Studies on a CoQ10 binding protein and CoQ10 synthesizing enzymes

(コエンザイム Q10 結合タンパク質とコエンザイム Q10
生合成系酵素の解析)

Tiezhong CUI

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Abbreviations

BN-PAGE: blue native polyacrylamide gel electrophoresis

CoQ: coenzyme Q

Dds: decaprenyl diphosphate synthase

Dlp1: D-less polyprenyl diphosphate synthase

DM: dodecyl maltoside

DMAPP: dimethylallyl diphosphate

$DMQH_2$: demethoxyubiquinol

DPP: decaprenyl diphosphate

Dps: decaprenyl diphosphate synthase

FPP: farnesyl diphosphate

GPP: geranyl diphosphate

GST: glutathione S-transferase

HexPP: hexaprenyl diphosphate

IPP: isopentenyl diphosphate

IPTG: isopropyl-1-thio-β-D-galactoside

IspB: octaprenyl diphosphate synthase

MEP: 2-C-methyl-D-erythritol-4-phosphate

MVA: mavalonate

OPP: octaprenyl diphosphate

PDS: prenyl diphosphate synthase

PHB: *p*-hydroxybenzoate

PM: pombe minimal

PMU: pombe minimal medium with uracil

Q: coenzyme Q

SPP: solanesyl diphosphate

SPS: solanesyl pyrophosphate synthase

TLC: thin layer chromatography

UQ: ubiquinone

Chapter 1

General Introduction

Coenzyme Q (also called CoQ, Q, ubiquinone or UQ) is a naturally occurring coenzyme, which was firstly discovered by Frederick L. Crane in 1957 [1]. Coenzyme Q distributes widely in living organisms, and is composed of benzoquinone ring and an isoprenoid chain of varying length [2]. For instance, human and *Schizosaccharomyces pombe* produce Q-10 [3,4], mouse and *Zea mays* produce Q-9 [3], *Escherichia coli* produces Q-8 [5], and *Saccharomyces cerevisiae* produces Q-6 [6]. In eukaryotes, Q is located predominantly at the mitochondrial inner membrane, where its primary role is in the transfer of electrons from the membrane-bound dehydrogenases to *bc1* complex of the electron transport chain [7]. Besides, Q acts as an antioxidant that protects from lipid peroxidation by scavenging free radicals directly and/ or by regenerating levels of tocopherol [8]. Bacteria possess several structurally different quinones, among which Q, menaquinone, and demethylmenaquinone are the most common. These quinones are found in the cytoplasmic membrane, where they participate as electron carriers in respiration and in disulfide-bond formation [2]. Q participates in aerobic respiration, whereas menaquinone and demethylmenaquinone have roles in anaerobic respiration.

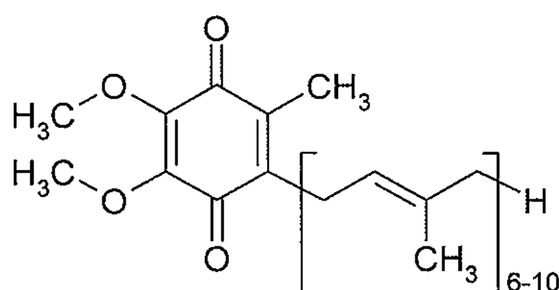


Fig. 1.1 Structure of coenzyme Q.

The length of side chain differs among living organisms, with 6 to 10 isoprene units.

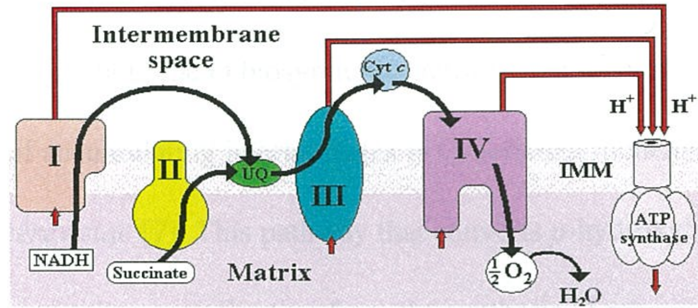


Fig. 1.2 Q in the electron transport chain.

Q accepts electron from NADH and succinate dehydrogenases (complex 1 and 2), and donates them to the *bc1* complex (complex 3) in the mitochondrial respiratory chain.

1.1 Biosynthesis of coenzyme Q in microorganisms

Current knowledge about the Q biosynthetic pathway in eukaryotes is mostly derived from characterization of accumulating intermediates in Q-deficient mutants strains of budding yeast *Saccharomyces cerevisiae* [7]. This pathway that converts *p*-hydroxybenzoate (PHB) into Q includes eight steps in the yeast. Starting from the synthesis of precursor PHB, the isoprenoid chain is condensed and transferred to benzoate ring of PHB. Then the ring is modified with multiple reactions, including methylations, decarboxylation and hydroxylations, to mature Q. So far, most of genes (if additional ones are present) involved in the Q biosynthetic pathway has been identified, but some of them have unknown functions [9]. All the Q synthetic proteins (Coq1-Coq9) localize to the mitochondria in yeast and their import are dependent on a mitochondrial membrane potential. Although *coq* genes are functional in diverse aspects, the absence of any *coq* gene exhibits similar phenotype in yeast, such as inability of growth in the media with non-fermentable carbon source, dramatic reduce in mitochondrial activities and elevated sensitivity to radicals [10,11]. Following is a brief discussion about the functions of Coq protein in the biosynthesis of Q.

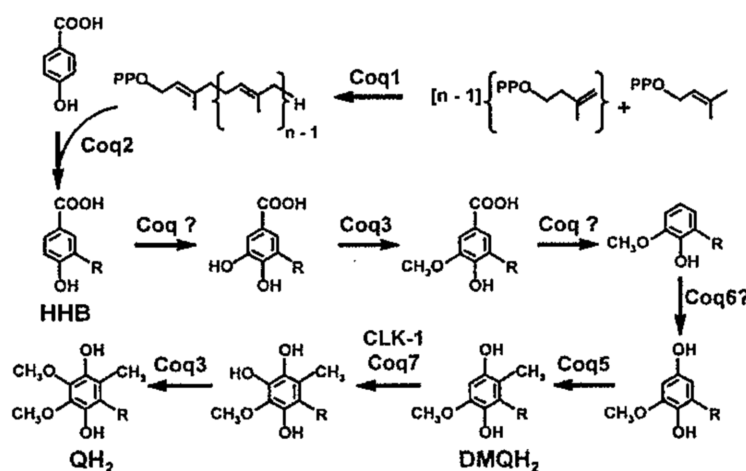


Fig. 1.3 Q biosynthetic pathway in budding yeast [7].

Almost all of the *coq* genes have been identified. Still, the function of Coq4, Coq8 and Coq9 are not known yet, and the reaction steps with a question mark are need to be clarified.

1.1.1 Coq1

Coq1 and its homologs encode prenyl diphosphate synthase, which is responsible for the synthesis of isoprenoid-side chain of Q. The detailed information of these enzymes will be discussed later.

1.1.2 Coq2

Coq2 encodes PHB:polyprenyl diphosphate transferase that catalyses the condensation of PHB with the isoprenoid chain [12,13]. This enzyme generally displays a lack of substrate specificity, because purified UbiA (the Coq2 ortholog in *E. coli*) accepts isoprenoid tail with length ranging from n=2 to n=9 [14]. Also, the purified enzyme from *Pseudomonas putida* has fairly wide substrate specificities [15]. However, the specificity was shown to be influenced by Mg^{2+} concentration in whole yeast extracts [12]. Analysis of the amino acid sequence of the *S. cerevisiae* Coq2 protein revealed two conserved putative substrate binding domains found in a family of poly-prenyltransferases, six potential membrane spanning domain, and a typical mitochondrial targeting sequence [12]. *In vitro* import studies demonstrated that the polypeptide is imported and fully processed within the mitochondria [16]. The recent data indicates that the Coq2 protein behaves as an integral membrane protein associated to the inner mitochondrial membrane, facing the matrix side.

1.1.3 Coq3

Coq3 encodes an *O*-methyltransferase, which is required for two *O*-methylation steps in the Q biosynthetic pathway [17]. Homologs of the *coq3* gene in rat, *A. thaliana*, and human can functionally complement the absence in yeast [18-20], indicating the conservation of *coq3* in a wide range of organisms. The Coq3 protein contains four regions that are conserved in a large family of methyltransferases utilizing S-adenosylmethionine (SAM or AdoMet) as the methyl

donor and requiring a divalent cation [21]. As other Coq polypeptides, Coq3 contains a mitochondrial targeting sequence, and is imported and processed into the mature form in the mitochondria in a membrane-potential-dependent manner. The localization analysis showed that Coq3 is a peripheral protein associated to the matrix side of the inner mitochondrial membrane [22].

1.1.4 Coq4

Coq4 is absolutely required for the biosynthesis of Q, but its enzymatic function has not been elucidated [23]. The amino acid sequence of Coq4 does not share significant homology with protein domains or motifs with known enzymatic activity. However, the absence of Coq4 leads to a reduced level of Coq3 and Coq7 proteins, and Coq4 co-migrates with Coq3, Coq6 and Coq7 as a high molecular mass complex, indicating that the Coq4 protein has a structural role in the Q biosynthetic complex [22,24].

1.1.5 Coq5

Coq5 catalyzes the only *C*-methylation step in Q biosynthesis [25]. The amino acid sequence of Coq5 contains four motifs present in a large family of AdoMet-dependent methyltransferases. *In vitro C*-methyltansferase assays with the farnesylated analogs of the corresponding intermediates confirmed that Coq5 is required for the conversion of 2-methoxy-6-polyprenyl-1,4-benzoquinone to 2-methoxy-5-methyl-6-polyprenyl-1,4-benzoquinone. The localization analysis demonstrated that Coq5 is peripherally associated with inner mitochondrial membrane on the matrix side [25]. Also, the recent data indicated that Coq5 is a component of Q biosynthetic complex [26].

1.1.6 Coq6

Coq6 encodes a putative monooxygenase responsible for the addition of hydroxy group to

4-hydroxy-3-polyprenyl benzoic acid and/or 6-methoxy-2-polyprenyl phenol, two uncharacterized hydroxylation steps in Q biosynthesis [27]. This idea came from the amino acid sequence analyses of Coq6 protein, which contains three conserved domains: an ADP-binding fingerprint, a motif with a putative dual function in FAD/NAD(P)H binding and a consensus sequence that binds to the ribityl moiety of FAD. Yet, the direct proof should be provided to confirm this idea. Similarly, Coq6 is peripherally associated with the matrix side of the inner mitochondrial membrane.

1.1.7 Coq7

Coq7 is a well-characterized monooxygenase required for the hydroxylation of 5-demethoxyubiquinol ($DMQH_2$) in several organisms [24,28,29]. The direct proof is that the deletion of *coq7* resulted in the accumulation of DMQ in *S. pombe*. The Coq7 protein belongs to a family of di-iron-binding oxidases containing a conserved motif, FXXH, for the iron ligands [24]. Moreover, expression of *coq7* homologs from *C. elegans*, rat, or human were shown to rescue the yeast *coq7* null mutant for growth on non-fermentable carbon sources [30,31], indicating functional conservation across species. The previous studies held different opinions toward the location of Coq7 in mitochondria. Clarke and coworkers showed that Coq7 is peripherally associated to the inner membrane [24]. On the contrary, an earlier work exhibited that Coq7 is an interfacial membrane protein [32]. Thus, the true nature of the Coq7 protein-membrane association awaits a structure determination for Coq7 or one of its homolog. The recent data also indicated that Coq7 is involved in the formation of Q biosynthetic complex [22].

1.1.8 Coq8

Coq8 was initially identified as *ABC1* (activity of *bc1* complex) for its ability to partially

suppress the cytochrome *b* translation defect due to the mutation of the *cbs2* gene, which encodes a translational activator of cytochrome *b* [33]. It was shown that the respiratory complex 2, 3 and 4 of the *abc1* null mutant were thermo-sensitive and addition of exogenous Q could partially compensate for the respiratory deficiency, indicating that *abc1* is required for the conformation and activity of *bc1* complex and its neighboring complexes [34]. However, in a later report Do exhibited that *abc1* is identical to *coq8* and is required for the Q biosynthesis [35]. Also, the pleiotropic defects of *abc1* observed previously are derived from the Q deficiency. Coq8 has been classified as a putative protein kinase on the presence of kinase conserved motifs in its primary structure. Recent results in fact demonstrated that Coq8 phosphorylates Coq3, and for that reason it is considered to be a regulator of Coq enzymes [36].

1.1.9 Coq9

Coq9 is an protein required for the biosynthesis of Q with unknown function [10]. Coq9 homologs are distributed across species, but it does not have homology to proteins with known function. The recent data indicate that Coq9 was co-sendimented with Coq3 and Coq5 and might be involved in the formation of Coq complex [26]. Also, the localization analysis demonstrated that Coq9 protein is a peripheral membrane protein associated with the matrix side of the mitochondrial inner membrane.

1.1.10 A complex theory

There are several well-characterized complexes in mitochondria, such as complexes of dehydrogenases, *bc1* complex and ATP synthase, so it is not surprising to regard that cells employ a complex for Q biosynthesis. The complex allows channeling intermediates, enhance catalytic efficiency, and provide a better coordination. This seems to be the case in Q

biosynthesis as well.

The existence of a Coq complex came from several lines of evidence. First, each of *S. cerevisiae coq3* to *coq9* mutants predominantly accumulates the same earlier intermediates HHB, the product of Coq2p, instead of the corresponding diagnostic intermediate [10]. Also, the levels of Coq3, Coq4, Coq6, Coq7 and Coq9 polypeptides are significantly decreased in mitochondria isolated from any of the other *coq* null mutants [24]. Second, the biochemical analyses provide physical evidence for the model of Q biosynthetic complex. Size exclusion chromatography (gel filtration) coupled with *O*-methyltransferase assays of the supernatant from digitonin-solubilized mitochondria demonstrates that Coq3, Coq4, Coq6 and Coq7 polypeptides co-elutes as a high molecular mass complex with Coq3 *O*-methyltransferase activity [24]. Third, the recently popular application of Blue Native PAGE brought us further evidence of Coq complex. The data showed that Coq2, Coq3, Coq4, and Coq9 proteins co-migrate as a high molecular mass complex or complexes [24]. Moreover, Coq9-HA was recently demonstrated to physically interact with Coq4, Coq5, Coq6, and Coq7 proteins via co-precipitation. All together, these results support the existence of a multi-subunit Q biosynthetic complex.

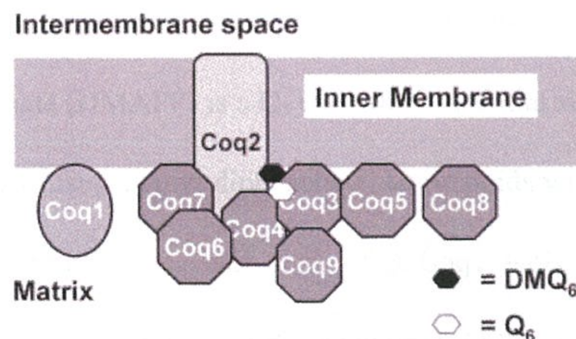


Fig. 1.4 A model of the mitochondrial Q biosynthetic protein complex in *S. cerevisiae* [7]. The putative complex contains six Coq polypeptides which are peripherally associated with the inner membrane and a spanning intergral membrane Coq protein, Coq2.

1.2 Biosynthesis of Q isoprenoid chain

The isoprenoid side chain of Q is synthesized through the mevalonate (MVA) pathway in eukaryotes, but is synthesized through the MEP (2-C-methyl-D-erythritol-4-phosphate, or non-mevalonate) pathway in prokaryotes or in plant chloroplasts [37]. DMAPP (dimethylallyl diphosphate), the isomer of IPP (isopentenyl diphosphate) is a primer for the condensation of IPP to construct isoprenoid with a certain length. A side chain longer than C_{25} and shorter than C_{55} is general for Qs in nature.

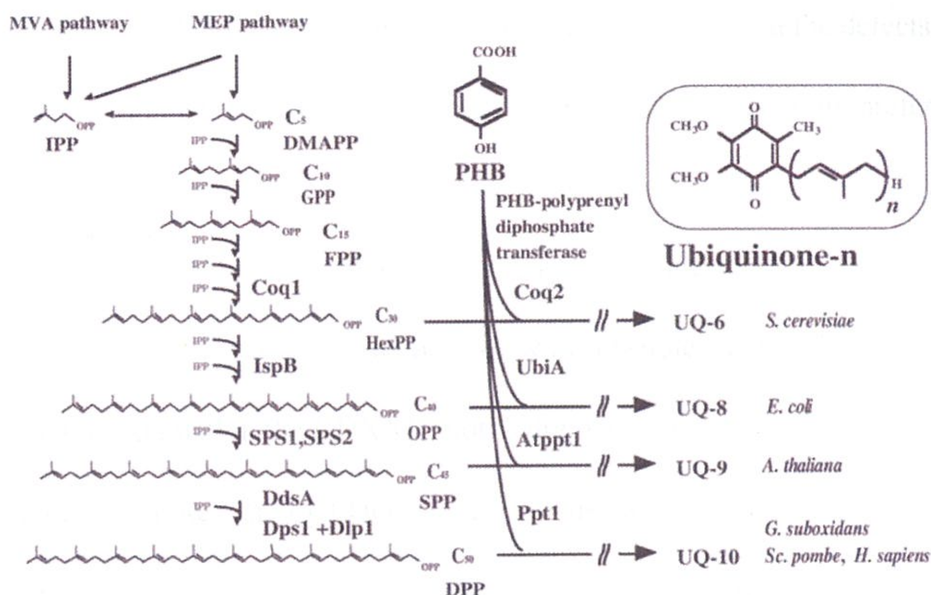


Fig. 1.4 Synthesis of the isoprenoid side-chain of ubiquinone.

Dimethyl allyl diphosphate (DMAPP) is a C_5 unit compound that serves as the precursor to condense multiple units of isopentenyl diphosphate. Isoprenoids with more than C_{25} units are generally used for the synthesis of the side-chain of Q. Coq1, IspB, SPS1 (or SPS2) and the Dps1–Dlp1 complex are hexaprenyl (HexPP), octaprenyl (OPP), nonaprenyl and decaprenyl (DPP) diphosphate synthases that produce the isoprenoid side-chains of Q-6, Q-8, Q-9 and Q-10, respectively. PHB-polyprenyl diphosphate synthase condenses PHB and prenyl

diphosphate. MEP, methylerythritol phosphate; MVA, mevalonic acid.

Prenyl diphosphate synthases (PDS) determine the length of isoprenoid chain and thus the species of Q. For instance, according to the specificities of enzymes, *S. cerevisiae* has six units of isoprene in its Q side chain, *Candida utilis* has seven units, *E. coli* has eight units, mice and *Arabidopsis thaliana* have nine units, and *Schizosaccharomyces pombe* and humans have 10 units [2]. However, the length of isoprenoid chain seems not crucial for the functions of Q, because Qs with diverse isoprene units can be replaceable in *E. coli* and yeast cells [38]. For example, expression of PDSs producing 6 to 10 isoprene units recovered the defects of *S. cerevisiae coq1* null mutant respectively, although the native one was slightly preferred.

The PDS responsible for the side chain of Q is *trans*-PDS, which shares seven conserved regions and two aspartate-rich motifs DDXXD in amino sequences [39]. The two DDXXD motifs are responsible for the catalysis and binding with substrates in association with Mg^{2+}. Alteration of Asp to Ala within the DDXXD motifs dramatically affects the kinetics of PDS. For the binding of substrates, the first DDXXD is responsible for the FPP or its products, and the second one for IPP [40,41]. The most important site for the determination of the length was the fifth amino acid position from the first aspartate-rich region, as proposed from the analysis of FPP synthase and GGPP synthase [42,43]. It was also found that the formation of an IspB dimer is essential for the determination of the length. The structure of IspB in *Thermotoga maritima* was recently solved and was found to be very similar to that of FPP synthase [44].

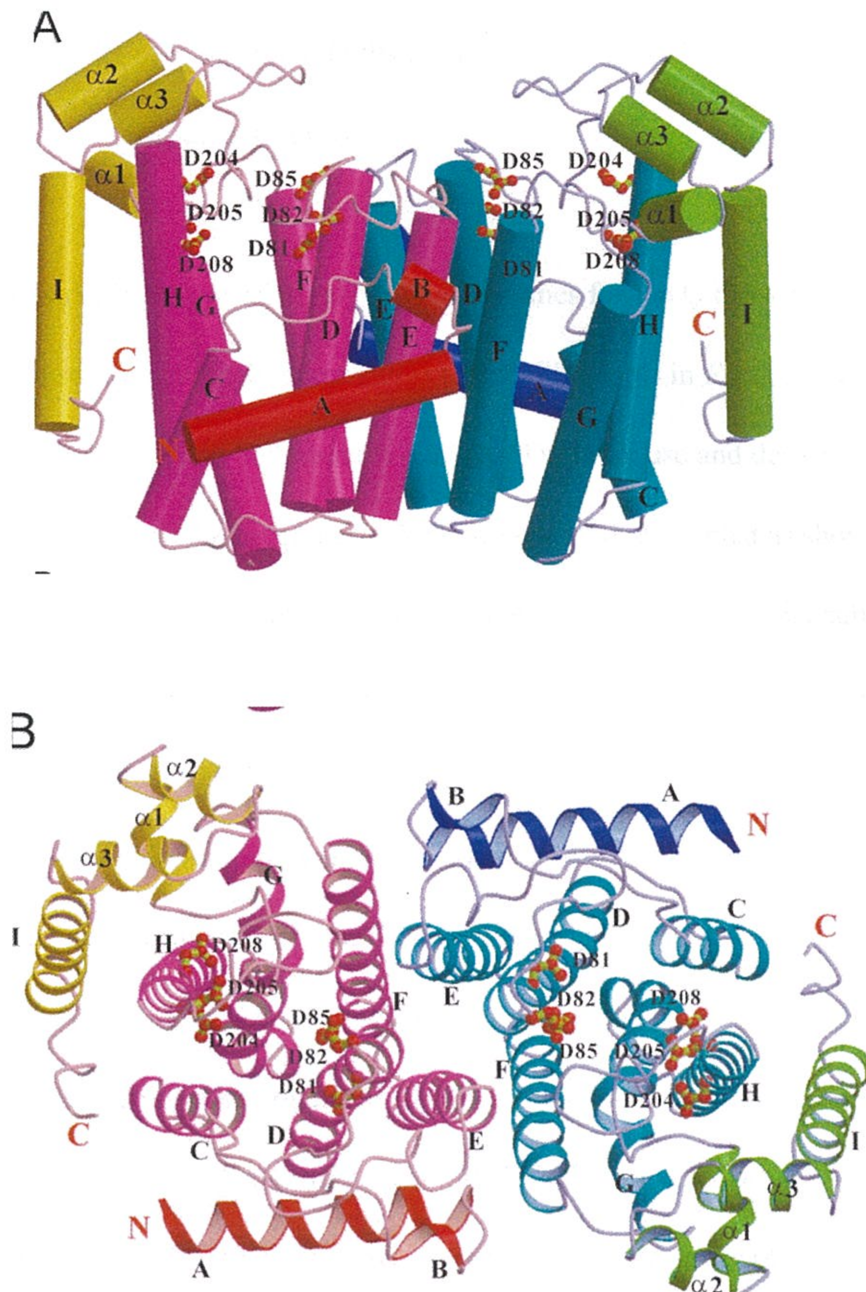


Fig. 1.5 *A*, the side view model of *T. maritima* OPPs is shown using a cylinder diagram. Two identical subunits are associated into a dimer by forming a four-layer helix bundle. The N-terminal α helical hairpins in the outer layers are shown in *blue* and *red* for the two individual subunits, whereas the eight helices in the inner layers are shown in *cyan* and *magenta*. Two small peripheral domains are colored *green* and *yellow*. *Black arrows* indicate the location of the active site with the Asp residues in the two DD*XX*D motifs shown in *red*. In

B, the top view model of *T. maritima* OPPs is shown using a ribbon diagram. The color and styles shown here are the same as above.

On the other side, PDSs are relatively diverse enzymes for the Q biosynthesis. They have two types of constitution, namely homomer (i.e. octa-PDS IspB in *E. coli* [5] and hexa-PDS Coq1 in *S. cerevisiae* [6]) and heteromer (i.e. nona-PDS in mouse and deca-PDS in *S. pombe* and human [3]). Although the homomeric PDSs were well documented as shown above, the heteromeric ones are still poorly understood. The heteromeric PDSs consist subunit 1 bearing a very high identity in amino sequences with other homomeric PDSs, and subunit 2 (Dlp1, D-less PDS) lacking the DDXXD motif. Both subunits are essential for the Q biosynthesis. In an animal model, mutation in mouse *dlp1* was found to be the cause of renal disease [45]. Human genetic disorder in the *dps1* and *dlp1* were reported to be the cause of Leigh syndrom [46]. Then, a question was raised why higher organisms employ a heteromeric PDS, rather than a homomeric one in lower organisms, for the synthesis of the dispensable CoQ. Very recently, Zhang *et al* reported an artificial deca-PDS in fission yeast, which was composed of budding yeast Coq1 and fission yeast Dps1. This work supports the evolutionary conservation of PDS and indicates that the cellular environment is also important for the activity of PDS [6].

1.3 Regulation of Q function and biosynthesis

Compared with the clarity of Q biosynthesis, its regulation is far from clearance. It was believed that Q transfers electron in a protein-mediated manner within the mitochondrial dehydrogenases [47-49]. Most of the other Q, however, exists in a Q pool in the mitochondrial membrane [50]. It is not clear yet that how the Q pool is regulated in the mitochondrial membranes. Recently, the finding of a Q-binding protein brings us some clues [51]. The protein was termed as Coq10, following the other nine Q biosynthetic proteins (Coq1-Coq9), but the data excluded the possibility that Coq10 is required for the biosynthesis of Q. Similar to previously characterized Coq mutants, the yeast *coq10* mutant exhibits defective NADH-, succinate-ubiquinone reductases and ubiquinone-cytochrome c reductase, which can be restored to near wide-type level by addition of Q2. Unlike the other *coq* mutants, however, the *coq10* mutant grows slowly on medium containing non-fermentable carbon source. Interestingly, the slow-growing phenotype of the *coq10* mutant on medium containing ethanol and glycerol was partially rescued by exogenous Q6 supplementation or multi-copy expression of the *coq2, coq7* or *coq8* genes to increase the synthesis of Q. This suggests that the endogenous Q in a *coq10* mutant is not so functional as that in wild-type cells and higher levels of Q can complement the absence of the *coq10* gene, indicating a regulatory role of Coq10 for the function of Q. Also, the purified Coq10 from yeast cells contained Q6, which supports Coq10 is a Q-binding protein [51]. What's the real function of Coq10? Sequence analysis of yeast Coq10 protein, as well as its homologs in *Caulobacter crescentus* and other eukaryotes, identifies it as a member of the protein superfamily containing the START domain. The solution structure of the *C. crescentus* homolog of Coq10 identified a hydrophobic tunnel which in other START family members functions in binding cholesterol,

polyketides, or phospholipids. Because polypeptides belonging to this superfamily have been shown to be involved in lipid binding and trafficking, it is likely that Coq10 protein may function in transport and/or directing newly synthesized Q to its correct location in the mitochondrial electron transport chain.

In *S. cerevisiae*, Q levels are directly correlated to mitochondrial development and oxygen availability [52], so the biosynthesis of Q can be regulated by the respiratory robustness. As reported, biosynthesis of Q was found to be highest in aerobically grown cells and barely detectable in anaerobic cells [7]. Also, when *S. cerevisiae* cells were grown in aerobic batch cultures, the amounts of Q varied depending on carbon catabolites [53]. High glucose concentration inhibited Q biosynthesis to a higher degree than similar concentration of galactose, a non-repressing fermentable carbon source. Consistently, Q production was greatly increased in media containing non-fermentable carbon source, when catabolite repression is at the minimum. To clarify the underlying mechanism of carbon source on Q production, the expression of *coq* genes was systematically analyzed. The studies have demonstrated that mRNA levels of *coq3, coq4, coq5* and *coq7* genes were higher in yeasts grown in glycerol-containing media than in cultures containing fermentable dextrose [54,55]. The amount of Coq7 polypeptide was significantly increased by growth on media containing ethanol [24]. The future work need to address the mechanism that how the carbon catabolites control the Q biosynthesis and the dynamics of the multi-subunit Q complex.

1.4 Composition of this thesis

In this thesis, Chapter 1 introduces a detailed background knowledge and current progress of the related research. Chapter 2 reports a novel artificial prenyl diphophate synthase with an unexpected strucutre. Chapter 3 describes a Q-binding protein required for multiple functions of Q in fission yeast. Finally, in chapter 5 the author concludes this thesis.

Chapter 2

A subunit of decaprenyl diphosphate synthase stabilizes octaprenyl diphosphate synthase in *E. coli* by forming a high-molecular weight complex

2.1 Abstract

The length of the isoprenoid-side chain in ubiquinone, an essential component of the electron transport chain, is defined by poly-prenyl diphosphate synthase, which comprises either homomers (e.g., IspB in *Escherichia coli*) or heteromers (e.g., Dps1 and Dlp1 in *Schizosaccharomyces pombe* and humans). I found that expression of either *dlp1* or *dps1* recovered the thermo-sensitive growth of an *E. coli* $ispB^{R321A}$ mutant and restored IspB activity and production of Coenzyme Q-8. IspB interacted with Dlp1 (or Dps1), forming a high molecular weight complex that stabilized IspB, leading to full functionality.

2.2 Introduction

Q, which is composed of a benzoquinone ring and an isoprenoid tail, is an essential factor for aerobic respiration in living cells. The Q biosynthetic pathway in eukaryotes (the synthesis of a prenyl tail, the combination of the quinone moiety with the prenyl tail, and a series of modifications to the quinone backbone [2]) has been elucidated mainly in *Saccharomyces cerevisiae* [7]. Much data exists showing that this pathway (apart from the synthesis of the prenyl tail) is conserved in a wide range of eukaryotes [56]. The length of the prenyl tail varies between different organisms. For instance, *S. cerevisiae* has 6 isoprene units in its Q side chain, whereas *Escherichia coli* has 8, mice have 9, and both *Schizosaccharomyces pombe* and humans have 10 [9]. However, the length of the isoprenoid chain seems not to be crucial to function because Q in *E. coli* and yeast cells can be replaced with molecules containing side-chains of varying lengths with no adverse effects [38,57,58].

It is known that poly-prenyl diphosphate synthase (poly-PDS), which defines the length of the ubiquinone tail, is either homomeric (i.e., octa-PDS IspB in *E. coli* [59] and hexa-PDS Coq1 in *S. cerevisiae* [6]), or heteromeric (i.e., nona-PDS in mouse, and deca-PDSs in *S. pombe* and human [3,4]). Although the homomeric poly-PDSs are well documented, heteromeric poly-PDSs are not. The heteromeric poly-PDSs consist of two subunits: subunit 1, which has an amino acid sequence with high homology to other *trans*-PDSs; and subunit 2 (Dlp1, D-less PDS), which lacks the DDXXD motifs [4]. This raises two questions: why do higher organisms employ a heteromeric PDS, rather than a homomeric one, for the synthesis of Q; and does the heteromeric poly-PDS have advantages over the homomeric one?

In this study, I identified and characterized two novel, artificial, poly-PDSs, namely the IspB-Dps1 complex and the IspB-Dlp1 complex, in *E. coli*. Although fission yeast Dps1 or

Dlp1 are not themselves functional in *E. coli*, they can bind to IspB, forming a high-molecular weight complex that promotes its stability. Thus, my data show that heteromeric PDS has advantages over the homomeric PDS, and might represent an evolutionary trend.

2.3 Materials and methods

Materials

DNA markers and restriction enzymes were obtained from TOYOBO (Osaka, Japan). Protein markers were obtained from Fermentas Life Sciences (Ontario, Canada) and Oriental Yeast (Tokyo, Japan). Antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Isopentenyl diphosphate (IPP) and all-*E*- farnesyl diphosphate (FPP) were obtained from Sigma Chemical Co. (St. Louis, MO, USA). [1-^{14}C]IPP (1.96 TBq·mol^{-1}) was obtained from Amersham (Little Chalfont, UK). Kieselgel 60 F254 TLC plates were purchased from Merck (Rahway, NJ, USA). Reversed-phase LKC-18 thin-layer plates were obtained from Whatman (Maidstone, UK). The Blue Native-PAGE NOVEX Bis-Tris Gel System and the NativeMark Unstained Protein Standard were obtained from Invitrogen (Osaka, Japan). Blue Native-PAGE was performed according to the manufacturer's instructions.

Plasmid construction

To construct pBQ-His-ispB or pBQ-His-ispB^{R321A}, the primers 5'-CGGATCCGATGAATTTAGAAAAAATC-3' and 5'-CGAAGCTTGGCCATGGGCGCG-3' were used to amplify *ispB* from pKO56 [56] and pBRA(R321A). The amplified fragments were first cloned into the *Bam*HI and *Hin*dIII sites of a pQE-31 vector (Qiagen), and the fragments containing *His$_6$-ispB* and *His$_6$-ispB^{R321A}* were digested with *Eco*RI and *Hin*dIII, before cloning into the same restriction sites in pBluescript II KS+ (Stratagene). To construct pSTVK-msps1, *mSPS1* was released from the pBmSPS1plasmid using *Eco*RI and *Kpn*I [3], and cloned into the same sites in pSTVK28. To construct pSTVK-hdps1, *hDPS1* was digested from pGEX-hdps1 using *Bam*HI and *Xba*I [3],

and cloned into the same sites in pSTVK28.

To construct pGKO56-R321A, site-directed mutagenesis was performed using a QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene, Tokyo, Japan) as previously described [60]. Briefly, a pair of reverse-complementary primers (5'-CATCGCTGTTCAAGCCGATCGTTAATCC-3', only the forward primer shown) was used to amplify pGKO56. The amplified PCR products were self-ligated, and the plasmid was recovered from *E. coli* to obtain pGKO56-R321A. The sequences of the mutants were confirmed using an ABI3100 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

Purification of GST-IspB from E. coli

GST-IspB, GST-IspB^{R321A} and GST were purified as previously described [6].

Q extraction and PDS assay

Q was extracted and measured as previously described [60]. PDS activity was measured as previously described [3].

Table 1. Plasmids used in this study. Ap, ampicillin; Km, kanamycin.

Plasmids	Characteristics	Sources
pSTVK-msps1	Km, full-length mouse *SPS1* in pSTV28K	This study
pSTVK-hdps1	Km, full-length human *DLP1* in pSTV28K	This study
pBQ-His-ispB	Ap, His_6 with full-length *ispB* in pBluescript II KS+	This study
pBQ-His-ispB^{R321A}	Ap, His_6 with full-length *ispB*^{R321A} in pBluescript II KS+	This study
pBRA(R321A)	Ap, 2.5-kb fragment including full-length *ispB*^{R321A} in pBluescript	[5]
pSTVK-His-dps1	Km, His_6 with full-length *dps1* in pSTVK28	[6]
pSTVK-His-dlp1	Km, His_6 with 1.1-kb fragment including full-length *dlp1* in pSTVK28	Lab stock
pSTVmDLP1	Km, full-length mouse *DLP1* in pSTVK28	[3]
pSTVhDLP1	Km, full-length human *DLP1* in pSTVK28	[3]
pGEX-1	Amp, *tac* promoter, GST tag, high expression vector	Amersham
pGKO56	Amp, full-length *ispB* gene in pGEX-1X	[5]
pGKO56-R321A	Amp, full-length *ispB*^{R321A} gene in pGEX-1X	This study
pSTVK-His-hDPS1	Km, His_6 with full-length human *DPS1* in pSTVK28	This study
pSTVK-HIS-hDLP1	Km, His_6 with full-length human *DLP1* in pSTVK28	[3]

2.4 Results

Expression of dps1 or dlp1 reversed the thermo-sensitivity of the E. coli ispB^{R321A} mutant.

It is known that the *ispB* gene is essential for *E. coli* growth [58] and that Arg321 is important for the thermo-stability of IspB; so *ispB*^{R321A} mutants grow very slowly at 43°C [5]. Surprisingly, when *S. pombe dps1* was expressed in the *ispB*^{R321A} mutant, the growth of the co-expressed cells was similar to wild-type cells (Fig. 2.1). *S. pombe dlp1* also reversed the thermo-sensitivity of the *ispB* mutant in a similar manner (Fig. 2.1). Also, I observed that both human and mouse *mDLP1* and *hDLP1,* but not *mSPS1* or *hDPS1*, rescued the growth of the *ispB*^{R321A} mutant at 43°C (Fig. 2.1), indicating the functional conservation of Dlp1 in a broad range of organisms. Because neither Dlp1, nor Dps1 alone can function as an active PDS, these observations suggest that both Dps1 and Dlp1 are capable of supporting the IspB activity in *E. coli*.

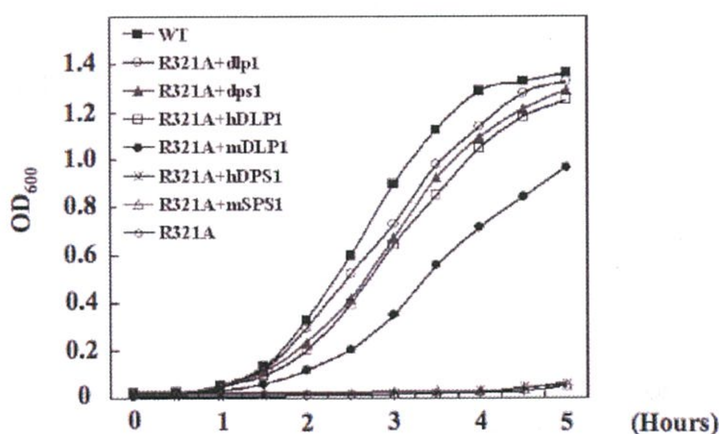


Fig. 2.1 *dps1* or *dlp1* reverses the thermo-sensitivity of an *E. coli ispB*^{R321A} mutant. An overnight culture of the indicated strains was diluted to OD_{600} = 0.02, and the cells were incubated with vigorous shaking at 43°C. Cell mass was calculated every 30 minutes. *E. coli* strain KO229 (Δ*ispB* harboring an *ispB*-containing plasmid) [58] was used as the wild-type

control. The plasmid pKA3 in the KO229 *E. coli* strain was replaced by pBRA (R321A) in order to express the mutant *ispB*. Expression of *dps1, mSPS1*, or *hDPS1* from *S. pombe*, mouse, or human, respectively, was achieved by introduction of pSTVK-His-dps1, pSTVK-msps1, or pSTVK-hdps1 into the mutant *ispB* cells. Expression of *dlp1, mDLP1*, or *hDLP1* from *S. pombe*, mouse, or human, respectively, was achieved by the introduction of pSTVK-His-dlp1, pSTVmDLP1, or pSTVhDLP1 into the mutant *ispB* cells.

IspB plays a major role in Q synthesis in the dps1 or dlp1 co-expressed cells.

I then analyzed the Q-species in the co-expressed cells to ascertain the role played by Dps1 or Dlp1 within the complex, as *dps1* and *dlp1* are responsible for the synthesis of Q-10, and *ispB* for the synthesis of Q-8. The expression of *dps1* or *dlp1* by the $ispB^{R321A}$ mutant did not change the ubiquinone species at either 37˚C (data not shown), or 43˚C (Figs. 2.2C and D); also, the Q-8 levels in all the samples were similar (Fig. 2.2). This suggests that IspB plays a major role in Q-8 synthesis within the co-expressed cells, and that the contribution of Dps1 or Dlp1 may be to assist the function of IspB.

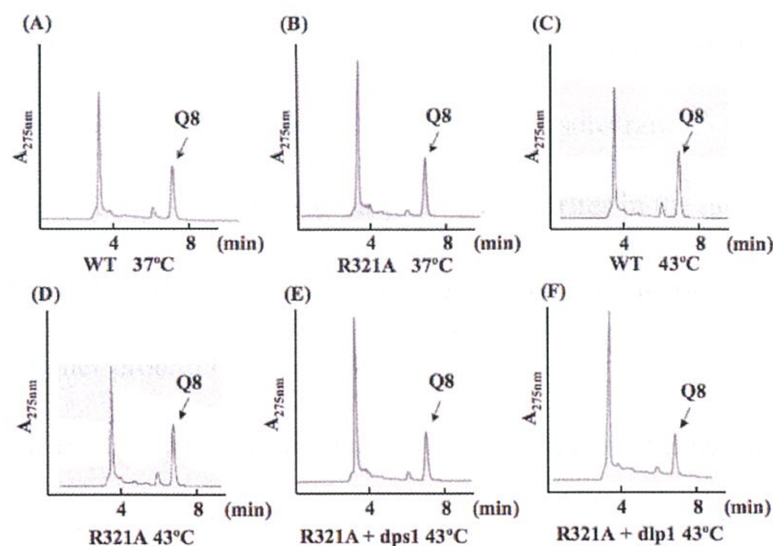


Fig. 2.2 Expression of *dps1* or *dlp1* does not alter the Q species in *E. coli*.

Both the wild-type and the *ispB*^{R321A} *E. coli* strains were incubated at the indicated temperatures and ubiquinone was extracted. To test the effect of *dps1* and *dlp1* on ubiquinone synthesis, the plasmid harboring *dps1* or *dlp1* was introduced into the mutant *ispB*^{R321A} cells.

Low PDS activity in the early growth phase of the ispB^{R321A} mutant leads to its thermo-sensitivity, and is complemented by dps1 or dlp1.

To further analyze the roles of Dps1 and Dlp1, and the reason for defects in the IspB mutant, I looked at the enzymatic activities of poly-PDSs at both 30°C and 43°C, and at several points in the growth phase, by measuring the intensity of incorporated radioactive products. As expected, neither *dps1*, nor *dlp1* affected the activity of the mutant IspB at 30°C (Fig. 2.3A, left). However, the expression of *dps1* or *dlp1* led to a 5-fold increase in activity at 43°C at OD_{600} = 0.4 (Fig. 2.3A, right). Because neither *dps1*, nor *dlp1* changed the level of IspB expression (Fig. 2.3B), it is reasonable to suppose that *dps1* or *dlp1* promotes the stability of the mutant IspB via a physical interaction. Interestingly, at the later point in the growth phase, the PDS activity of the mutant cells increased very quickly and was similar to that of the co-expressed cells at 43°C (Fig. 2.3A right). This may have been due to increased IspB levels after OD_{600} = 0.4 (Fig. 2.3B) and the limited availability of substrate. Consistently, the thermo-sensitivity of the IspB mutant was only observed earlier in the growth phase, and the mutant cells showed a wild-type-like generation time in the exponential phase. Thus, these data indicate that higher protein levels can compensate for the structural defects within the IspB mutant. To the contrary, the low activity of the mutant IspB during the early phase of growth could not support normal growth, and so led to the thermo-sensitivity of the mutant

cells.

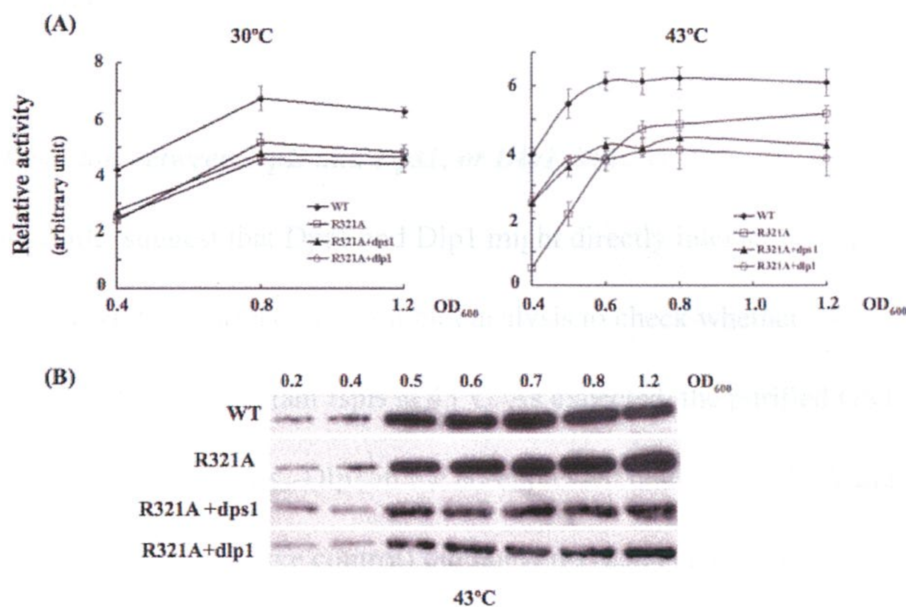


Fig. 2.3 Analysis of PDS activity and IspB protein levels.

(A) Growth phase-specific activity of PDSs in the *E. coli* cells.

The *E. coli* cells were grown at both 30˚C and 43˚C and collected at the indicated OD_{600} values. The PDS activity of *E. coli* was assayed at both 30˚C and 43˚C using [1-^{14}C]IPP and FPP as substrates. After removal of excess [1-^{14}C]IPP, the amount of radioactivity in the products was measured to give an indication of PDS activity. The strains used were: WT, Δ*ispB* harboring pBQ-His-ispB; R321A, Δ*ispB* harboring pBQ-His-ispB^{R321A}; R321A+dps1, Δ*ispB* harboring pBQ-His-ispB^{R321A} and pSTVK-His-dps1; and R321A+dlp1, Δ*ispB* harboring pBQ-His-ispB^{R321A} and pSTVK-His-dlp1. The data represent the means ± SD of five separate measurements.

(B) Phase-specific IspB levels in the *E. coli* cells. *E. coli* cells were grown at 43˚C, and collected at the indicated OD_{600} values. Equal amounts of the crude extracts obtained from the cells were used for immunoblot analysis with an anti-penta-His antibody (diluted 1:1000). The

bands corresponding to His_6-IspB or His_6-IspB^{R321A} were then compared. The same strains were used as in (A).

Physical interaction between IspB and Dps1, or Dlp1, in E. coli

The above results suggest that Dps1 and Dlp1 might directly interact with and stabilize IspB in *E. coli*. Next, I performed Western blot analysis to check whether Dps1 or Dlp1 was co-immunoprecipitated with mutant IspB at 43˚C. As expected, the purified GST-IspB^{R321A} bound to either His_6-Dps1 or His_6-Dlp1 in the co-expressed cells (Figs. 2.4A and B, lane 1), and the purified GST (the negative control) did not (Figs. 2.4A and B, lane 4). The binding was not limited to the IspB mutant. The wild-type GST-IspB bound to His_6-Dps1 or His_6-Dlp at both 43˚C and 37˚C. This confirms that Dps1 and Dlp1 reverse the thermo-sensitivity of the IspB mutant cells by increasing the stability of IspB.

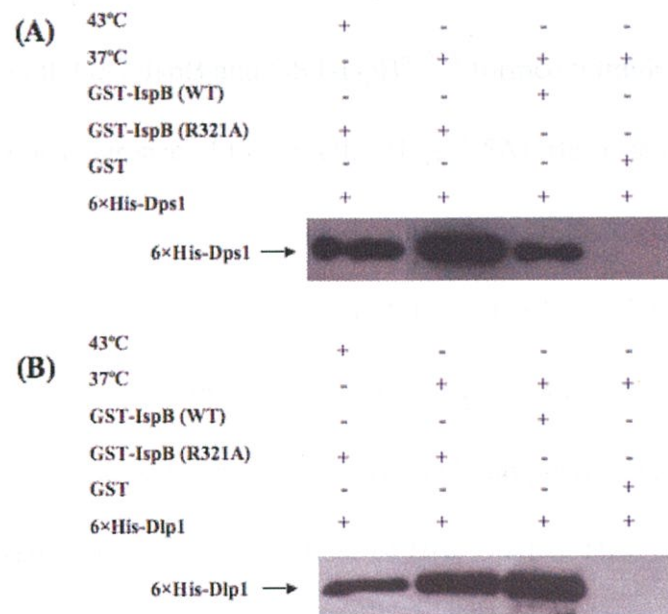


Fig. 2.4 Physical interactions of Dps1-IspB and Dlp1-IspB in *E. coli*

(A) Dps1 binds to IspB in *E. coli. E. coli* DH5α cells harboring the corresponding plasmids

were grown at the indicated temperatures. The purified GST-IspB, GST-IspB^{R321A} and GST samples were then subjected to Western blot analysis using an anti-penta His antibody to examine the co-precipitation of Dps1 with IspB. pGKO56 and pGKO56-R321A were used to express GST-IspB and GST-IspB^{R321A}, respectively. pGEX-1 was used to express the GST protein and pSTVK-His-dps1 for His$_6$-Dps1.

(B) Dlp1 binds to IspB in *E. coli*. As in (A), except that the *dps1* expression plasmid was replaced by the *dlp1* expression plasmid, pSTVK-His-dlp1.

IspB-Dps1 or IspB-Dlp1 form high-molecular weight complexes in E. coli

As shown previously, both the native Dps1-Dlp1 complex and the artificial Coq1-Dps1 complex form a heterotetramer in *S. pombe* [3,4,6]. To investigate how Dps1 and Dlp1 interact with IspB, I employed Blue Native PAGE to analyze these complexes. First, I looked at the homomeric IspB complex, which is known to form a homodimer *in vitro* [5]. Unexpectedly, both GST-IspB and GST-IspB^{R321A} formed tetramers, dimers and polymers, as judged by the molecular size of the proteins (Fig. 2.5A). Because Blue Native PAGE maintains proteins in a more 'native' state compared with the cross-linking technique, these data imply that tetramers might be the main form of IspB found in *E. coli*. It also indicates that mutation of R321A does not prevent IspB from forming these high order structures. We also analyzed the size of the IspB-Dps1 and IspB-Dlp1 complexes. Because the expression levels of GST-IspB were much higher than those of His$_6$-Dps1 or His$_6$-Dlp1 in the co-expressed cells, the amounts of the IspB-Dps1 or IspB-Dlp1 complex are not comparable with those of the IspB complex. Therefore, immunoblotting must be used to detect the heteromeric complexes. To my surprise, the molecular weight of both the IspB-Dps1 and the IspB-Dlp1

complexes was approximately 800 kDa (Fig. 2.5B, lane 2 and 3). Clearly, these were novel heteromeric poly-PDSs, and were quite different from the heterotetramers seen previously. In addition, we found that a human Dps1 homolog did not form such a complex with IspB (Fig. 2.5B, lane 4). These data suggest a structural difference between *S. pombe* Dps1 and its mammalian homologs, which is supported by a report showing that *S. pombe* Dps1 cannot be complemented by mammalian Dlp1 [3].

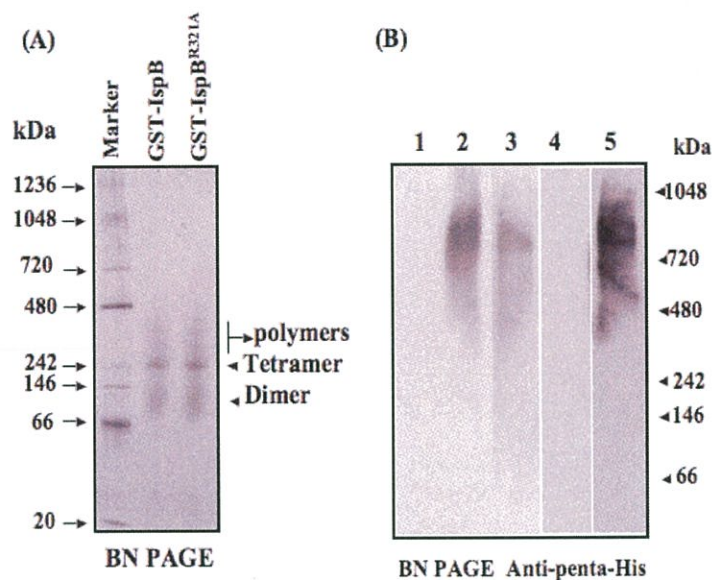


Fig. 2.5 High-molecular weight complexes formed by Dps1-IspB and Dlp1-IspB.

(A) IspB forms a tetramer in *E. coli*. Δ*ispB* harboring pGKO56 and Δ*ispB* harboring pGKO56-R321A were grown at 43°C and used to purify wild-type and mutant IspB, respectively. The purified GST-IspB was then analyzed using the Blue Native PAGE system on 4–16% Bis-Tris gels. Molecular weight markers range in size from 20 to 1,200 kDa.

(B) Dps1 and Dlp1 form high-molecular weight complexes with IspB. GST-IspB was prepared as described in (A) from Δ*ispB* harboring pGKO56 (lane 1), Δ*ispB* harboring pGKO56 and pSTVK-His-dps1 (lane 2), Δ*ispB* harboring pGKO56 and pSTVK-His-dlp1

(lane 3), Δ*ispB* harboring pGKO56 and pSTVK-His-hDPS1 (lane 4), and Δ*ispB* harboring pGKO56 and pSTVKHIShDLP1 (lane 5), and analyzed by Blue Native PAGE. The native gel was then transferred onto a PVDF membrane and an anti-penta-His antibody (diluted 1:500) was used to detect the complexes.

2.5 Discussion

Analysis of an *E. coli ispB* mutant led me to find an unexpected role for heteromeric PDSs in this study. Dlp1 or Dps1 from *S. pombe* formed a large complex with IspB and supported the growth of a temperature sensitive *ispB*^{R321A} *E. coli* mutant. Although Dps1 or Dlp1 from *S. pombe* cannot function alone to restore the enzyme activity of IspB in these mutants, they are able to do so by forming a complex with IspB. These observations indicate that Dlp1 and Dps1 retain the ability to enhance inactive PDS through stabilization. It is noteworthy that the mammalian poly-PDSs are slightly different from their *S. pombe* counterparts, as illustrated by the fact that mammalian *dps1* (or *sps1*) failed to restore temperature sensitivity in the IspB^{R321A} mutant.

Zhang et al recently reported an artificial PDS, which was composed of *S. cerevisiae* Coq1 and *S. pombe* Dps1 [6], and provided evidence that a component of heteromeric PDS supports the activity of homomeric PDS under certain conditions. In this study, I show that a subunit of heteromeric PDS stabilizes the homomeric PDS and restores its enzyme activity. However, unlike the data presented in the previous work showing that Dps1 played a role in heteromeric PDS formation, this study shows both Dlp1 and Dps1 can play a role in the stabilization of an artificial PDS.

Homomeric PDSs such as *E. coli* IspB [5], *S. cerevisae* Coq1 [6], *Gluconobacter suboxydans* DdsA [61], *Trypanosoma cruzi* [62], and *Arabidopsis* SPS1, 2 [63] are found in many organisms. However, heteromeric PDSs are only found in a small number of organisms, including *S. pombe*, mice, and human. Although the role played by Dps1 and Dlp1 in the heteromeric PDSs is currently unknown, we do know that each component requires its partner for activity [6]. It is not yet clear why some organisms, including mammals, require

heteromeric poly-PDS. The elucidation of the crystal structure of heteromeric enzymes will bring us closer to unraveling the evolution of poly-PDSs.

Chapter 3

Coq10, a mitochondrial coenzyme Q binding protein, is required for proper respiration in *Schizosaccharomyces pombe*

3.1 Abstract

It has been widely accepted that coenzyme Q (Q) exists freely in the mitochondrial membrane. However, the recent identification of a mitochondrial Q binding protein, termed Coq10, in budding yeast has the potential to change our current view of Q status in membranes. Here, I studied the counterpart of budding yeast Coq10 (also termed Coq10) in fission yeast. Fission yeast *coq10*-null mutants exhibited a similar, albeit less severe, phenotype as Q-deficient fission yeast, including the requirement for antioxidants for proper growth on minimal medium, increased sensitivity to H_2O_2, high levels of H_2S production, and a deficiency in respiration. The *coq10* null mutant produced nearly normal levels of Q10, suggesting that *coq10* does not belong to the group of Q biosynthetic genes. To elucidate the role of Coq10, we expressed recombinant *coq10* in *Escherichia coli*, and found that Q8 was present in purified recombinant Coq10. Mutational analysis of 13 conserved residues of Coq10 revealed that two hydrophobic amino acid residues, leucine 63 (L63) and tryptophan 104 (W104) play an important role in Coq10 binding to Q. An L63A/W104A double mutant of Coq10 exhibited lower Q-binding activity than either of the single mutants, and was unable to complement the *coq10* deletion in fission yeast. In light of the observation that a human Coq10 ortholog was able to functionally compensate for the absence of *coq10* in fission yeast, my results suggest that the role of Coq10 is important for proper respiration in a variety of organisms.

3.2 Introduction

Coenzyme Q (Q or ubiquinone) is an essential electron carrier in the respiratory chain of eukaryotic cells and most prokaryotes. Biosynthesis of Q occurs in the mitochondria in eukaryotes and in cytosolic membranes in prokaryotes. The biosynthetic pathway of Q has been elucidated primarily through genetic analysis of *Escherichia coli* and *Saccharomyces cerevisiae* mutants [2, 7, 37]. Q synthesis is initiated with the synthesis of polyisoprenyl diphosphate and the subsequent combination of polyisoprenyl diphosphate with *p*-hydroxybenzoate. The benzoquinone ring is then multiply modified to generate Q. To date, nine genes (*coq1-coq9*) have been identified that are involved in Q biosynthesis in *S. cerevisiae*, and orthologous genes in *Schizosaccharomyces pombe* [2, 4-6], *Caenorhabditis elegans* [29-31] and humans [3, 46] have been partly characterized. It has been suggested that Coq polypeptides form a complex in the mitochondria in *S. cerevisiae*, and complexes that lack any of the components are unstable [7]. This is one reason why the functions of some of the components are as yet unknown.

The fission yeast *S. pombe* is a well-studied model organism. It is easy to handle, and many essential genes are conserved in fission yeast and mammals. *S. pombe* produces coenzyme Q10 [3], a natural Q species that is also found in humans, and is thus viewed as a potential tool for the commercial production of Q10. Coenzyme Q10 is used worldwide as a food supplement, and increasing the production of Q10 through genetic engineering in a variety of hosts has been a strong focus in the field of applied biology [64]. There are some differences between the Q biosynthetic pathways of *S. pombe* and *S. cerevisiae*. First, polyisoprenyl synthase in *S. pombe* is a heterotetramer composed of two subunits, Dps1 and Dlp1 [4], similar to mouse and human [3], but different from the homodimeric (or homotetrameric)

structure in *S. cerevisiae* [6]. Second, the disruption of certain Q synthetic genes in *S. pombe* results in the production of different intermediates (unpublished data in our group) than those found in *S. cerevisiae*, in which the specific early intermediate 3-hexaprenyl-4-hydroxybenzoic acid (HHB) accumulates [7]. Third, the role of Q in some biological processes differs between *S. pombe* and *S. cerevisiae*. For example, Q reduction is coupled to sulfide oxidation in *S. pombe* [11], but not in *S. cerevisiae*. These results have prompted our interest in exploring the multiple functions of Q in *S. pombe*.

It is generally accepted that most Q is distributed freely in membranes [50]. However, Barros, M.H. et al [51] recently suggested that Coq10, a Q-binding protein in *S. cerevisiae*, might be involved in the transport of Q from its synthetic site to its functional site. These results not only strengthen our knowledge of the regulation of Q, but also challenge the current model that has existed for a long time that Q is a free lipid molecule in membranes. These findings prompted me to examine Coq10 function in other organisms to gain further insight into this important protein. Here, I have carried out a functional characterization of Coq10 in *S. pombe*. My results indicate that *S. pombe* Coq10 is a Q binding protein, and is required for the proper function of Q in several processes. I propose that Coq10 is a universal binding protein of Q.

3.3 Experimental procedures

Materials

Restriction enzymes and other DNA-modifying enzymes were purchased from Takara (Kyoto, Japan) and New England Biolab Japan, Inc. (Tokyo, Japan). *n*-Dodecyl ß-D-maltoside was purchased from Sigma Chemical Co. (St Louis, MO, USA). Ni-NTA agarose was purchased from QIAGEN (Tokyo, Japan). The human *coq10A* cDNA clone was purchased from Invitrogen Corp. (Tokyo, Japan).

Strains, plasmids and media

E. coli strain DH5α and the vectors pBluescript II SK+/-, pT7Blue-T (Novagen, Darmstadt, Germany), pET28c (Novagen), pREP1 [65], pREP81 and pSLF272 [65] were used for the construction of plasmids. Yeasts were grown in YE (0.5% yeast extracts, 3% glucose) or PM minimal medium with appropriate supplements, as previously described [66]. The concentration of supplemented amino acids, adenine and uracil was 75μg/ml.

DNA manipulations

Cloning, restriction enzyme analysis and the preparation of DNA plasmids were performed as described previously [66]. PCR was carried out as previously described [67]. Nucleotide sequences were determined using the dideoxynucleotide chain-termination method and an ABI377 DNA sequencer. For the expression of *coq10* in *S. pombe* and *E. coli,* two oligonucleotides, coq10-N and coq10-C, were used to amplify a full-length *coq10* cDNA from the *S. pombe* genome. The resulting *coq10* sequence was cloned into pT7Blue-T to generate pT7-coq10. An *Nde* I - *Bam* HI fragment containing *coq10* was subcloned into the corresponding sites of pREP1, pREP81 and pET28c to generate pREP1-coq10, pREP81-Scoq10 and pET28c-coq10, respectively. For the expression of *coq10-GFP*, two primers, coq10-Ntag and coq10-Ctag, were used to amplify the *coq10* cDNA, and then the

amplified fragment was cloned into the *Xho* I and *Bgl* II sites of pSLF272a to generate pSLF272a-coq10. To construct pREP1-Hucoq10A, two oligonucleotides, Hucoq10-N and Hucoq10-C, were used to amplify the predicted *Hucoq10A* open reading frame (ORF) from a human *coq10A* cDNA clone. The resulting fragment was cloned into the *Sal* I and *Bam* HI sites of pREP1 to generate pREP1-Hucoq10A.

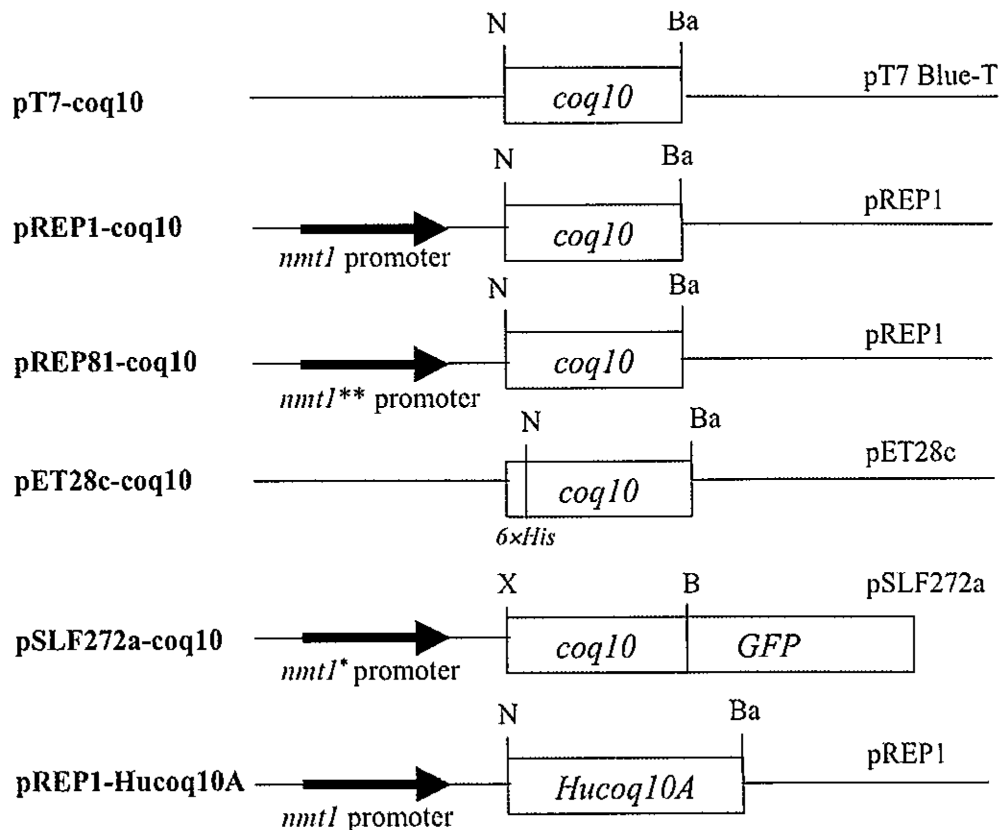


Fig. 3.0 Plasmids used in this study.

pT7-coq10, pREP1-coq10 and pREP81-coq10 contained full-length *coq10*. pET28c-coq10 contained full-length *coq10* fused to 6×His at the N-terminus. pSLF272a-coq10 contained full-length *coq10* fused to GFP at the C-terminus. pREP1-Hucoq10A contained the putative ORF of *Hucoq10A*. Of the nmt1, nmt1* and nmt1** promoters, nmt1 is the strongest and nmt1** is the weakest.

Gene disruption

PCR-based gene targeting was performed as previously described [28]. Briefly, two pairs of primers, coq10-A and coq10-B, and coq10-C and coq10-D, were used to amplify the upstream and downstream regions of *coq10*. The amplified *coq10* fragments were used as long primers to amplify a *kanMX6* module with flanking *coq10* homologous sequences. The amplified fragment was introduced into *S. pombe* cells by homologous recombination and cells were screened by G418 (Sigma, Chemical Co., St Louis, MO, USA) resistance. A pair of checking primers, nb2 and coq10-check, was used to confirm that the chromosomal *coq10* gene was replaced. The resulting deletion mutant was termed CZ48.

Extraction and measurement of Q from S. pombe

Q was extracted as described previously [4]. Briefly, crude lipid extracts were analyzed by normal phase thin layer chromatography (TLC) with authentic coenzyme Q10 as the standard. Normal-phase TLC was carried out on a Kieselgel 60 F_{254} plate with benzene/acetone (97:3 v/v). The band containing coenzyme Q10 was collected from the TLC plate following UV visualization and extracted with chloroform/methanol (1:1 v/v). The samples were dried and resolved in ethanol. Purified Q was further analyzed by HPLC with ethanol as the solvent.

Measurement of extracellular sulfide

Hydrogen sulfide was measured as the production of PbS from lead acetate. A quantitative determination of sulfide was performed by the methylene blue method, with minor alterations [11]. Briefly, *S. pombe* was grown in YES or PMU medium (50ml) to late log phase. Cultures (0.5 ml) were mixed with 0.1 ml of 0.1% dimethylphenylenediamine (in 5.5 M HCl) and 0.1 ml of 23 mM $FeCl_3$ (in 1.2 M HCl). The samples were incubated at 37°C for 5 minutes.

Following centrifugation at 13,000 rpm for 5 min, the supernatant was removed, and absorbance at 670 nm was measured using a blank consisting of reagents alone.

Mitochondrial staining and fluorescence microscopy

Mitochondria were stained using the mitochondria-specific dye MitoTracker Red^{FM} (Invitrogen). Cells were suspended in 10 mM HEPES (pH7.4) containing 5% glucose, and then MitoTracker Red^{FM} was added to a final concentration of 50 nM. After standing for 15 min at room temperature, cells were visualized by fluorescence microscopy using a BX51 microscope (Olympus, Tokyo, Japan) (1,000 x magnification). Fluorescence of GFPS65A was observed at an excitation wavelength of 485 nm. Fluorescence images were obtained using a digital camera (DP70, Olympus) connected to the microscope.

Purification of recombinant 6×His-Coq10 fusion protein

An overnight culture (10 ml) of *E. coli* BL21(DE3)pLysS harboring pET28c-coq10 was used to inoculate a 500 ml culture of peptone-phosphate enriched medium [48] containing 10 mM $MgCl_2$, 440 mM sorbitol, 2.5 mM betaine, 50 μg/ml kanamycin and 36 μg/ml chloromycetin. Cultures were incubated at 37°C with vigorous shaking until the optical density (OD_{660nm}) reached 0.6. The culture was cooled to 25°C and isopropyl β-D-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM. Growth was allowed to continue for 3 hours at 25°C and then the cells were collected by centrifugation at 6,000 g for 10 min. The cell pellets were stored at -20°C until use. Cell paste was suspended in 5 ml of lysis buffer (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole, pH 8.0). Protease inhibitor cocktail (EDTA free) (Roche Corp., Basel Switzerland) was added to the cell suspension, according to the manufacturer's instructions, and then the cells were disrupted by sonication (Cosmo Bio, Co. Ltd., Tokyo, Japan) for 8 min with 50% duty cycles. After

sonication, unbroken cells and inclusion bodies were removed by centrifucation at 12 000 *g* for 20 min, and the supernatant was centrifuged at 120, 000 *g* for 90 min. The pellets (membrane fraction) were suspended at 5 mg of protein/mL in lysis buffer. After addition of *n*-Dodecyl ß-D-maltoside (DM) at a concentration of 0.4%, the sample was incubated for 30 min with slow stirring, and centrifuged at 120, 000 *g* for 90 min. The supernatant was removed and mixed with an equal volume of pretreated Ni-NTA agarose. The gel mixture was gently shaken for 2 h at 4°C and then packed into a column. The beads were washed using 3×10 column volumes of wash buffer (50 mM NaH_2PO_4, 300 mM NaCl, pH 8.0) containing 20 mM imidazole. The 6xHis-Coq10 fusion protein was eluted from the column using 5 x 0.5 column volumes of elution buffer (50 mM NaH_2PO_4, 300 mM NaCl, 250 mM imidazole, 0.02% DM, pH 8.0). The Coq10 mutant proteins were purified using the same procedure.

Extraction and measurement of Q from purified proteins

The amount of Q bound to purified Coq10 was determined according to the method of Yamashita *et al* [47], with some modifications. Briefly, 0.25 µg of Q6 were added to purified protein as an internal control. The mixture was treated with a 5-fold volume of methanol and incubated at 70°C for 10 min with frequent agitation. Denatured protein was removed by centrifugation at 13,000 rpm for 3 min. The resulting supernatant was mixed with one-fold hexane. The upper phase was dried and resolved in 100 µl ethanol for HPLC analysis.

Site-directed mutagenesis

Site-directed mutagenesis was performed using the QuickChange II XL Site-Directed Mutagenesis Kit (Agilent Technol. Inc., CA, USA), according to the manufacturer's instructions. Briefly, for the construction of the single mutants, pT7-coq10 was used as the template for the PCR reactions. The amplified PCR products were self-ligated in *E. coli* and

the sequences of the *coq10* mutants were confirmed. The plasmids were digested with *Nde*I and *Bam*HI and the resultant fragments were subcloned into the corresponding sites of pREP81 and pET28c to generate the mutant *coq10* expression plasmids for *S. pombe* and *E. coli*, respectively. pT7-coq10^{L63A} was used as the template instead of pT7-coq10 to construct the double mutant, *coq10*$^{L63A\text{-}W104A}$. The sequences of all the substitution mutants were confirmed by sequence analysis.

3.4 Results

Cloning of *coq10* and construction of *S. pombe* Δ*coq10* mutants

I identified an orthologous gene of *S. cerevisiae COQ10* in the *S. pombe* genome through the GeneDB hosted by the Sanger Institute [http://www.genedb.org/]. The predicted amino sequence of the gene (SPCC16A11.07) exhibited medium sequence identity (21%) with Coq10 of *S. cerevisiae*, and we designated it as *S. pombe* Coq10. I searched the National Center for Biotechnology Information (NCBI) database [http://www.ncbi.nlm.nih.gov/] and found that orthologous *coq10* genes are widely distributed among a broad range of species. However, to date, only *coq10* of *S. cerevisiae* has been characterized [51].

To analyze the function of *S. pombe coq10*, I amplified *coq10* sequences from the *S. pombe* genome by PCR using specific primers, and used the amplified sequences to construct a deletion mutant strain, *S. pombe* Δ*coq10*, in which *coq10* was replaced by the *kanMX6* module. The strain resistance to G418 and analysis of the deletion using PCR and specific primers confirmed that *coq10* was successfully deleted.

Fig. 3.1 Alignment of the Coq10A homologous from *Homo sapiens* (H.s), Coq10 from *Xenopus laevis* (X.l.), Coq10A from *Drospohila melanogaster* (D.m.), Coq10 (At4g17650) from *Arabidopsis thanliana* (A.t.), Coq10 from *S. cerevisiae* (S.c.) and Coq10 from *S. pombe* (S.p.). Identical amino acids are highlighted in black. Asterisks indicate substituted amino acids.

Q production in Δ*coq10* cells

To investigate the role of *coq10* in Q biosynthesis, I compared the Q levels of Δ*coq10* and wild type strains. The levels of Q10 in *S. pombe* Δ*coq10* were comparable to the wild type strain (Fig. 3.2), which suggested that *coq10* is not required for Q biosynthesis in *S. pombe*. This result was consistent with a previous report in which the production of Q6 was nearly normal in a *coq10* deletion mutant of *S. cerevisiae* [51].

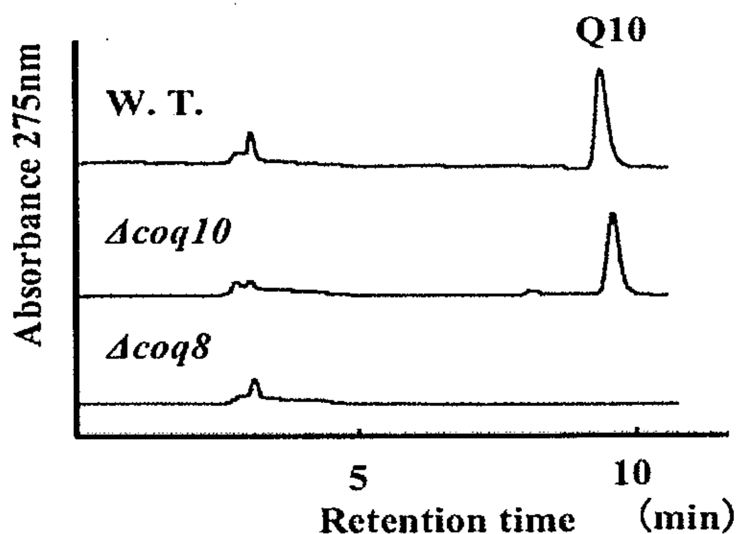


Fig. 3.2 Coenzyme Q10 levels in *S. pombe*.

Cultures (100 ml) in mid-log phase of wild type (PR110), Δ*coq10* (CZ48) and Δ*coq8* (NBp17)

strains were subjected to extraction for the analysis of Q levels. Q was first separated by TLC, and then 1:20 of each extract was further analyzed by HPLC.

Phenotype of *S. pombe Δcoq10*

The growth of *S. pombe* Q-deficient mutants on minimal medium is slower than wild type cells, and the addition of an antioxidant, such as glutathione or cysteine, can overcome this growth defect [4, 6]. Growth on medium that contains a non-fermentable carbon source is minimally detected. Similar to the Q-deficient mutant Δ*coq8*, Δ*coq10* exhibited growth retardation on pombe minimal (PM) medium, although to a lesser extent than Δ*coq8* cells (Fig. 3.3A). The addition of cysteine to the medium improved the growth of both strains (Fig. 3.3B). When I added glutathione to minimal medium, the growth of both Δ*coq10* and Δ*coq8* strains was recovered, which confirmed the antioxidant-dependent phenotype of these strains (Fig. 3.3C). These results suggested that the lack of Coq10 in Δ*coq10* mutants results in the impaired function of Q10 as compared to wild type cells. In support of this, Δ*coq10* mutants displayed an intermediate sensitivity to Cu^{2+} and H_2O_2 as compared to wild type and Δ*coq8* mutants (Fig. 3.4). However, unlike Δ*coq8* [11] and other Q-deficient mutants [28], which are highly sensitive to both Cu^{2+} and H_2O_2 (Fig. 3.4), Δ*coq10* exhibited a sharp sensitivity to H_2O_2, but not to Cu^{2+}, similar to the respiration mutant Δ*cyc1* (cytochrome *c*). This difference may help to distinguish Q-deficient mutants from respiration mutants. *S. pombe* possesses a Q sulfide reductase, heavy metal tolerance protein 2 (Hmt2), which links Q reduction and sulfide oxidation. Due to the presence of this enzyme, Q-deficient mutants accumulate abnormally high amounts of sulfide. The level of sulfide in Δ*coq10* mutants was much higher (21nM/A_{600}) than wild type cells (2 nM/A_{600}), although lower than Δ*coq8* cells (35 nM/A_{600}). These results

confirmed that *coq10* is required for functional Q.

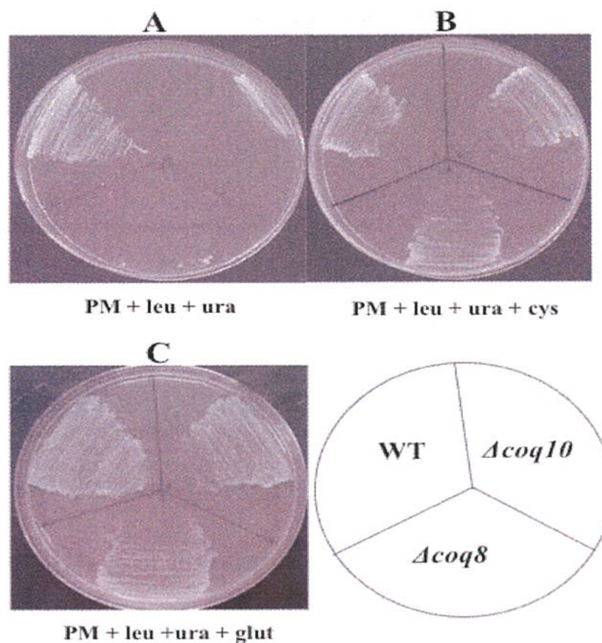
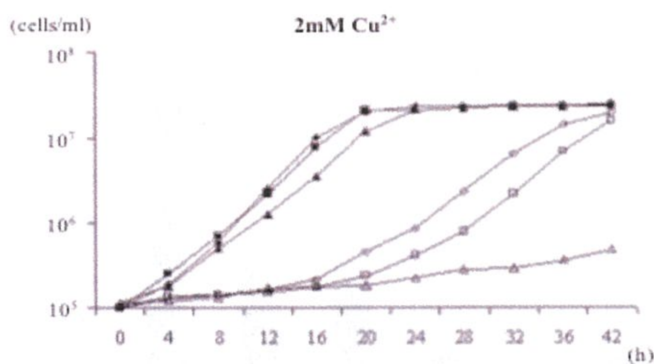


Fig. 3.3 Growth recovery of Δ*coq10* mutants on minimal media by the addition of antioxidants.

Wild type, Δ*coq10* and Δ*coq8* strains were grown on (A) PM medium supplemented with 75 μg/ml leucine and uracil, or (B) PM medium supplemented with leucine, uracil and 200 μg/ml cysteine. (C) The indicated strains were grown as described for (B), with the exception that glutathione was used instead of cysteine. All strains were grown for 2 days at 30°C.



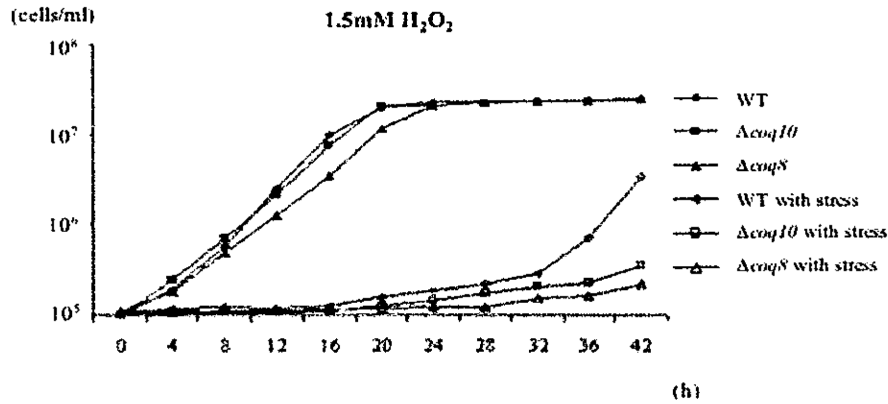


Fig. 3.4 Sensitivity of Δ*coq10* mutants to oxygen radical producers.

Wild type, Δ*coq10* and Δ*coq8* strains were pre-grown, and then placed in fresh YES media with 2 mM Cu^{2+}, 1.5 mM H_2O_2, or nothing. Cell number was counted at 4 h intervals.

Since Q plays an essential role in respiration, I analyzed the respiration competency of Δ*coq10* mutants by measuring oxygen consumption (Fig. 3.5). The level of O_2 uptake by Δ*coq10* mutants was similar to Δ*cyc1* and Δ*dps1* (decarprenyl diphosphate synthase) mutants, which indicated that *coq10* is crucial for respiration. Taken together, these results suggested that *coq10* is required for multiple functions of Q, and that the role of *coq10* in Q-dependent biological processes is distinct from the one of other Q biosynthetic genes. The phenotype of the Δ*coq10* mutants is closer to that of respiration deficient mutants of *S. pombe.*

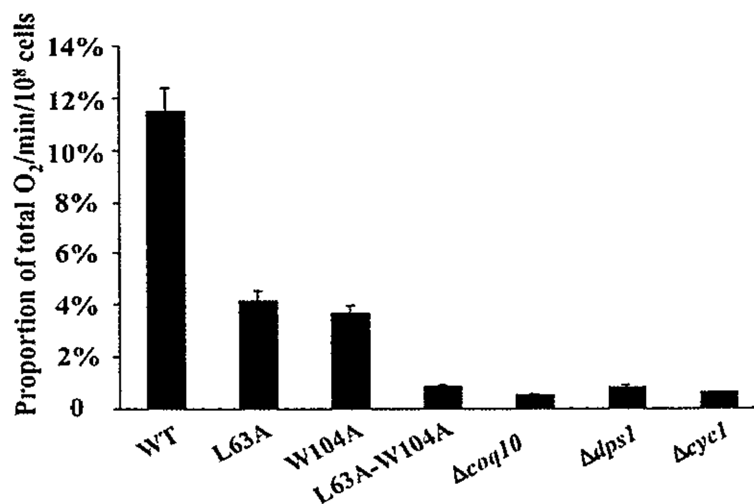


Fig. 3.5 *coq10* is required for respiration of *S. pombe*.

Oxygen consumption was measured in cells taken from YES cultures at mid-log phase. Cells were washed twice with phosphate buffered saline (PBS), and then 1 x 10^8 cells were resuspended in 100 μl of PBS containing 2% glucose. Analysis was carried out using a YSI model 53 oxygen monitor (Yellow Springs Instrument Co., Inc.). Oxygen consumption was measured as the loss of oxygen in 3 ml of PBS. For the analysis of the point mutants of Δ*coq10*, *S. pombe* Δ*coq10* was transformed with multi-copy expression plasmids for *coq10*^{L63A}, *coq10*^{W104A} or *coq10*$^{L63A\text{-}W104}$ (pREP81). The data represents the averages of three measurements.

Identification of key functional amino acids in Coq10

To identify the amino residues of Coq10 that might be involved in the binding of Coq10 to CoQ, I first identified 13 highly conserved hydrophobic amino acids by sequence alignment of Coq10 orthologs (Fig. 3.1). I then constructed individual alanine substitution mutants of these amino acids. Among the single mutants, expression of L63A and W104A *coq10* mutants in a *coq10*-null background resulted in defective respiration (Fig. 3.5) and growth retardation on minimal medium compared to wild-type cells (Fig. 3.6A). The single L63A and W104A mutants and the double mutant exhibited other defects as well, similar to the phenotype of Δ*coq10* mutants, including the requirement for antioxidants for proper growth on minimal medium (Fig. 3.6A) and increased sulfide production (Fig. 3.6B). Thus, the L63A/W104A double mutant was almost non-functional in the background of a *coq10* deletion mutant.

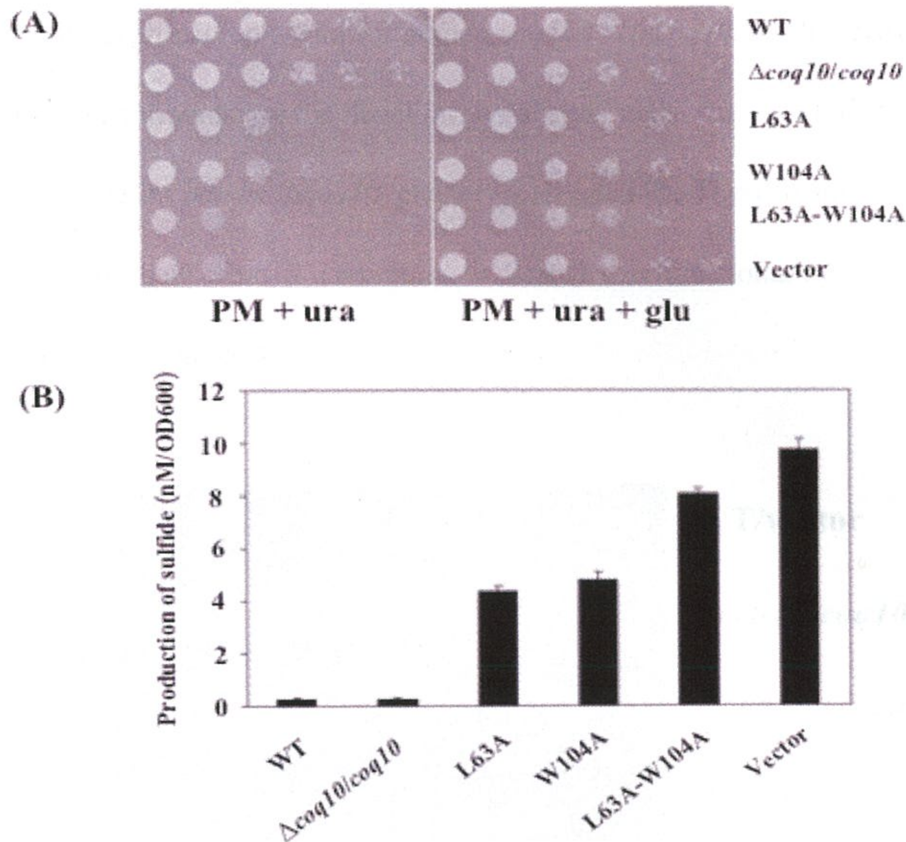


Fig. 3.6 L63 and W104 are required for functions of Coq10.

(A) Wild-type cells harboring pREP81, or *Δcoq10* harboring pREP81-coq10, pREP81-coq10^{L63A}, pREP81-coq10^{W104A}, pREP81-coq10$^{L63A\text{-}W104A}$ or pREP81 (vector), as indicated, were spotted onto PMU minimal medium with or without glutathione. The cells were incubated at 30°C for 3 days, and growth was observated.

(B) Cells were grown on PMU medium and extracellular sulfide levels were measured as described in Experimental procedures. The strains are as described for (A). The data represent the mean and SD of three measurements.

Complementation of the *Δcoq10* mutation by human *coq10*.

I searched the NCBI database and found that *coq10* is widely distributed in many organisms. To investigate the conservation of Coq10 function between fission yeast and humans, I

constructed an expression plasmid for human *coq10A* (*Hucoq10A*). The recovery of growth of *Hucoq10A* transformants to the level of wild type cells suggested that *Hucoq10A* fully complements the *S. pombe* Δ*coq10* phenotype (Fig. 3.7). These results, together with the complementation data from *S. cerevisiae*, indicated that functional homologs of Coq10 are present in yeast and humans.

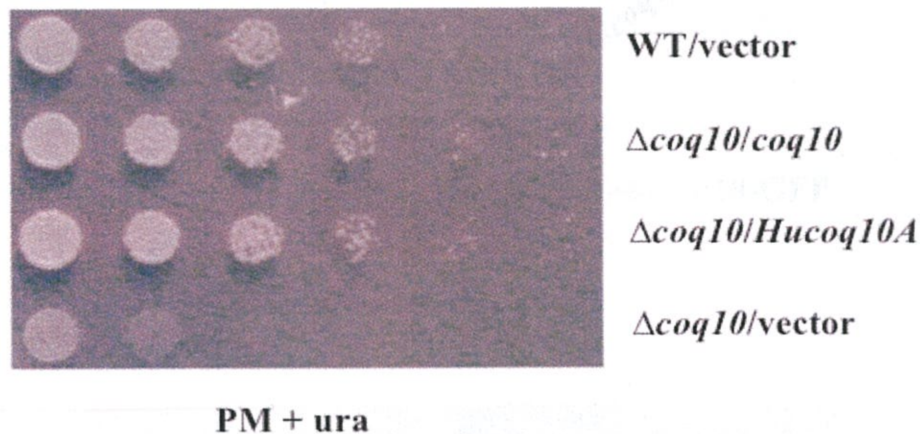


Fig. 3.7 Complementation of Δ*coq10* by *Hucoq10A*.

Wild type cells harboring pREP1, or Δ*coq10* mutants harboring pREP81-coq10, pREP1-Hucoq10A and pREP1, as indicated, were spotted onto PMU minimal medium. The cells were incubated at 30°C for 3 days and growth was observed.

Coq10 localizes to mitochondria in *S. pombe*

Q synthetic proteins and Coq10 have been shown to localize to the mitochondria in *S. cerevisiae* [51]. To further investigate the role of Coq10 in respiration in fission yeast, I constructed an expression plasmid for a green fluorescent protein (GFP) fusion protein of Coq10 (Coq10-GFP), and analyzed the localization of Coq10-GFP in Δ*coq10* mutants. The expression of *Coq10-GFP* in *S. pombe* was confirmed by Western blot (Fig. 3.8A). The

expression of Coq10-GFP complemented the phenotype of Δ*coq10* mutants, which indicated that that Coq10-GFP is functional in these cells. When I observed the cells by fluorescence microscopy, Coq10-GFP co-distributed with Mitotracker Red, a mitochondrial marker (Fig. 3.8B), indicating that Coq10 localizes to the mitochondria in *S. pombe*.

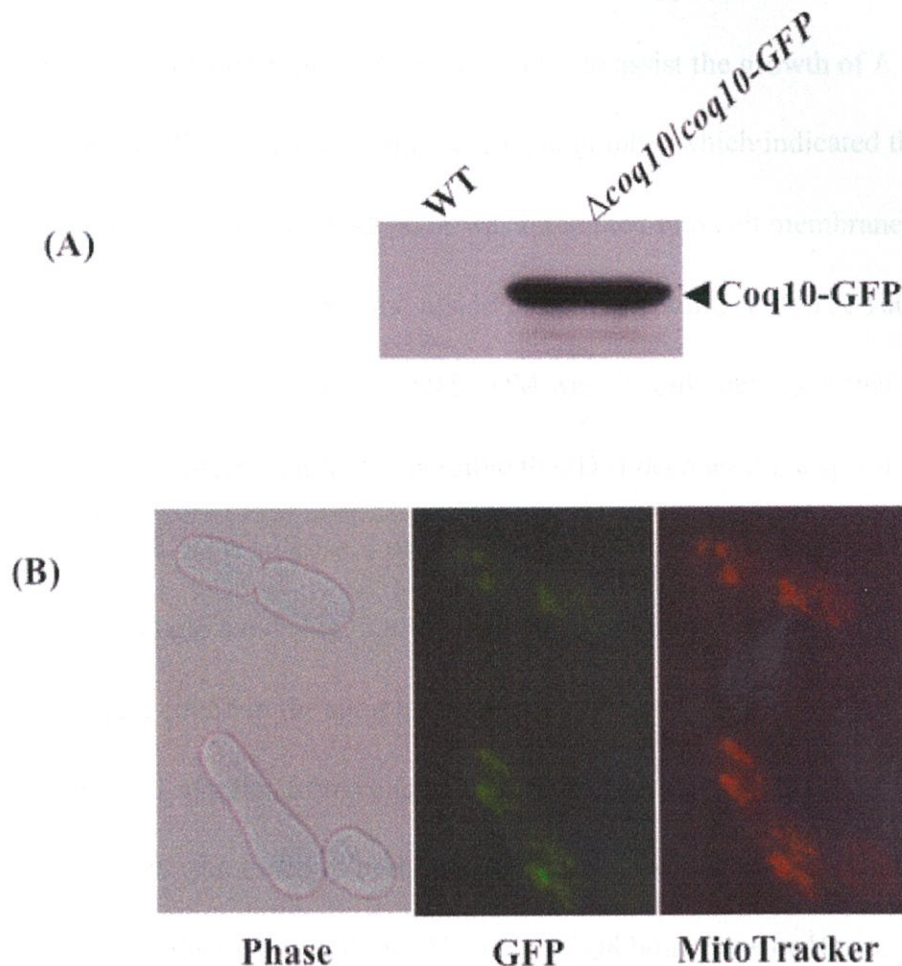


Fig. 3.8 Co-localization of Coq10-GFP with a mitochondria-specific dye.

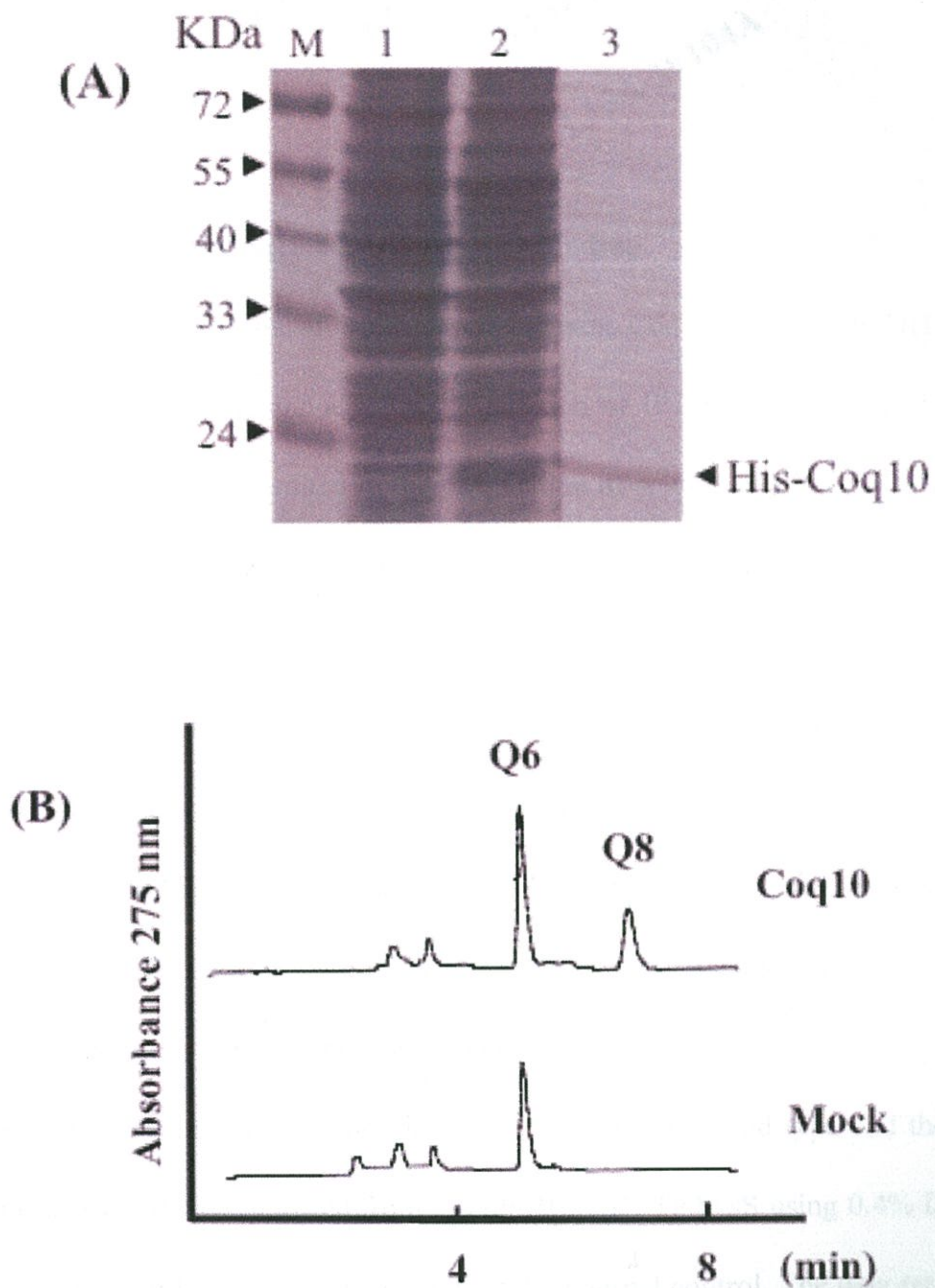
(A) The expression of Coq10-GFP was confirmed by Western blot analysis using an anti-GFP antibody (Roche). (B) Phase contrast image of Δ*coq10* mutant cells expressing Coq10-GFP (Phase), and fluorescence images of the same cells showing the location of Coq10-GFP (GFP), and mitochondrial staining by Mito-Tracker red (MitoTracker).

Expression, purification and analysis of recombinant Coq10 from *E. coli*

For the analysis of purified Coq10, I constructed a histidine (6xHis) fusion protein of Coq10 (*6×His-Coq10*) and expressed it in *E. coli*. The addition of IPTG to transformed cells resulted in the expression of a 20 kDa protein (Fig. 3.9A). *E. coli* that expressed *coq10* showed a slightly faster growth rate as compared to wild type cells under low rotation speed, which suggested that Coq10 functions in some way to assist the growth of *E. coli*. I was unable to purify 6×His-Coq10 by affinity chromatography, which indicated that most of the fusion protein formed inclusion bodies, or was integrated into cell membranes. I then screened several detergents to try and purify the fusion protein, including Triton X-100, Tween-20, Chaps, NP-40 and dodecyl maltoside (DM). DM was the only detergent that enabled effective recovery of fusion protein. While it is possible that DM decreased the specific binding of the fusion protein to Ni-NTA agarose, I was able to resolve the Coq10 fusion protein by SDS-PAGE as a single band (Fig. 3.9A). Both Q6, an internal control, and Q8, a native *E. coli* CoQ species, were found in the samples containing purified Coq10, whereas only Q6 was detected in purified samples from cells that expressed the control vector alone (Fig. 3.9B). In this experiment, Q6 was added to both samples just before extraction of Q as an internal control to calculate the quantity of Q8. The ratio of Q8 bound to Coq10 was 0.81 mol/mol, which is close to a 1: 1 ratio, under my experimental conditions (Fig. 3.9). These results confirmed that *S. pombe* Coq10 is a Q-binding protein, and that it probably binds to Q in 1: 1 ratio in nature.

I then tested the Q-binding ability of mutated Coq10s, which are described in Figs 3.5 and 3.6. I expressed each of the single mutants of L63A and W104A, as well as an L63A/W104A double mutant, as 6×His-Coq10 fusion proteins in *E. coli*, and measured the amount of Q

associated with the purified proteins. The L63A and W104A single mutants exhibited decreased binding to Q, (60% and 43%, respectively, of that bound by wild type Coq10). Binding to Q was further decreased in the L63A/W104A double mutant (30% of that of wild type protein). These results indicated that L63 and W104 are critical residues for binding of CoQ by Coq10, and are important for the functions of Coq10 as shown in Figs 3.5 and 3.6.



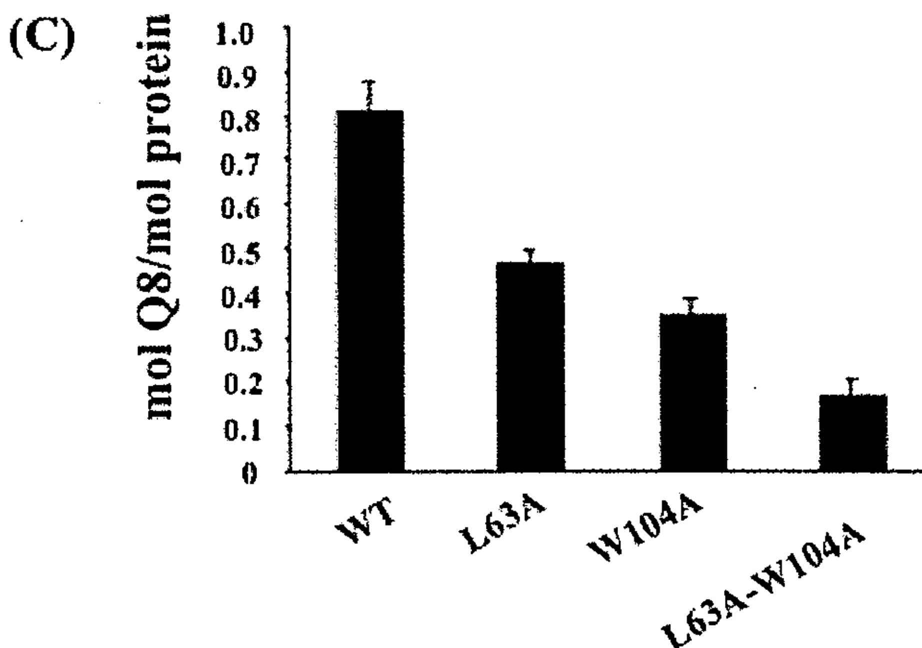


Fig. 3.9. L63 and W104 of Coq10 contribute to Q-binding.

(A) 12.5% SDS-PAGE analysis of isolated recombinant Coq10. *E. coli* BL21(DE3)pLysS harboring pET28c-coq10 were induced by the addition of IPTG (0.5 mM) at 25°C for 3 h (lane 2). The addition of DM resulted in the purification of soluble 6×His-Coq10 (lane 3). M, protein marker; Lane 1, crude lysate before induction; Lane 2, crude lysate after induction; Lane 3, purified 6×His-Coq10.

(B) Coq10 binds to Q8. Purified His-Coq10 was used for the extraction of Q. The extracts were analyzed by HPLC (Shimadzu Co., Japan) at a flow rate at 1 ml/min. As a negative control, *E. coli* BL21(DE3)pLysS harboring pET28c (control empty vector) were analyzed in parallel. To calculate the binding ratio of Coq10 to Q, authentic Q6 as an internal control was mixed with purified protein before the extraction of Q8.

(C) L63 and W104 are involved in the binding of Coq10 to Q. Wild type and the indicated point mutants of Coq10 were purified from *E. coli* BL21(DE3)pLysS using 0.4% DM. Bound Q8 was analyzed by HPLC using authentic Q6 as the internal control. The data represents the

averages of three measurements.

3.5 Discussion

In the current study, I carried out a functional characterization of *S. pombe* Coq10, a Q-binding protein in yeast. Q was detected in complex with purified Coq10, consistent with the idea that *S. pombe* Coq10 is a Q-binding protein, similar to its role in *S. cerevisiae*. Disruption of *coq10* in *S. pombe* did not appear to affect Q10 levels in cells (Fig. 3.2), which ruled out a role of *coq10* in Q biosynthesis. However, Δ*coq10* mutants displayed a typical *S. pombe coq* mutant phenotype, including the requirement for antioxidants for growth on minimal medium (Fig. 3.3), increased sulfide accumulation (Fig. 3.5), sensitivity to H_2O_2, and defects in respiration (Fig. 3.6). The underlying mechanisms of these phenotypes most likely do not involve the minor decrease in Q10 levels in Δ*coq10* mutants, because very low levels of Q were able to restore the growth of Δ*dps1* mutants that expressed orthologous genes from other organisms [3, 63]. Alternatively, the deletion of *coq10* may decrease the functional activity of Q, resulting in a Q-deficient phenotype. Q is not only an antioxidant, but also a pre-oxidant. Q becomes toxic in cells if the oxidized form of Q accumulates, thus Q-binding proteins must be needed to protect cells from damage due to this pre-oxidant property of Q. My results suggest that Coq10 is a universal component in the regulation of Q function. In other words, Q function in membranes is a protein-mediated process.

The over-expression of *COQ2*, *COQ7* or *COQ8* partially complements the Δ*COQ10* mutation in *S. cerevisiae*, and this is accompanied by higher levels of Q synthesis. Because the overexpression of *ppt1 (coq2)*, *coq7* or *coq8* in *S. pombe* is inhibitory for cell growth, it was difficult to assess the effect of these genes in the Δ*coq10* mutant background.

Through the use of a detergent-based purification scheme, I was able to purify sufficient amounts of recombinant Coq10 from *E. coli* for protein analysis. The binding ratio of purified

Coq10 to Q was 0.81 mol Q/mol protein, which suggests that the natural ratio of Coq10 to the bound Q is 1:1. While the use of a detergent in the purification process most likely affected the ability of Coq10 to bind to Q, the Q-binding properties of wild type and mutant Coq10 could be distinguished. In addition, this ratio is an improvement over the reported binding of *S. cerevisiae* Coq10 to Q, in which the ratio of Coq10 bound to Q6 was 0.032-0.034 mol Q/mol protein [51]. I also tried to express Coq10 in *S. pombe* in order to analyze the binding of purified Coq10 to Q10. However the over-expression of *coq10* inhibited the growth of *S. pombe*, and the yield of purified Coq10 was very low, making it difficult to assess the Q binding properties of Coq10 from *S. pombe*.

I identified two amino acids of Coq10 that were important for Coq10 function. Mutation of L63 and W104 abolished the ability of Coq10 to complement the *coq10* deletion mutation, and lowered, but did not completely inhibit, the Q-binding activity of Coq10. The components of the electron transfer system NADH-ubiquinone reductase [47], succinate-ubiquinone reductase [68] and ubiquione-cytochrome *c* reductase [48] have been reported to have Q-binding subunits. Those are necessary for the electron transfer mediated by Q in each reaction. Other types of Q-binding proteins have also been identified in bacterial RegB [69], and recently in human urinal saposin B [70]. However, the alignment of all those Q binding proteins with Coq10 did not reveal any clear conserved amino acids motifs. Coq10 has been categorized in Pfam PF03364 [http://pfam.sanger.ac.uk/] as an enzyme involved in polyketide synthesis, and as a lipid binding and transport protein. Based on sequence alignment, there appears to be many eukaryotic orthologs of Coq10, as well as orthologous proteins in bacteria whose functions are not known. Very recently, Barros *et al* [71] modeled the structure of Coq10 from *S. cerevisiae* and *S. pombe*, and indicated that a hydrophobic tunnel exists in

Coq10 for binding to Q. Furthermore, they verified that L63 and W104 in *S. pombe* are in this tunnel and important for the Q binding.

Human coenzyme Q deficiency leads to neural and muscular diseases, and patients possessing a genetic disorder in Q biosynthetic genes have been cumulatively discovered [46]. The characterization of *S. pombe coq10* provides new phenotypic characteristics for analysis, and I have shown that *coq10* function is conserved between yeasts and human (Fig. 3.7). Thus, my results should help identify and characterize medical cases related to genetic deficiencies of *coq10.*

In conclusion, Coq10 is a conserved Q-binding protein that is essential for proper function of the electron transfer system, possibly by assisting in the transfer of Q from one site to another in the mitochondrial membranes of eukaryotes.

Chapter 4 References

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Chapter 5 Conclusions

From the above results, I drew the conclusions as following:

(1) *E. coli* IspB is able to form a high-molecular weight complex with fission yeast Dlp1 or Dps1 to strengthen its stability, suggesting the conservation and potential evolutionary trend of PDS.

(2) Coq10 is not required for the biosynthesis of Q, but is required for the functional roles of Q in every aspect. Coq10 is exclusive of the complexes of dehydrogenase in mitochondria and Coq10 binds to Q *in vivo.* Thus, the current data present the hypothesis that Q functions in a protein-mediated manner in mitochondrial membranes.

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Summary

Coenzyme Q (CoQ or Q) plays an essential role in the electron transport chain, so its biosynthesis and regulation is very important for us to understand this basic metabolic route. The Q biosynthetic pathway has been elucidated in yeast and *E. coli*. To date, most of *COQ* genes (if additional ones exist) have been identified. The present focus of Q biosynthetic pathway is the functional characterization of *COQ* genes with unknown function and how the Coq polypeptides work in the organisms. On the other hand, its regulation is far from clearance relative to the Q biosynthesis. Especially, it is not known how the Q pool in the mitochondria is maintained and regulated. In the thesis, my works addressed some aspects of the above questions.

In chapter 1, I described a general introduction of these studies.

In chapter 2, I described an artificial polyprenyl diphosphate synthase (PDS). PDS is responsible for the synthesis of side chain of CoQ and thus defines the CoQ species. PDS is classified as homomer (such as IspB in *E. coli* and Coq1 in *S. cerevisiae*) and heteromer (such as Dps1 and Dlp1 in *S. pombe* and human). Although Dps1 shares high sequence similarity with known homomeric PDS and Dlp1 does not, Dps1 still needs Dlp1 as the partner to be functional in *S. pombe* and human. Surprisingly, when *dps1* or *dlp1* was expressed in an *E. coli* R321A temperature-sensitive mutant, the strain showed a wild type-like growth at the restricted temperature, and IspB activity was restored with production of CoQ8. The further assays indicated that IspB interacted with Dlp1 (or Dps1) to form a high molecular weight complex that stabilized IspB, leading to full functionality. These results indicate that a

non-functional subunit of PDS retains the ability to regain the enzymatic function, thus providing important insights into the mechanism and evolution of PDS.

In the Chapter 3, I described a Q-binding protein required for the multiple functions of Q in *S. pombe*. It has been widely accepted that coenzyme Q (Q) exists freely in the mitochondrial membrane. However, the recent identification of a mitochondrial Q binding protein, termed Coq10, in budding yeast has the potential to change our current view of Q status in membranes. Here, the author studied the counterpart of budding yeast Coq10 (also termed Coq10) in fission yeast. Fission yeast *coq10*-null mutants exhibited a similar, albeit less severe, phenotype as Q-deficient fission yeast, including the requirement for antioxidants for proper growth on minimal medium, increased sensitivity to H_2O_2, high levels of H_2S production, and a deficiency in respiration. The *coq10* null mutant produced nearly normal levels of Q10, suggesting that *coq10* does not belong to the group of Q biosynthetic genes. To elucidate the role of Coq10, I expressed recombinant *coq10* in *Escherichia coli*, and found that Q8 was present in purified recombinant Coq10. Mutational analysis of 13 conserved residues of Coq10 revealed that two hydrophobic amino acid residues, leucine 63 (L63) and tryptophan 104 (W104) play an important role in Coq10 binding to Q. An L63A/W104A double mutant of Coq10 exhibited lower Q-binding activity than either of the single mutants, and was unable to complement the *coq10* deletion in fission yeast. In light of the observation that a human Coq10 ortholog was able to functionally compensate for the absence of *coq10* in fission yeast, the results suggest that the role of Coq10 is important for proper respiration in a variety of organisms.

From the above results, I drew the conclusions as following: (1) *E. coli* IspB is able to form a high-molecular weight complex with fission yeast Dlp1 or Dps1 to strengthen its stability, suggesting the conservation and potential evolutionary trend of PDS. (2) Coq10 is not required for the biosynthesis of Q, but is required for the functional roles of Q in every aspect. Coq10 is exclusive of the complexes of dehydrogenase in mitochondria and Coq10 binds to Q *in vivo*. Thus, the current data present the hypothesis that Q functions in a protein-mediated manner in mitochondrial membranes.

要旨

コエンザイム Q（CoQ、Q）は、電子伝達系における必須因子であり、その合成や制御系の研究は細胞内代謝系を理解する上で非常に重要である。これまで Q 合成系の研究は大腸菌と出芽酵母を中心に行われてきており、今までに多くの *COQ* 遺伝子が同定されている。現在では特に、機能未知の *COQ* 遺伝子の機能解明と Coq 複合体の解析が行われている。一方、Q 合成の制御系は未だ完全には解明されていない。特に、ミトコンドリアにおける Q プールがどのように維持、制御されているかは不明である。本論文では、上記の未解明な点の解明を目指して研究を行った。

まず、人工的なポリプレニル二リン酸合成酵素（PDS）の解析を行なった。PDS は Q の側鎖合成と鎖長の決定を担っている。PDS は、ホモ型（大腸菌の IspB や出芽酵母の Coq1 など）とヘテロ型（分裂酵母やヒトなどの Dps1 と Dlp1）に分類される。Dps1 のアミノ酸配列は、これまでに知られているホモ型の PDS と保存性が高く、Dlp1 はそれほど保存性が高くないが、Dps1 の活性の発現には Dlp1 が必要である。驚くべきことに、大腸菌の $IspB^{R321A}$ 変異型温度感受性株で *dps1* か *dlp1* 遺伝子を発現させると、高温で野生型と同じ増殖を示し IspB 活性の回復と Q8 の合成が見られた。さらに、IspB と Dlp1（または Dps1）は、高分子量複合体を形成し IspB を安定化することで機能回復していると考えられた。Dlp1（または Dps1）は単独で機能しないのにもかかわらず、温度感受性 IspB の機能を回復することができることから、PDS の機能と進化における重要な知見が今回得られた。

次に、分裂酵母の Q の機能性に重要な役割を果たす Q 結合タンパク質について解析を行なった。コエンザイム Q はミトコンドリア膜にフリーな状態で存在しているとこれまで考えられてきたが、出芽酵母で Coq10 と呼ばれるミトコンドリア局在の Q 結合タンパク質が発見され、これまでの知見を揺るがす可能性が示唆された。そこで著者は、分裂酵母の Coq10 タンパク質の解析を行った。分裂酵母の *coq10* 破壊株は、最少培地での生育に抗酸化剤が必要であることや過酸化水素に対する感受性、硫化水素の発生、呼吸欠損などの、少し弱いながらも他の Q 欠損株と同様の表現型を示した。*coq10* 破壊株はほとんど野生型と変わらない Q10 合成能を持つことから、*coq10* 遺伝子は Q 合成そのものには関与していないと考えられる。Coq10 の機能解明のため、著者は大腸菌で *coq10* 遺伝子を発現させ、Coq10 が Q8 と結合することを証明した。他生物種の Coq10 と保存性の高い 13 個のアミノ酸のうち 63 番目のリジンと 104 番目のトリプトファンが Q 結合に重要な役割をしていることを示した。さらに、これらの二重変異体（L63A/W104A）は、それぞれの変異体より Q 結合能が減少し、*coq10* 破壊

株での相補能も消失することを見いだした。ヒトCoq10は、分裂酵母*coq10*破壊株で機能することから、Coq10は様々な生物で呼吸鎖の機能に重要であることが示唆された。

以上の結果から、以下の結論が得られた。(1) 分裂酵母Dps1やDlp1は大腸菌IspBと高分子複合体を形成することで安定性を強化できることから、PDSの保存性や進化において機能性が維持されていることが示唆された。(2) Coq10は、Q合成には関与していないが、Qが機能する上で重要な役割を果たしていることを明らかにした。Coq10タンパク質はQに結合し、ミトコンドリアの呼吸鎖の正常な機能に必須であることを示した。以上の結果から、著者はCoq10タンパク質がミトコンドリア膜における呼吸鎖複合体の維持あるいは電子伝達系に必須な機能を有しているという説を提起している。

List of publications

Tie-Zhong Cui, Tomohiro Kaino, Makoto Kawamukai. A subunit of decaprenyl diphosphate synthase stabilizes octaprenyl diphosphate synthase in *Escherichia coli* by forming a high-molecular weight complex.

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