prog.* **20**, 51-56.
31. Zahiri, H. S., Noghabi, K. A. & Shin, Y. C. (2006) Biochemical characterization of the decaprenyl diphosphate synthase of *Rhodobacter sphaeroides* for coenzyme Q10 production, *Appl Microbiol Biotechnol.* **73**, 796-806.
32. Shimizu, N., Koyama, T. & Ogura, K. (1998) Molecular cloning, expression, and characterization of the genes encoding the two essential protein components of *Micrococcus luteus* B-P 26 hexaprenyl diphosphate synthase, *J Bacteriol.* **180**, 1578-1581.
33. Koike-Takeshita, A., Koyama, T., Obata, S. & Ogura, K. (1995) Molecular cloning and nucleotide sequences of the genes for two essential proteins constituting a novel enzyme system for heptaprenyl diphosphate synthesis, *J. Biol. Chem.* **270**, 18396-18400.
34. Koike-Takeshita, A., Koyama, T. & Ogura, K. (1998) Intersubunit structure within heterodimers of medium-chain prenyl diphosphate synthases. Formation of a hybrid-type heptaprenyl diphosphate synthase, *J Biochem.* **124**, 790-797.
35. Kaur, D., Brennan, P. J. & Crick, D. C. (2004) Decaprenyl diphosphate synthesis in *Mycobacterium tuberculosis*, *J Bacteriol.* **186**, 7564-7570.
36. Ferella, M., Montalvetti, A., Rohloff, P., Miranda, K., Fang, J., Reina, S., Kawamukai, M., Bua, J., Nilsson, D., Pravia, C., Katzin, A., Cassera, M. B., Aslund, L., Andersson, B., Docampo, R. & Bontempi, E. J. (2006) A solanesyl-diphosphate synthase localizes in glycosomes of *Trypanosoma cruzi*, *J Biol Chem.* **281**, 39339-39348.
37. Tonhosolo, R., D'Alexandri, F. L., Genta, F. A., Wunderlich, G., Gozzo, F. C., Eberlin, M. N., Peres, V. J., Kimura, E. A. & Katzin, A. M. (2005) Identification, molecular cloning and functional characterization of an octaprenyl pyrophosphate synthase in intra-erythrocytic stages of *Plasmodium falciparum*, *Biochem J.* **392**, 117-126.
38. Ashby, M. N. & Edwards, P. A. (1990) Elucidation of the deficiency in two yeast coenzyme Q mutants. Characterization of the structural gene encoding hexaprenyl pyrophosphate synthetase, *J Biol Chem.* **265**, 13157-13164.
39. Saiki, R., Nagata, A., Uchida, N., Kainou, T., Matsuda, H. & Kawamukai, M. (2003) Fission yeast decaprenyl diphosphate synthase consists of Dps1 and the newly characterized Dlp1 protein in a novel heterotetrameric structure, *Eur. J. Biochem.* **270**, 4113-4121.
40. Jun, L., Saiki, R., Tatsumi, K., Nakagawa, T. & Kawamukai, M. (2004) Identification and subcellular localization of two solanesyl diphosphate synthases from *Arabidopsis thaliana*, *Plant Cell Physiol.* **45**, 1882-1888.
41. Hirooka, K., Bamba, T., Fukusaki, E.-i. & Kobayashi, A. (2003) Cloning and kinetic

characterization of *Arabidopsis thaliana* solanesyl diphosphate syntahse, *Biochem J.* **370**, 679-686.
42. Asai, K.-i., Fujisaki, S., Nishimura, Y., Nishino, T., Okada, K., Nakagawa, T., Kawamukai, M. & Matsuda, H. (1994) The identification of *Escherichia coli ispB* (*cel*) gene encoding the octaprenyl diphosphate synthase, *Biochem Biophys Res Commun.* **202**, 340-345.
43. Uchida, N., Suzuki, K., Saiki, R., Kainou, T., Tanaka, K., Matsuda, H. & Kawamukai, M. (2000) Phenotypes of fission yeast defective in ubiquinone production due to disruption of the gene for *p*-hydroxybenzoate polyprenyl diphosphate transferase, *J. Bacteriol.* **182**, 6933-6939.
44. Gin, P. & Clarke, C. F. (2005) Genetic evidence for a multi-subunit complex in coenzyme Q biosynthesis in yeast and the role of the Coq1 hexaprenyl diphosphate synthase, *J Biol Chem.* **280**, 2676-2681.
45. Zhang, Y. W., Li, X. Y. & Koyama, T. (2000) Chain length determination of prenyltransferases: both heteromeric subunits of medium-chain (E)-prenyl diphosphate synthase are involved in the product chain length determination, *Biochemistry.* **39**, 12717-12722.
46. Burke, C. C. & Croteau, R. (2002) Interaction with the small subunit of geranyl diphosphate synthase modifies the chain length specificity of geranylgeranyl diphosphate synthase to produce geranyl diphosphate, *J. Biol. Chem.* **277**, 3141-3149.
47. Ye, Y., Fujii, M., Hirata, A., Kawamukai, M., Shimoda, C. & Nakamura, T. (2007) Geranylgeranyl diphosphate synthase in fission yeast is a heteromer of farnesyl diphosphate synthase (FPS), Fps1, and an FPS-like protein, Spo9, essential for sporulation, *Mol Biol Cell.* **18**, 3568-3581.
48. Kawamukai, M., Gerst, J., Field, J., Riggs, M., Rodgers, L., Wigler, M. & Young, D. (1992) Genetic and biochemical analysis of the adenylyl cyclase-associated protein, cap, in *Schizosaccharomyces pombe*, *Mol Biol Cell.* **3**, 167-180.
49. Ozoe, F., Kurokawa, R., Kobayashi, Y., Jeong, H., Tanaka, K., Sen, K., Nakagawa, T., Matsuda, H. & Kawamukai, M. (2002) The 14-3-3 proteins Rad24 and Rad25 negatively regulate Byr2 by affecting its localization in *Schizosaccharmyces pombe*, *Mol. Cell. Biol.* **22**, 7105-7109.
50. Kawamukai, M., Ferguson, K., Wigler, M. & Young, D. (1991) Genetic and biochemical analysis of the adenylyl cyclase of *Schizosaccharomyces pombe.*, *Cell Regul.* **2**, 155-164.

Table 1 Strains and plasmids used in this study

Strain/Plasmid	Phenotype	Source/Ref.
Strains		
E. coli DH5α	F^-, *recA*1, *gyrA*96, *thi*-1, *supE*44, *relA*1, *mcrA*$^-$	[18]
E. coli KO229/pKA3	Cm^r; Sp^r; *ispB*::*cat*; harboring pKA3	[18]
S. cerevisiae SP1	*MATa leu2 ura3 trp1 his3 ade8 can1*	[48]
S. cerevisiae YKK6	*URA3*$^+$, *COQ1*::*URA3*	[17]
S. pombe PR110	*h*$^+$, *leu1-32, ura4-D18*	[14]
S. pombe RS312	*h*$^+$, *leu1-32, ade6-M210, ura4-D18 _dlp1*::*ura4*	[39]
S. pombe KS10	*h*$^+$, *leu1-32, ade6-M216, ura4-D18 _dps1*::*ura4*	[3]
S. pombe KMR1	*h*$^+$, *leu1-32, ade6-M216, ura4-D18 _dlp1*::*ura4*::*ADE2,*	This study
S. pombe LA1	*h*^{90}, *leu1-32, ade6-M210, ura4-D18 _dlp1*::*ura4*::*ADE2, _dps1*::*kanMx6*	This study
Plasmids		
pREP1-DLP1	Ap^r, *nmt1* promoter, full-length *dlp1* in pREP1	[39]
pQE31-dps1	Ap^r, full-length *dps1* in pQE31	[39]
pHADPS1	Ap^r, *nmt1* promoter, full-length *dps1* in pSLF173	[39]
pGSTDLP1	Ap^r, *nmt1* promoter, full-length *dps1* in pDS473a	[39]
pSTV28-HIS-dps1	Km^r, HIS_6 with full-length *dps1* in *Sal* I site of pSTV28	This study
YEp13M4-COQ1-dps1	Ap^r, 159bp *COQ1* mitochondrial import signal ahead of *dps1* in YEp13M4	This study
pT7-COQ1	Ap^r, cDNA *COQ1* in pT7Blue T	This study
pBSSK-COQ1	Ap^r, *Bam*H I-*Sma* I fragment of full-length *COQ1* in pBluescript SK(+)	This study
pGEX-COQ1	Ap^r, *Bam*H I-*Eco*R I fragment of full-length *COQ1* in pGEX-KG	This study
pREP1-TP45-COQ1	Ap^r, *nmt1* promoter, TP45-*Bam*H I-*Sma* I fragment of full-length *COQ1* in pREP1	This study
pDS473-COQ1	Ap^r, *nmt1* promoter, *Bam*H I-*Sma* I fragment of full-length *COQ1* in pDS473a	This study

Ap, ampicillin; Cm, chloramphenicol; Km, kanamycin, Sp, spectinomycin.

Table 2 Oligonucleotide primers used in this study

Primer name	Description
COQ1-BamHI	5’-CCGGATCCCATGTTTCAAAGGTCTGGC-3’
COQ1-SmaI	5’-GCCCCCGGGTTACTTTCTTCTTGTTAGTATAC-3’
COQ1-BamHI-TP45	5’-CCGGATCCATGTTTCAAAGGTCTGGC-3’
COQ1-EcoRI	5’-CGAATTCTTACTTTCTTCTTGT-3’
COQ1-a	5’-CAGTGAATTCGAGCTCGGTACCC-3’
COQ1-b	5’-ATACATACTGAATCATCATCTCCTTCGAG-3’
dps1-a	5’- ATGATTCAGTATGTAT-3’
dps1-b	5’-ATAAGGCGCATTTTTCTTCAAAGCTTTCACTTCTTTCTCG-3’

Figure Legends

Figure 1. Alignment of the amino acid sequences of *S. cerevisiae* Coq1, *E. coli* IspB, *S. pombe* Dps1, and hDps1. (A) (1) hexa-PDS (Coq1) from *S. cerevisiae* (accession no. J05547), (2) octa-PDS (IspB) from *E. coli* (accession no. NP417654), (3) a component of deca-PDS (Dps1) from *S. pombe* (accession no. D84311), and (4) a component of deca-PDS (hDps1/PDSS1) from humans (accession no. AB210838). Seven highly conserved regions (I~VII) among the long-chain poly-PDSs are indicated by underlines. Two aspartate-rich motifs in domains II and VI, which are considered to be the substrate binding sites in polyprenyl diphoshpate, are denoted by boxes. (B) Confirmation of *S. cerevisiae COQ1* cDNA by RT-PCR. RNAs were prepared from a *S. cerevisiae* W3031A strain with a QIAGEN RNeasy Mini Kit. RT-PCR was performed with a pair of primers for the *S. cerevisiae COQ1* gene by using Promega AccessQuick RT-PCR System. lane 1, λDNA/*Hind*III digest marker; lane 2, *COQ1* amplified from genomic DNA; lane 3, *COQ1* mRNA amplified from W3031A with RT; lane 4, *COQ1* mRNA amplified from W3031A without RT; lane 5, *COQ1* mRNA amplified from YKK6 (Δ*COQ1*); lane 6, 100 bp ladder.

Figure 2. Complementation and UQ extraction of an *E. coli ispB* disruptant by expressing *COQ1*. Ubiquinone extracted from *E. coli* was first separated by normal-phase TLC and further analyzed by HPLC with standard UQ-10 (A). Ubiquinone was extracted from the strain DH5α harboring pBlueScript SK (B), DH5α harboring pBSSK-COQ1 (C), KO229 harboring pBSSK-COQ1 (D), DH5α harboring pGEX-COQ1 (E), KO229 harboring pGEX-COQ1 (F), or pGEX-COQ1 and pSTV28-HIS-dps1 (G). The *COQ1* gene complemented the *ispB* disruptant and UQ-6 was detected from the recombinant *E. coli* (D).

Figure 3. Growth of RS312, KS10, or LA1 on minimal medium by expressing *COQ1*. RS312 (*dlp1::ura4*) (A), KS10 (*dps1::ura4*) (B), or LA1 (*dlp1::ura4::ADE2, dps1::kanMx6*) (C) harboring indicated plasmids were grown for 3 d at 30°C on PM or PMA containing 75 μg/ml adenine. RS312 restored its growth by pREP1-TP45-COQ1 on PMA medium and grew as well as wild-type fission yeast, while KS10 or LA1 did not. All strains restored their growth when supplemented with cysteine

(400 μg/ml) on the same medium.

Figure 4. Ubiquinone species in fission yeast *dps1* and *dlp1* disruptants expressing *COQ1*. Ubiquinone was extracted from RS312 (Δ*dlp1*) harboring plasmid pREP1 (B), pREP1-TP45-COQ1 (C), or pRDLP1 (D). UQ-10 was used as the standard (A). Ubiquinone was also extracted from LA1 (Δ*dlp1* Δ*dps1*) harboring plasmid pHA-dps1 and pDS473-COQ1 (E), pDS473-COQ1 (F), pHA-dlp1 and pDS473-COQ1 (G), pREP1-Hudps1 and pDS473-COQ1 (H), or pREP1-Hudps1 and pREP2-Hudlp1 (I). Crude UQ was separated by a TLC plate and then loaded onto an HPLC. LA1 harboring pHADPS1 and pDS473-COQ1 (E) produced mainly UQ-10 and a small amount of UQ-9. However, no UQ was detected from LA1 harboring pDS473-COQ1 (F), pHA-dlp1 and pDS473-COQ1 (G), or pREP1-Hudps1 and pDS473-COQ1 (H).

Figure 5. Interaction of Coq1 and Dps1 in fission yeast and *E. coli*. (A) Crude proteins were extracted from LA1 harboring various plasmids and incubated with Glutathione Sepharose 4B at 4°C for 60 min. The purified samples were employed for Western blot analysis with GST- or HA-antibodies to examine the binding of Coq1 and Dps1. Proteins extracts from LA1 harboring pDS473-dlp1 and pHADPS1 (lane 1), pDS473 and pHADPS1 (lane 2), pDS473-COQ1 and pHADPS1 (lane 3), pDS473-COQ1 and pHA-dlp1 (lane 4). (B) Co-immunoprecipitation analysis of Coq1 and Dps1 in *E. coli*. Recombinant cells of DH5α harboring pGEX-Dps1 and pSTV28-HIS-dps1 (lanes 1 and 2), or KO229 harboring pGEX-COQ1 and pSTV28-HIS-dps1 (lanes 3 and 4) were harvested after induction by 1 mM IPTG at 37°C for 3 h. Crude proteins were extracted from those strains by sonication and purified by Glutathione Sepharose 4B at 4°C for 60 min. Crude enzymes (lanes 1 and 3) and the purified samples (lanes 2 and 4) were subjected for immunoblotting analysis with an anti-GST or an anti-His antibody.

Figure 6. Detection of UQ species in *S. cerevisiae* expressing both *COQ1* and *dps1*. Ubiquinone was extracted from wild type *S. cerevisiae* SP1 (B), SP1 harboring plasmid YEp13M4-COQ1-dps1 (C), *COQ1* deletion mutant (YKK6) harboring YEp13M4-COQ1-dps1 (D), or YKK6 (E). Ubiquinone was first separated by TLC and then by HPLC with the standard UQ-10 (A). UQ-6 was detected from wild-

type (B and C), while no UQ was detected from the *COQ1* disruptant expressing only the *dps1* gene (D).

Figure 7. The product catalyzed by the PDS was comprised of Coq1 and Dps1. The *in vitro* enzymatic reaction of Coq1 and Dps1 co-expressed in fission yeast (A) or *E. coli* (B) was carried out with [^{14}C]IPP and FPP as substrates and cell extracts as the crude enzyme source. The products were hydrolyzed with phosphatase, and then separated by reversed-phase TLC. The crude extracts analyzed in the lanes are as follows: (A) lane 1, LA1 harboring pHADPS1 and pDS473-COQ1; lane 2, wild type PR110. (B) lane 1, *E. coli* DH5α: lane 2, DH5 α harboring pGEX-COQ1; lane 3, *ispB* disruptant (KO229) harboring pGEX-COQ1 and pSTV28-HIS-dps1; lane 4, KO229 harboring pGEX-COQ1. Arrows indicate the major products synthesized by PDSs. GG, all-E-geranylgeraniol; S, all-E-solanesol; Ori, origin; S.F., solvent front; HexOH, hexaprenol (C_{30}); OOH, octaprenol (C_{40}); DOH, decaprenol (C_{50}).

Figure 8. Molecular size of the Coq1-Dps1 complex determined by Blue Native-PAGE. The purified Coq1-Dps1 protein was prepared according to the manufacturer's instructions. The unstained protein native marker ranged in size from 20 to 1200 kDa and was used as a standard. Unstained protein marker (lane 1); crude extraction from LA1 (lane 2); crude protein extracted from LA1 harboring pHADPS1 and pDS473-COQ1 (lane 3); purified Coq1-Dps1 protein (lane 4).

A

```
(1)                                                                MFQRSGAAHH IKLISSRRCR FKSSFAVALN    30
(3)                                                                         M IQYVYLKHMR KLWSLGKVRS    21
(4)  MASRWWRWRR GCSWKPAARS PGPGSPGRAG PLGPSAAAEV RAQVHRRKGL DLSQIPYINL VKHLTSACPN VCRISRFHHT    80

(1)  AASKLVTPKI LWNNPISLVS KEMNTLAKNI VALIGSGHPV LNKVTSYYFE TEGKKVRPLL VLLLSRALSE IPMTERNHLK   110
(2)                                                                       MN LEKINELTAQ DMAGVNAAIL    22
(3)  TVLRFSTTN- --RNASHLIK NELEQISPGI RQMLNSNSEF LEECSKYYTI AQGKQMRPSL VLLMSKATSL CHGIDRS--V    96
(4)  TPDSKTHSGE KYTDPFKLGW RDLKGLYEDI RKELLISTSE LKEMSEYYFD GKGKAFRPII VALMARACNI HHNNSRH---   157

(1)  IDKSDVPEDP IYSKPSQNQL FQRPASSISP LHILHGIKPL NPLTKGPEPL PEETFDKQRG ILPKQRRLAE IVEMIHTASL   190
(2)  ---EQLNSDV QLINQLGYYI VSGGGKRIRP MIAVLAARAV GYEGNA---- ---------- ----HVTIAA LIEFIHTATL    81
(3)  VGDKYIDDDD LRSFSTGQ-- ---------- ---------- ---------- ---------- ILPSQLRLAQ ITEMIHIASL   134
(4)  ---------- ---------- ---------- ---------- ---------- ---------- VQASQRAIAL IAEMIHTASL   177
                                        I
     DDXXD
(1)  LHDDVIDHSD TRRGRPSGNA AFTNKMAVLA GDFLLGRATV SISRLHNPEV VELMSNSIAN LVEGEFMQLK NTSIDADIDT   270
(2)  LHDDVVDESD MRRGKATANA AFGNAASVLV GDFIYTRAFQ MMTSLGSLKV LEVMSEAVNV IAEGEVLQLM NV--------   153
(3)  LHDDVIDHAN VRRGSPSSNV AFGNRRSILA GNFILARAST AMARLRNPQV TELLATVIAD LVRGEFLQLK NT--------   206
(4)  VHDDVIDDVS SRRGKHTVNK IWGEKKAVLA GDLILSAASI ALARIGNTTV ISILTQVIED LVRGEFLQL- ----------   246
          II                              III                              IV

(1)  IENGHKLLPV PSKKLEVKEH DFRVPSRQQG LQLSHDQIIE TAFEYYIHKT YLKTAALISK SCRCAAILSG ASPAVIDECY   350
(2)  ---------- --NDPDITEE N--------- ---------- -----YMRVI YSKTARLFEA AAQCSGILAG CTPEEEKGLQ   197
(3)  ---------M DPSSLEIKQS NF--------- ---------- ---DYYIEKS FLKTASLISK SCKASTILGQ CSPTVATAAG   256
(4)  ---------- --GSKENENE RF--------- ---------- ---AHYLEKT FKKTASLIAN SCKAVSVLGC PDPVVHEIAY   293
                                                                        V
                    DDXXD
(1)  DFGRNLGICF QLVDDMLDFT VSGKDLGKPS GADLKLGIAT APVLFAWKED PSLGPLISRN FSERGD---- VEKTIDSVRL   426
(2)  DYGRYLGTAF QLIDDLLDYN ADGEQLGKNV GDDLNEGKPT LPLLHAMHHG TPEQAQMIRT AIEQGNGRHL LEPVLEAMNA   277
(3)  EYGRCIGTAF QLMDDVLDYT SKDDTLGKAA GADLKLGLAT APVLFAWKKY PELGAMIVNR FNHPSD---- IQRARSLVEC   332
(4)  QYGKDVGIAF QLIDDVLDFT SCSDQMGKPT SADLKLGLAT GPVLFACQQF PEMNAMIMRR FSLPGD---- VDRARQYVLQ   369
                           VI

(1)  HNGIAKTKIL AEEYRDKALQ NLRDSLPESD ARSALEFLTN SILTRRK                                        473
(2)  CGSLEWTRQR AEEEADKAIA ALQ-VLPDTP WREALIGLAH IAVQRDR                                        323
(3)  TDAIEQTITW AKEYIKKAKD SLL-CLPDSP ARKALFALAD KVITRKK                                        378
(4)  SDGVQQTTYL AQQYCHEAIR EIS-KLRPSP ERDALIQLSE IVLTRDK                                        415
                                     VII
```

B

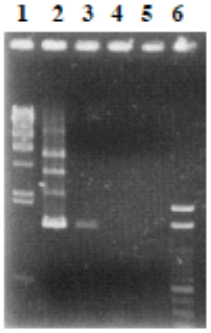


Fig. 1

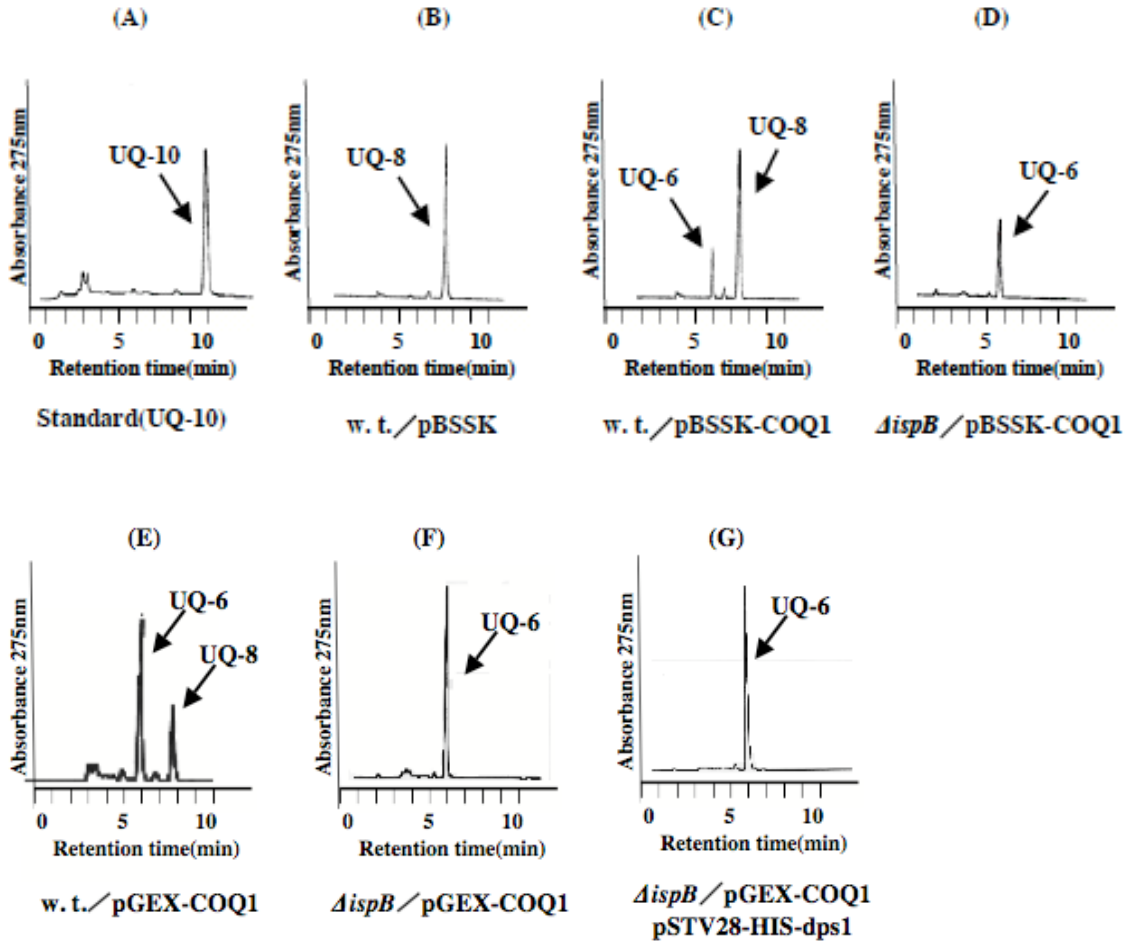

(A)
(B)
(C)
(D)
Absorbance 275nm
UQ-10
UQ-8
UQ-6
0
5
10
Retention time(min)
Standard(UQ-10)
w. t./pBSSK
w. t./pBSSK-COQ1
ΔispB/pBSSK-COQ1
(E)
(F)
(G)
w. t./pGEX-COQ1
ΔispB/pGEX-COQ1
ΔispB/pGEX-COQ1
pSTV28-HIS-dps1

Fig. 2

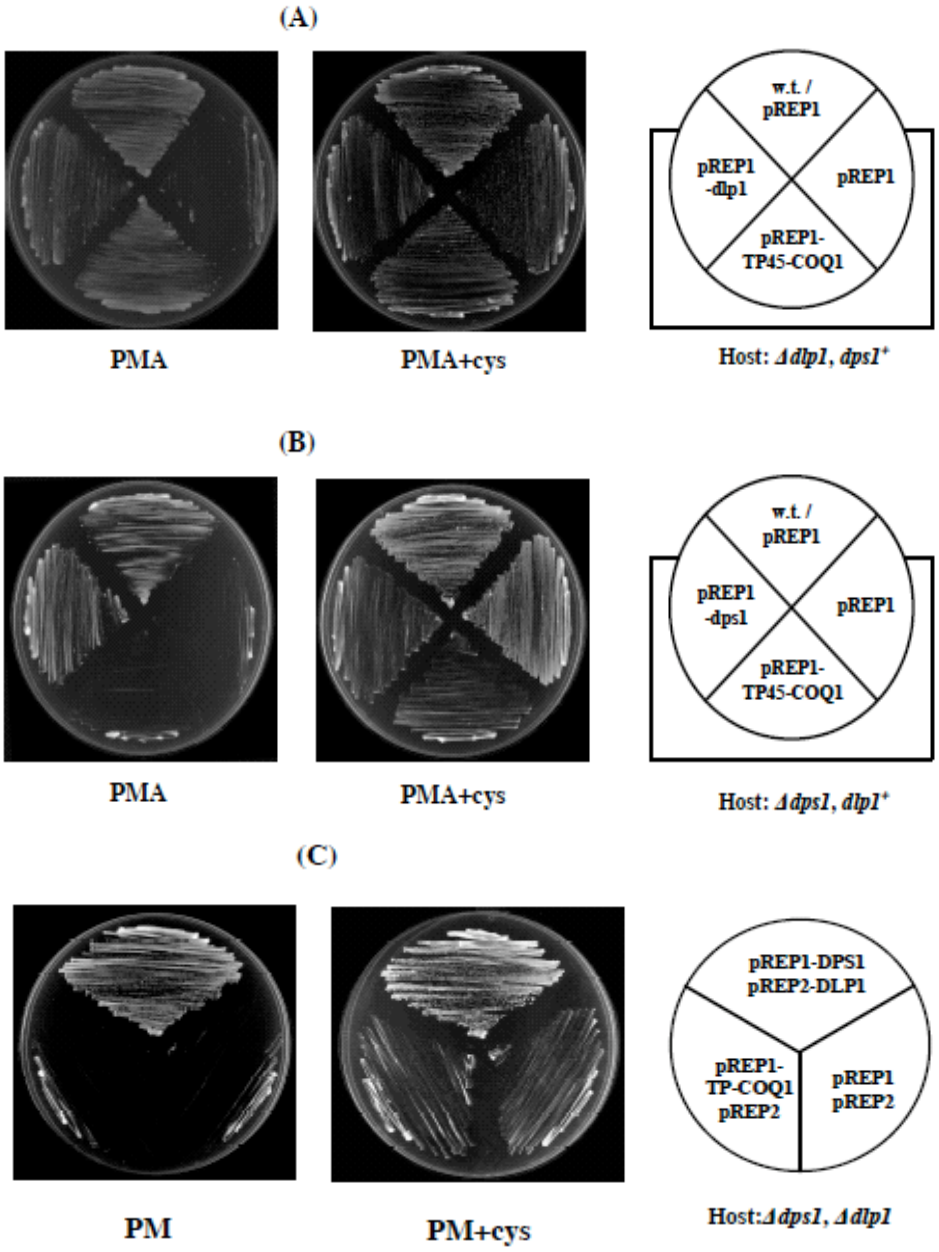

(A)
w.t. / pREP1
pREP1 -dlp1
pREP1
pREP1-TP45-COQ1
PMA
PMA+cys
Host: Δdlp1, dps1+
(B)
w.t. / pREP1
pREP1 -dps1
pREP1
pREP1-TP45-COQ1
PMA
PMA+cys
Host: Δdps1, dlp1+
(C)
pREP1-DPS1 pREP2-DLP1
pREP1-TP-COQ1 pREP2
pREP1 pREP2
PM
PM+cys
Host:Δdps1, Δdlp1

Fig. 3

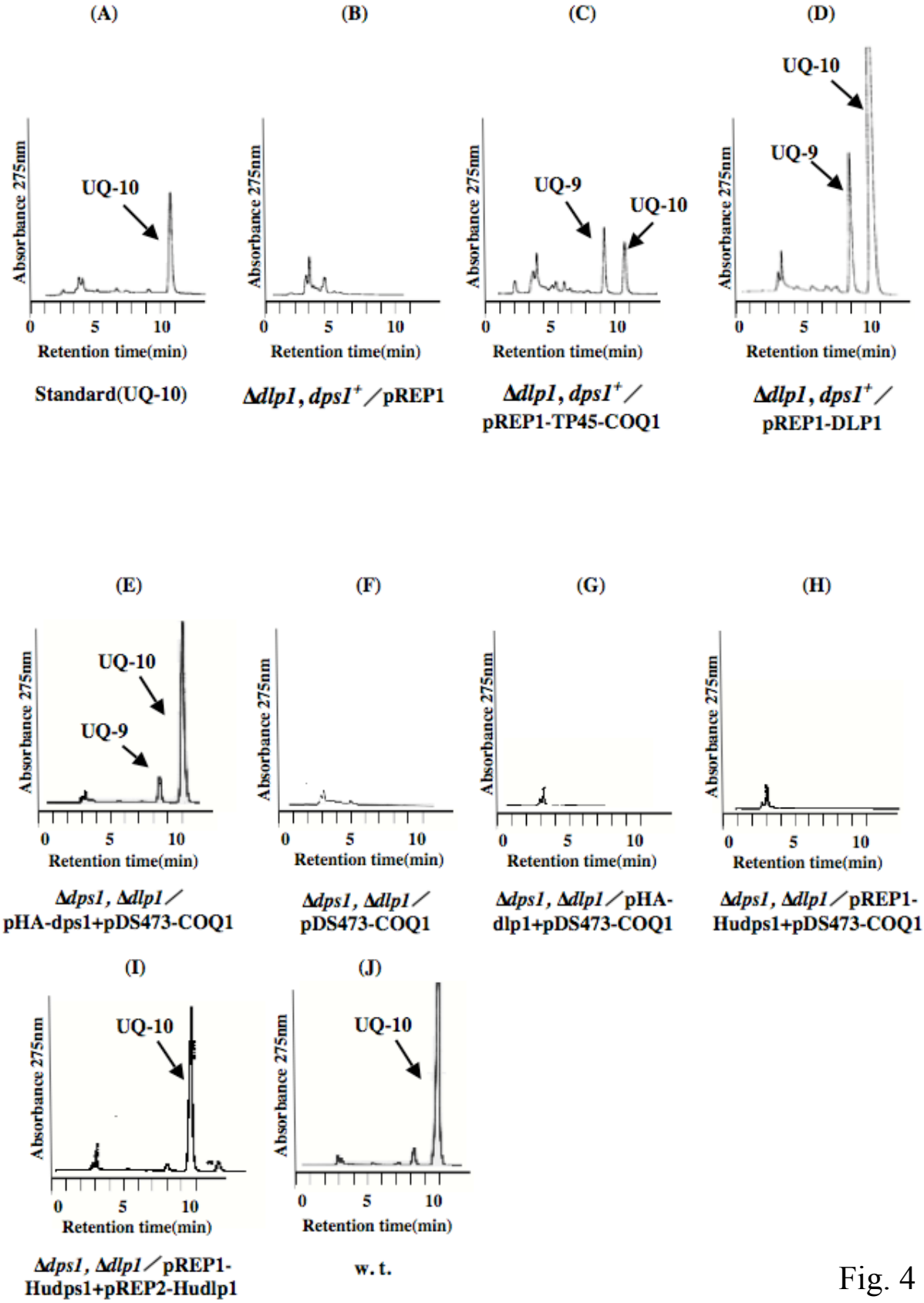
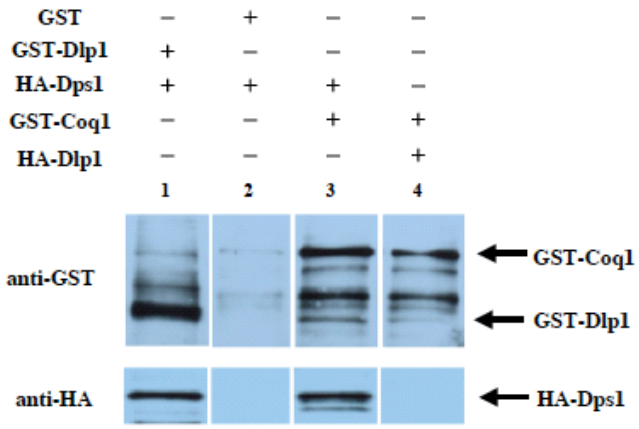

(A)
(B)
(C)
(D)
Absorbance 275nm
UQ-10
UQ-9
0
5
10
Retention time(min)
Standard(UQ-10)
Δdlp1, dps1+／pREP1
Δdlp1, dps1+／
pREP1-TP45-COQ1
Δdlp1, dps1+／
pREP1-DLP1
(E)
(F)
(G)
(H)
Δdps1, Δdlp1／
pHA-dps1+pDS473-COQ1
Δdps1, Δdlp1／
pDS473-COQ1
Δdps1, Δdlp1／pHA-
dlp1+pDS473-COQ1
Δdps1, Δdlp1／pREP1-
Hudps1+pDS473-COQ1
(I)
(J)
Δdps1, Δdlp1／pREP1-
Hudps1+pREP2-Hudlp1
w. t.

Fig. 4

A


GST
GST-Dlp1
HA-Dps1
GST-Coq1
HA-Dlp1
1
2
3
4
anti-GST
GST-Coq1
GST-Dlp1
anti-HA
HA-Dps1

B

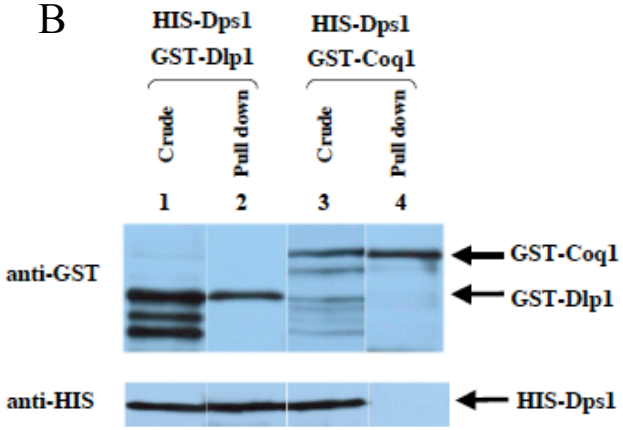

HIS-Dps1
GST-Dlp1
HIS-Dps1
GST-Coq1
Crude
Pull down
Crude
Pull down
1
2
3
4
anti-GST
GST-Coq1
GST-Dlp1
anti-HIS
HIS-Dps1

Fig. 5

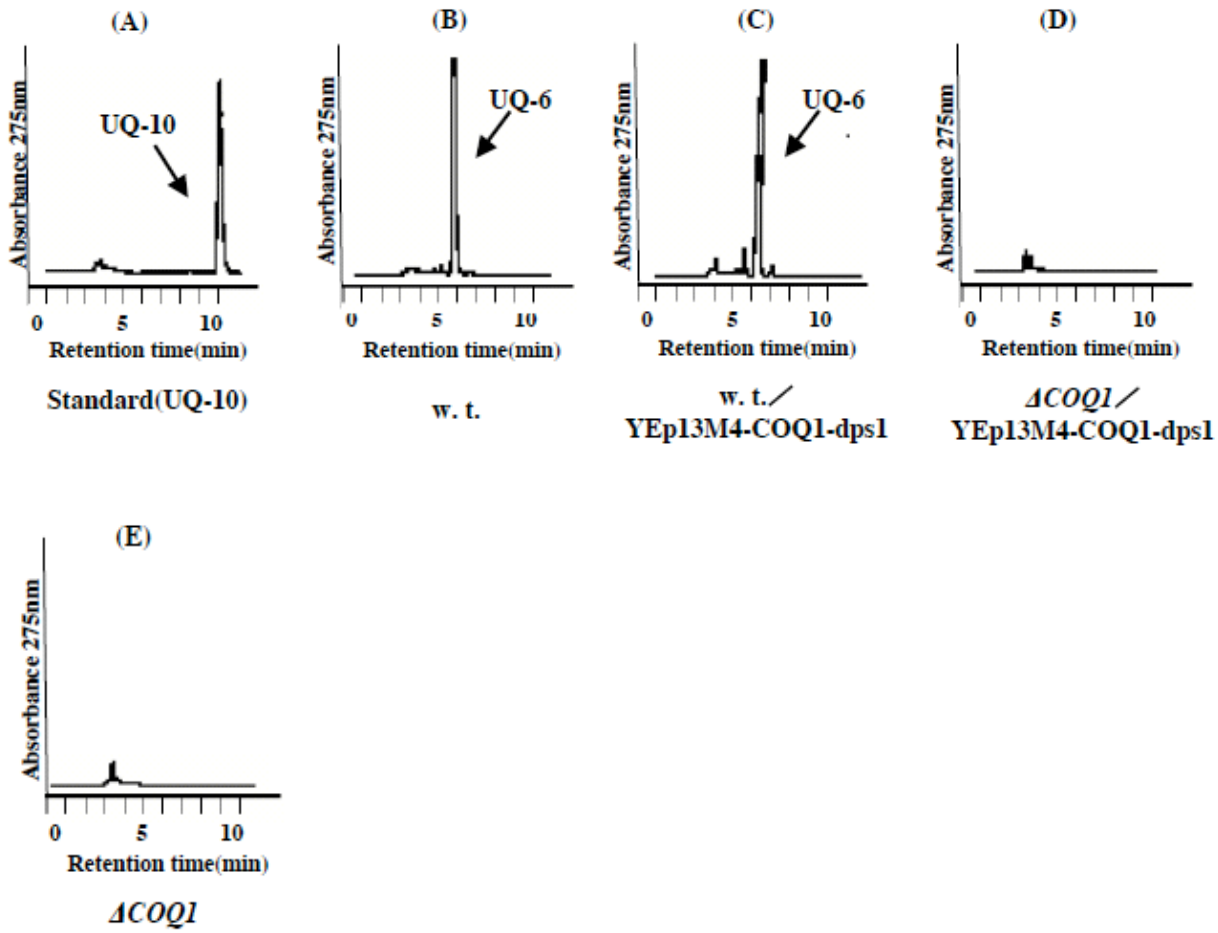

(A)
UQ-10
Absorbance 275nm
0
5
10
Retention time(min)
Standard(UQ-10)
(B)
UQ-6
Absorbance 275nm
0
5
10
Retention time(min)
w. t.
(C)
UQ-6
Absorbance 275nm
0
5
10
Retention time(min)
w. t./
YEp13M4-COQ1-dps1
(D)
Absorbance 275nm
0
5
10
Retention time(min)
ΔCOQ1/
YEp13M4-COQ1-dps1
(E)
Absorbance 275nm
0
5
10
Retention time(min)
ΔCOQ1

Fig. 6

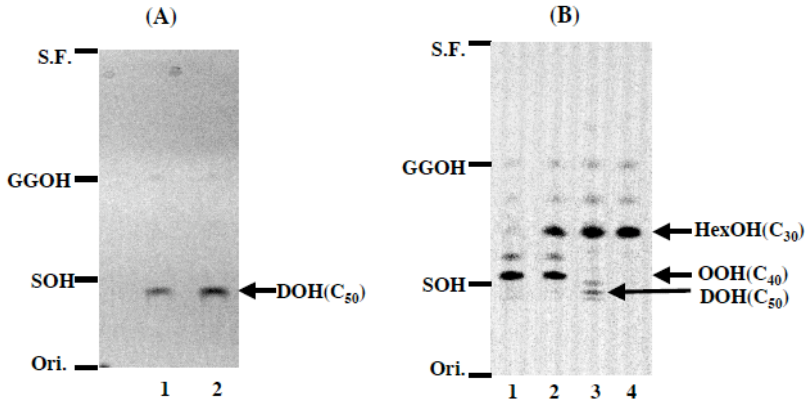

(A)
S.F.
GGOH
SOH
Ori.
DOH(C_{50})
1
2
(B)
S.F.
GGOH
SOH
Ori.
HexOH(C_{30})
OOH(C_{40})
DOH(C_{50})
1
2
3
4

Fig. 7

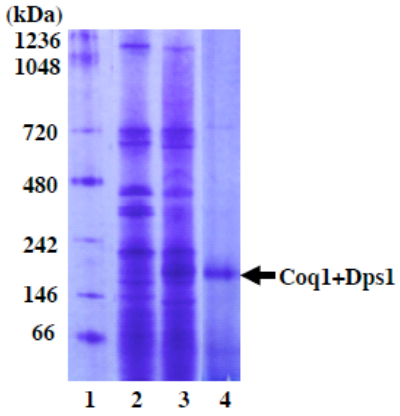


Fig. 8