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

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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., decaprenyl diphosphate synthase (Dps1) and D-less polyprenyl diphosphate synthase (Dlp1) in *Schizosaccharomyces pombe* and in humans). We 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.

Structured summary:
MINT-7385426: *Dlp1* (uniprotkb:Q86YH6) and *IspB* (uniprotkb:P0AD57) *physically interact* (MI:0915) by *blue native page* (MI:0276)
MINT-7385083, MINT-7385058: *IspB* (uniprotkb:P0AD57) and *IspB* (uniprotkb:P0AD57) *bind* (MI:0407) by *blue native page* (MI:0276)
MINT-7385413: *Dlp1* (uniprotkb:O13851) and *IspB* (uniprotkb:P0AD57) *physically interact* (MI:0915) by *blue native page* (MI:0276)
MINT-7385024: *IspB* (uniprotkb:P0AD57) *physically interacts* (MI:0915) with *Dps1* (uniprotkb:O43091) by *pull down* (MI:0096)
MINT-7385041: *IspB* (uniprotkb:P0AD57) *physically interacts* (MI:0915) with *Dlp1* (uniprotkb:O13851) by *pull down* (MI:0096)
MINT-7385388: *IspB* (uniprotkb:P0AD57) and *Dps1* (uniprotkb:O43091) *physically interact* (MI:0915) by *blue native page* (MI:0276)

1. Introduction

Ubiquinone, which is composed of a benzoquinone ring and an isoprenoid tail, is an essential factor for aerobic respiration in living cells. The ubiquinone 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 [1]) has been elucidated mainly in *Saccharomyces cerevisiae* [2]. Much data exists showing that this pathway (apart from the synthesis of the prenyl tail) is conserved in a wide range of eukaryotes [3]. The length of the prenyl tail varies between different organisms. For instance, *S. cerevisiae* has 6 isoprene units in its ubiquinone side chain, whereas *Escherichia coli* has 8, mice have 9, and both *Schizosaccharomyces pombe* and humans have 10 [4]. However, the length of the isoprenoid chain seems not to be crucial to function because ubiquinone in *E. coli* and yeast cells can be replaced with molecules containing side-chains of varying lengths with no adverse effects [5–7].

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* [8] and hexa-PDS Coq1 in *S. cerevisiae* [9]), or heteromeric (i.e., nona-PDS in mouse, and deca-PDSs in *S. pombe* and human) [10,11]. 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 motif

Abbreviations: Dlp1, D-less polyprenyl diphosphate synthase; Dps1, decaprenyl diphosphate synthase; FPP, farnesyl diphosphate; IPP, isopentenyl diphosphate; PDS, prenyl diphosphate synthase

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[10]. This raises two questions: why do higher organisms employ a heteromeric PDS, rather than a homomeric one, for the synthesis of ubiquinone; and does the heteromeric poly-PDS have advantages over the homomeric one?

In this study, we 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, our data show that heteromeric PDS has advantages over the homomeric PDS, and might represent an evolutionary trend.

2. Materials and methods

2.1. 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.

2.2. Plasmid construction

To construct pBQ-His-ispB or pBQ-His-ispB^{R321A}, the primers 5′-CGGATCCGATGAATTTAGAAAAAATC-3′ (forward) and 5′-CGAAGC TTGGCCATGGGCGCG-3′ (reverse) were used to amplify *ispB* from pKO56 [3] 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 pBmSPS1 plasmid using *Eco*RI and *Kpn*I [11], and cloned into the same sites in pSTVK28. To construct pSTVK-hdps1, *hDPS1* was digested from pGEX-hdps1 using *Bam*HI and *Xba*I [11], and cloned into the same sites in pSTVK28 (Table 1).

To construct pGKO56-R321A, site-directed mutagenesis was performed using a QuickChange II XL Site-Directed Mutagenesis Kit (Stratagene, Tokyo, Japan) as previously described [12]. Briefly, a pair of reverse-complementary primers (5′-CATCGCTGTTCAAGC CGATCGTTAATCC-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).

2.3. Purification of GST-IspB from E. coli

GST-IspB, GST-IspB^{R321A} and GST were purified as previously described [9].

2.4. Ubiquinone extraction and PDS assay

Ubiquinone was extracted and measured as previously described [12]. PDS activity was measured as previously described [11].

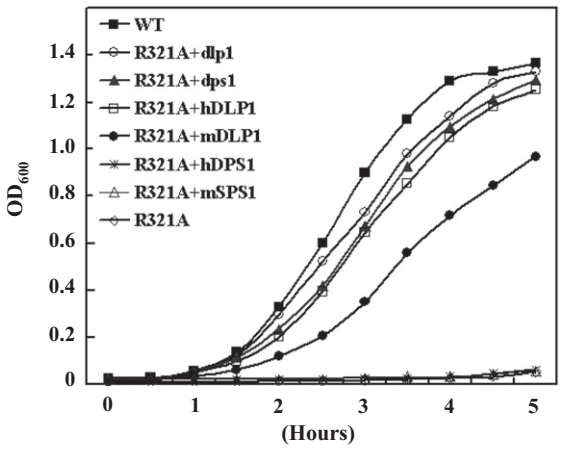

Fig. 1. *Dps1* or *dlp1* reverses the thermo-sensitivity of an *E. coli ispB*^{R321A} mutant. An overnight culture of the indicated strains was diluted to OD_{600} = 0.02, and the cells were incubated with vigorous shaking at 43 °C. Cell mass was calculated every 30 min. *E. coli* strain KO229 (Δ*ispB* harboring an *ispB*-containing plasmid) 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.

Table 1
Plasmids used in this study.

Plasmids	Characteristics	Sources
pSTVK-msps1	Km, full-length mouse *SPS1* in pSTV28K	This study
pSTVK-hdps1	Km, full-length human *DPS1* 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	[13]
pSTVK-His-dps1	Km, His_6 with full-length *dps1* in pSTVK28	[9]
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	[11]
pSTVhDLP1	Km, full-length human *DLP1* in pSTVK28	[11]
pGEX-1	Amp, *tac* promoter, GST tag, high expression vector	Amersham
pGKO56	Amp, full-length *ispB* gene in pGEX-1X	[13]
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	[11]

Ap, ampicillin; Km, kanamycin.

3. Results

3.1. 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 [7] and that Arg321 is important for the thermo-stability of IspB; so *ispB*^{R321A} mutants grow very slowly at 43 °C [13]. Surprisingly, when we expressed *S. pombe dps1* in the *ispB*^{R321A} mutant, the growth of the co-expressed cells was similar to wild-type cells (Fig. 1). *S. pombe dlp1* also reversed the thermo-sensitivity of the *ispB* mutant in a similar manner (Fig. 1). Also, we observed that both human *hDLP1* and mouse *mDLP1*, but not *hDPS1* or *mSPS1*, rescued the growth of the *ispB*^{R321A} mutant at 43 °C (Fig. 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 Dps1, and Dlp1 are capable of supporting the IspB activity in *E. coli*. We then focused on the role of *S. pombe* Dps1 and Dlp1 in the *E. coli ispB*^{R321A} mutant.

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

We 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 (Fig. 2C–F); also, the Q-8 levels in all the samples were similar (Fig. 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.

3.3. 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, we 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. 3A, left). However, the expression of *dps1* or *dlp1* led to a fivefold increase in activity at 43 °C at OD_{600} = 0.4 (Fig. 3A, right). Because neither *dps1*, nor *dlp1* changed the level of IspB expression (Fig. 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. 3A right). This may have been due to increased IspB levels after OD_{600} = 0.4 (Fig. 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 (data not shown). 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.

3.4. 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, we performed Western blot analysis to check whether Dps1 or Dlp1 was co-precipitated 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 (Fig. 4A and B, lane 1), and the purified GST (the negative control) did not (Fig. 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.

3.5. 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. pom-*

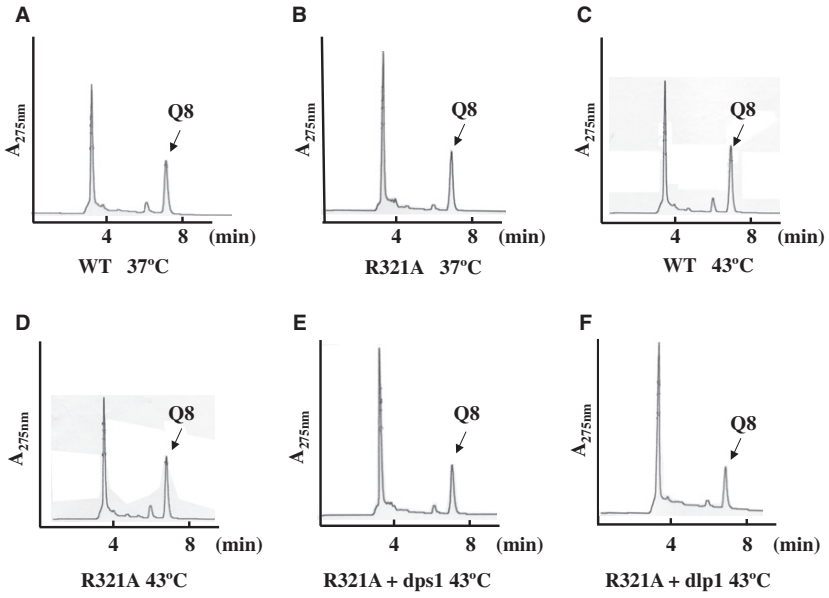

Fig. 2. Expression of *dps1* or *dlp1* does not alter the Q species in *E. coli*. Both the wild-type and the *ispB*^{R321A} *E. coli* strains were incubated at the indicated temperatures and ubiquinone was extracted. To test the effect of *dps1* and *dlp1* on ubiquinone synthesis, the plasmid harboring *dps1* or *dlp1* was introduced into the mutant *ispB*^{R321A} cells.

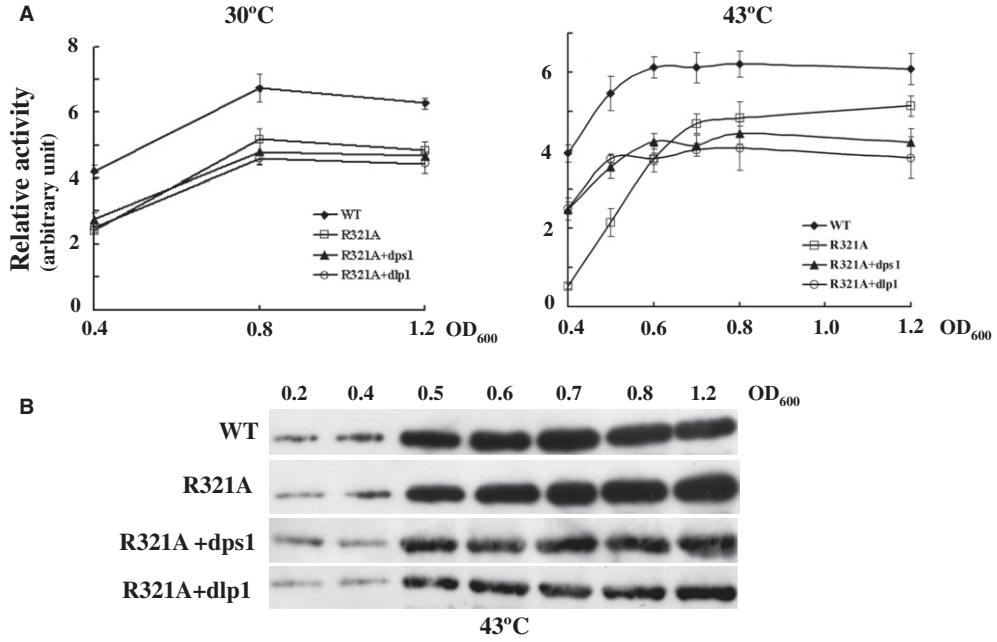

Fig. 3. Analysis of PDS activity and IspB protein levels. (A) Growth phase-specific activity of PDSs in the *E. coli* cells. The *E. coli* cells were grown at both 30 °C and 43 °C and collected at the indicated OD_{600} values. The PDS activity of *E. coli* was assayed at both 30 °C and 43 °C using [1–^{14}C]IPP 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 ± S.D. 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. 5 μg 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).

be [9,10]. To investigate how Dps1 and Dlp1 interact with IspB, we employed Blue Native PAGE to analyze these complexes. First, we looked at the homomeric IspB complex, which is known to form a homodimer in vitro [13]. Unexpectedly, both GST-IspB and GST-IspB^{R321A} formed tetramers, dimers and polymers, as judged by the molecular size of the proteins (Fig. 5A). Because Blue Native PAGE maintains proteins in a more 'native' state compared with

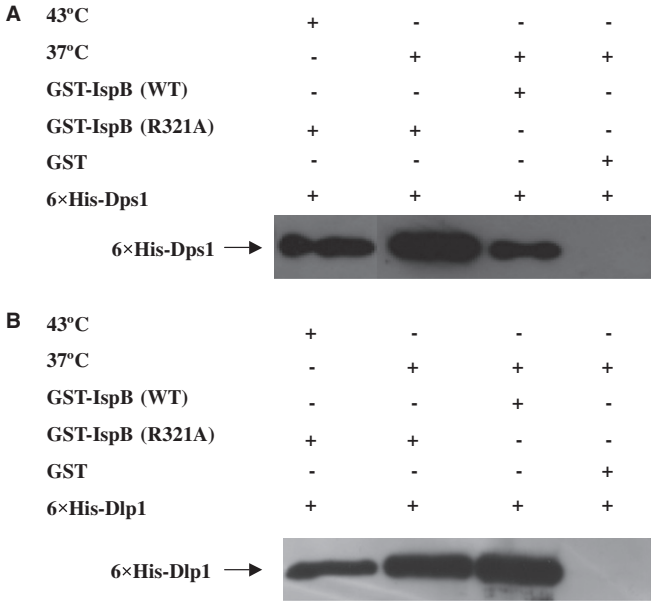

Fig. 4. Physical interactions of Dps1–IspB and Dlp1–IspB in *E. coli*. (A) Dps1 binds to IspB in *E. coli*. *E. coli* DH5α cells harboring the corresponding plasmids were grown at the indicated temperatures. The purified GST-IspB, GST-IspB^{R321A} and GST samples were then subjected to Western blot analysis 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.

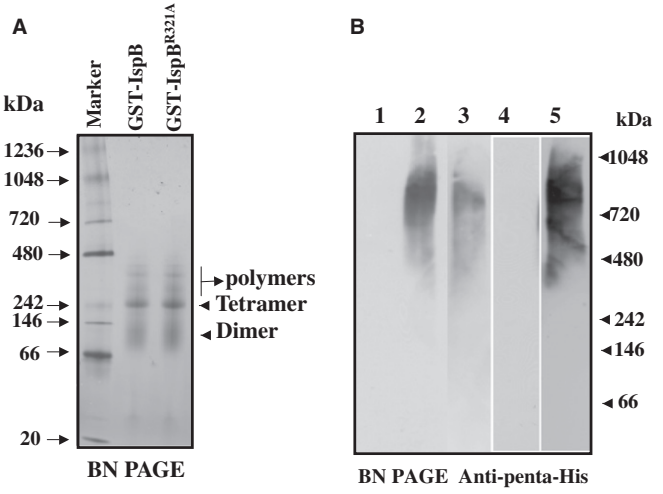

Fig. 5. High-molecular weight complexes formed by Dps1–IspB and Dlp1–IspB. (A) IspB forms a tetramer in *E. coli*. Δ*ispB* harboring pGKO56 and Δ*ispB* harboring pGKO56-R321A were grown at 43 °C and used to purify wild-type and mutant IspB, respectively. The purified GST-IspB was then analyzed using the Blue Native PAGE system on 4–16% Bis-Tris gels. Molecular weight markers range in size from 20 to 1200 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 pSTVK-HIS-hDlp1 (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.

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 our surprise, the molecular weight of both the IspB–Dps1 and the IspB–Dlp1 complexes was approximately 800 kDa (Fig. 5B, lanes 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. 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 the *S. pombe* Dps1 cannot form a complex with mammalian Dlp1.

4. Discussion

Analysis of an *E. coli ispB* mutant led us 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, they are able to restore the enzymatic activity of the $ispB^{R321A}$ mutant at 43 °C 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.

Our group recently reported an artificial PDS, which was composed of *S. cerevisiae* Coq1 and *S. pombe* Dps1 [9], and provided evidence that a component of heteromeric PDS supports the activity of homomeric PDS under certain conditions. In this study, we show that a subunit of heteromeric PDS stabilizes the homomeric PDS and restores its enzyme activity. However, unlike the data presented in our previous work showing that Dps1 played a role in heteromeric PDS formation [9], this study shows that both Dlp1 and Dps1 can play a role in the stabilization of an artificial PDS.

Homomeric PDSs such as *E. coli* IspB [13], *S. cerevisiae* Coq1 [9], *Gluconobacter suboxydans* DdsA [14], Trypanosoma cruzi [15], and *Arabidopsis* SPS1, 2 [16] are found in many organisms. However, heteromeric PDSs are only found in a small number of organisms, including *S. pombe* [10,17], mice, and humans [11]. Although the role played by Dps1 and Dlp1 in heteromeric PDSs is currently unknown, we do know that each component requires its partner for activity [9]. It is also not yet clear why some organisms, including mammals, require heteromeric poly-PDS. The elucidation of the crystal structures of heteromeric enzymes will bring us closer to unraveling the evolution of poly-PDSs.

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