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Biochemical characterization of the decaprenyl diphosphate synthase of *Rhodobacter sphaeroides* for coenzyme Q_{10} production

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Abstract Coenzyme Q_{10} (CoQ_{10}), like other CoQs of various organisms, plays indispensable roles not only in energy generation but also in several other processes required for cells' survival. In this study, a gene encoding for a decaprenyl diphosphate synthase (Rsdds) was cloned from *Rhodobacter sphaeroides* in *Escherichia coli*. The in vivo catalytic activity and product specificity of Rsdds were compared with those of a counterpart enzyme from *Agrobacterium tumefaciens* (Atdds) in *E. coli* as a heterologous host. In contrast with Atdds, Rsdds showed lower catalytic activity but higher product specificity for CoQ_{10} production, as indicated by the amount of CoQ_9 formation. The higher product specificity of Rsdds was also confirmed by utilizing both Rsdds and Atdds for in vitro synthesis of polyprenyl diphosphates. Thin layer chromatography indicated that the Rsdds enzyme resulted in relatively much less solanesyl diphosphate formation. The purified Rsdds catalyzed the addition of isopentenyl diphosphate to dimethyl allyl diphosphate, geranyl diphosphate, ω,*E,E*-farnesyl diphosphate (FPP), and ω,*E,E,E*-geranylgeranyl diphosphate as priming substrates. The kinetic parameters of V_{max} (pmol/min), K_M (μM), k_{cat} (1/min), and k_{cat}/K_M of the enzyme using FPP as the most appropriate substrate were determined to be 264.6, 13.1, 8.8, and 0.67, respectively.

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Introduction

Coenzyme Q (CoQ) is an essential component of the electron transport chain for aerobic respiration. In addition to the role that CoQ plays in energy generation, it has several other functions including the removal of reactive oxygen species, the regulation of gene expression, and the control of the redox status of cells by adjusting the NAD^+/NADH ratio (Bader et al. 1999; Battino et al. 1990; Crane 2001; Kawamukai 2002; Seballe and Poole 1999; Turunen et al. 2004). In humans, the administration of CoQ_{10} has proved to be promising in the prevention and treatment of several diseases, as well as aging symptoms (Matthews et al. 1998; Sharma et al. 2004; Somayajulu et al. 2005).

The CoQ structure is composed of a quinonoid head that is prenylated with an isoprenoid side chain (Fig. 1). The biosynthesis of CoQ begins with prenylation of 4-hydroxybenzoate and proceeds through several steps of ring modification reactions (Meganathan 2001). The formation of the isoprenoid side chain is catalyzed by a group of enzymes, called polyprenyl diphosphate synthases, which deploy an allylic diphosphate as a priming molecule for consecutive additions of isopentenyl diphosphate (IPP) molecules up to a certain length (Lange et al. 2000; Liang et al. 2002). The final length of the product is a characteristic that has been used for classification of polyprenyl diphosphate synthases (Ogura and Koyama 1998). Accordingly, these enzymes fall into four categories. The products of class I are short (C10, C15, and C20), those of class II are medium (C30 and C35), and those of

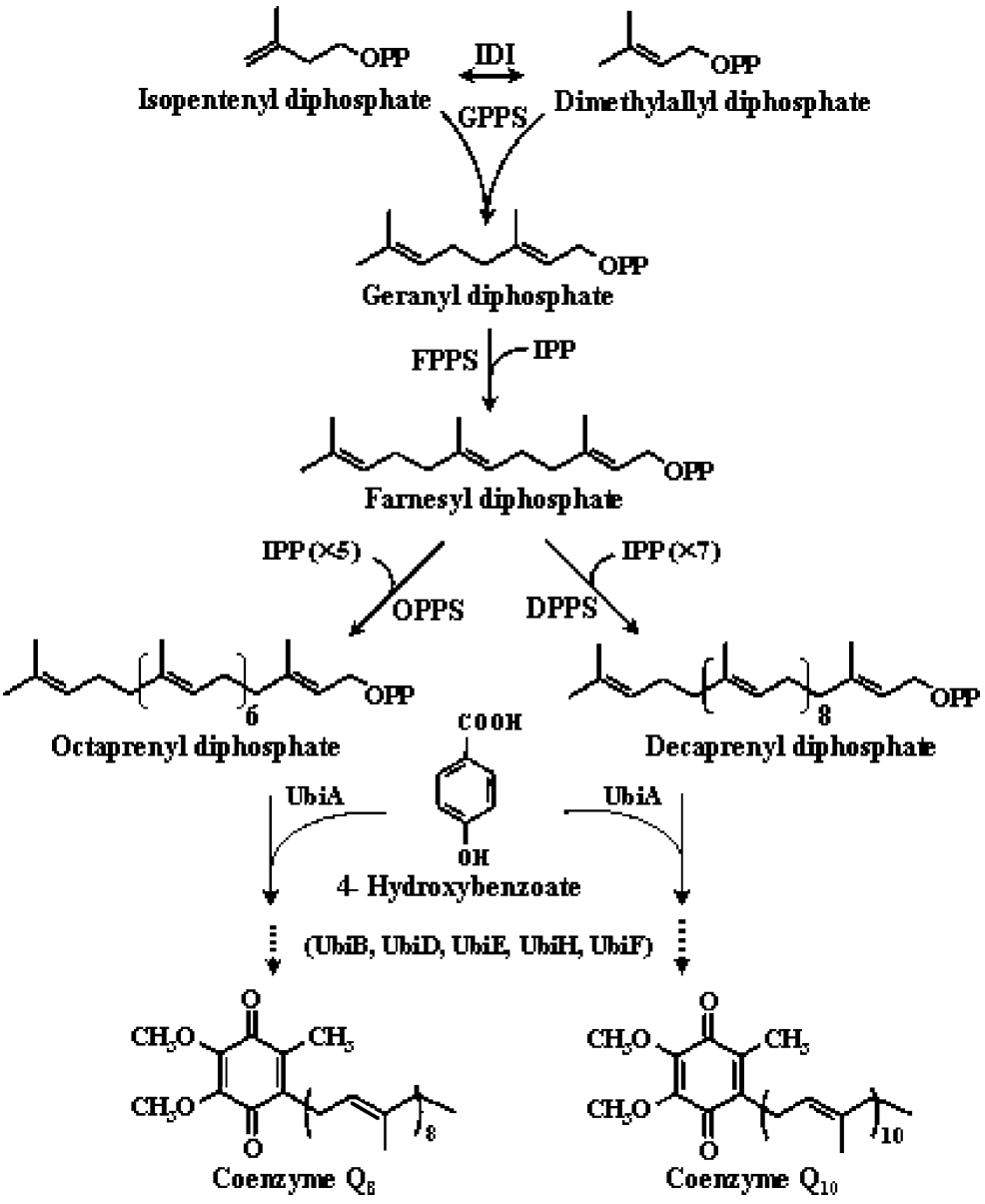

Fig. 1 Schematic representation of CoQ_{10} production in a recombinant *E. coli* harboring a heterologous *dds*A gene encoding DPPS enzyme. The enzymes indicated in this figure are designated as follows: *IDI* isopentenyl diphosphate isomerase, *GPPS* geranyl diphosphate synthase, *FPPS* farnesyl diphosphate synthase, *OPPS* octaprenyl diphosphate synthase, *DPPS* decaprenyl diphosphate synthase, and *UbiA* 4-hydroxy benzoic acid-polyprenyltransferase; UbiB, D, G, H, E, and F, are quinoid ring modification enzymes

class III are long (C40, C45, and C50) chain polyprenyl diphosphates. All the enzymes catalyze the formation of double bonds in *trans*-stereoconfiguration (*all-E*). The products of class IV polyprenyl diphosphate synthases are generally long and, in contrast to other classes, have double bonds in *cis*-stereoconfiguration.

The poly prenyl synthases of class II and III are responsible for biosynthesis of the isoprenoid side chain of CoQs in various organisms. Interestingly, the length of the isoprenoid chain determines the species specificity of CoQs (Okada et al. 1996). For instance, in *Escherichia coli*, *Saccharomyces cerevisiae*, and humans, the side chain is composed of eight, six, and ten IPP units, resulting in the formation of CoQ_8, CoQ_6, and CoQ_{10}, respectively.

Decaprenyl diphosphate synthase (DPPS), which catalyzes the synthesis of the CoQ_{10} side chain, has been isolated, cloned, and studied from several microbial species, including *Agrobacterium tumefaciens*, *Paracoccus denitrificans*, *Gluconobacter suboxydans*, and *Schizosaccharomyces pombe* (Lee et al. 2004; Takahashi et al. 2003; Okada et al. 1998a,b; Suzuki et al. 1997). The biosynthesis of the side chain appears to be a rate-limiting step and critical in CoQ production. It has been shown that overexpression of *ispB* encoding for octaprenyl diphosphate synthase results in significant increases in CoQ_8 production in *E. coli* (Zhu et al. 1995). Therefore, more extensive knowledge on the function and structure of DPPS enzymes is of importance with regard to the biotechnological production of CoQ_{10}.

Rhodobacter sphaeroides is a purple nonsulfur bacterium belonging to the phototrophic α-proteobacteria. In this study, we describe the purification and biochemical

characterization of a DPPS from *R. sphaeroides*. Because the enzyme catalyzes the formation of a C50 (all-*E*) isoprenoid which may serve as the side chain in CoQ_{10} biosynthesis, the in vivo catalytic activity and product specificity of the enzyme for CoQ_{10} production in *E. coli* were studied and compared with those of a counterpart enzyme from *A. tumefaciens*.

Materials and methods

Materials

Luria-Bertani (LB), tryptone, and yeast extract were purchased from Difco. Restriction enzymes and T4 DNA ligase were purchased from New England Biolabs. *Pfu* DNA polymerase was purchased from Promega. Plasmid preparation, gel extraction, and PCR purification kits were Qiagen products. CoQ_9 and CoQ_{10} were purchased from Sigma. *Escherichia coli* M15[pREP4] cells and pQE-30 vector were purchased from Qiagen (Table 1). [1-^{14}C] Isopentenyl diphosphate (2.1 GBq/mmol) was purchased from Amersham. Dimethylallyl diphosphate (DMAPP), farnesyl diphosphate (FPP), geranyl diphosphate (GPP), (all-*E*)-geranylgeranyl diphosphate (GGPP), and solanesol (all-*E*-nonaprenol) were purchased from Sigma. Reverse phase C18 thin-layer chromatography (TLC) plates were from Merck. Potato acid phosphatase was from Roche. All other materials were of analytical grade.

Cloning, expression, and purification of Rsdds

The DPPS gene (*rsdds*) was isolated from genomic DNA of *R. sphaeroides* 2.4.1 [American Type Culture Collection (ATCC) 17023] using PCR amplification. Recently, the genome sequence of *R. sphaeroides* was released by the Joint Genome Institute (http://genome.jgi-psf.org). An orthology search in the genome sequence of *R. sphaeroides* revealed an open reading frame (ORF) with high homology with known sequences of DPPS. The colony PCR technique was conducted for amplification of the ORF using rsdds-F2 and rsdds-R1 primers (Table 1). The amplified fragment was cut by *Eco*RI and *Sac*I and inserted into pTrc99A as a vector to make pTrsdds. For cloning of the *ddsA* gene from *A. tumefaciens* (ATCC 33970), PCR cloning was performed using atdds-1 and atdds-2 as forward and reverse primers (Table 1). The resulted fragment was cut with *Eco*RI and *Bam*HI and inserted into pTrc99a, resulting in pTatdds. The nucleotide sequences of *rsdds* and *atdds* genes were confirmed by sequencing.

For purification of the Rsdds enzyme, immobilized metal affinity chromatography (IMAC) was performed on an N-terminally His-tagged fusion enzyme. The *rsdds* gene was amplified by using rsdds-F3 and rsdds-R2, respectively, as forward and reverse primers so as to remove the start and stop codons and create *Bam*HI and *Hin*dIII sites upstream and downstream of the gene. The amplified fragment was inserted into pQE-30 to make pQrsdds, which was used for transformation of *E. coli* M15[pREP4] competent cells.

Table 1 Strains, plasmids, and primers used in this study

Name	Description	
Strains		
E. coli DH5α	[F′/*endA1 hsdR17* (rK–mK$^+$) *glnV44 thi-1 recA1 gyrA* (Nal^r) *relA1* ▲*(lacIZYA-argF)U169 deoR* 5(φ80*dlac*▲*(lacZ)M15*)]	(Gibco BRL)
E. coli M15	Nal^s, Str^s, Rif^s, RecA$^+$, Uvr$^+$, Lon$^+$, *lac*, *ara*, *gal*, *mtl*, *f*	(Qiagen)
E. coli PTA	*E. coli* DH5α harboring pTatdds	(This study)
E. coli PTR	*E. coli* DH5α harboring pTrsdds	(This study)
E. coli PQR	*E. coli M15* harboring pREP4 and pQrsdds	(This study)
Plasmids		
pTrc99A	P*trc* expression vector, pBR322 origin, lacI^q, Amp^r High copy	(Amersham Bioscience)
pQE30	T5 promoter, N-terminal His_6 tag, Amp^r	(Qiagen)
pREP4	*lac* repressor plasmid, Km^r	(Qiagen)
pTatdds	pTrc99A with *dds* gene from *A. tumefaciens*	(This study)
pTrsdds	pTrc99A with *dds* gene from *R. sphaeroides*	(This study)
pQrsdds	pQE30 with *dds* gene from *R. sphaeroides*	(This study)
Primers		
rsdds-F2	5′-ATAGAATTCAGGAGGTCATCGGGATGGGATTGGACGAGGTTTC-3′ (*Eco*RI)	
rsdds-R1	5′-TAAGAGCTCAAGGGATCAGGCGATGCGTTCGAC-3′ (*Sac*I)	
rsdds-F3	5′-ATAGGATCCGGATTGGACGAGGTTTCGCAAAAGC-3′ (*Bam*HI)	
rsdds-R2	5′-ATAAAGCTTGGCGATGCGTTCGACCACG-3′ (*Hin*dHIII)	
atdds-1	5′-GGAATTCTTGCCGCACAAGGCATCAG-3′ (*Eco*RI)	
atdds-2	5′C-CGGGATCCGCGTTAGTTGAGACGCTCGATG-3′ (*Bam*HI)	

The resulted recombinant *E. coli* PQR (Table 1) was grown overnight in 4 ml of LB broth containing 100 μg/ml of ampicillin and 30 μg/ml of kanamycin. Of the culture, 2.5 ml was subsequently used for inoculation of 50 ml of fresh LB broth in a 250-ml flask. The cells were grown at 30°C to an OD_{600} of 0.5 and induced with isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.2 mM. Incubation was continued for an additional 5 h at the same temperature. Cells were then harvested by centrifugation at 10,000 rpm for 20 min at 4°C. The pellet was resuspended in 5 ml of buffer A (25 mM Tris buffer, pH 7.5, containing 150 mM NaCl and 20 mM imidazol), and after the addition of lysozyme to a final concentration of 1 mg/ml, the suspension was left on ice for 30 min. The cells were disrupted using sonication (1 s on and 3 s off for 5 min at 35% amplitude); the resulted suspension was then centrifuged as before. The supernatant containing soluble fusion Rsdds enzyme was applied on a 5-ml IMAC column previously charged with Ni^{2+} and then washed and equilibrated with buffer A. The column was washed with three volumes of the same buffer so as to remove the unattached materials. The Rsdds fusion enzyme was then eluted using 2 vol of buffer B (25 mM Tris buffer, pH 7.5, containing 150 mM NaCl and 200 mM imidazol). The expression and purity of Rsdds enzyme were verified by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Coomassie brilliant blue 250R staining (Laemmli 1970). Protein concentrations were estimated using the Bio Rad protein assay reagent (Bradford) or routinely from the absorbance of enzyme preparations at 280 nm. Phylogenetic and molecular evolutionary analyses were conducted using MEGA version 3.0 (Kumar et al. 2004).

CoQ_{10} determination

For CoQ_{10} production, 5 ml of 2YT medium (tryptone, 1.6%; yeast extract, 1%; NaCl, 0.5%; ampicillin, 100 μg/ml) was used for overnight precultivation of recombinant *E. coli* strains in a test tube at 37°C. Main cultures were conducted by using the same medium supplemented with 0.5% glycerol and 0.01% 4-hydroxy benzoic acid (sodium salt, Sigma), either in 25-ml tubes containing 7 ml of medium or 250-ml flasks containing 25 ml of medium. Main cultures were inoculated to a starting OD_{600} of 0.1 and incubated at 30°C for 24 h in a rotary-shaking incubator at 200 rpm. Cell growth was measured at 600 nm and converted to dry cell weight (DCW) (gDCW/l) by using a standard curve.

The CoQ_{10} biosynthesis in the cultured cells was studied via high-performance liquid chromatography (HPLC). A 500-μl sample of each culture was centrifuged at 14,000 rpm by using a bench-top centrifuge. The pellet was washed once with 1 ml of distilled water and then with 1 ml of 20 mM Tris–HCl, pH 7.6. The washed cells were lysed by adding 450 μl of Cell Lytic B solution (Sigma) and 50 μl of egg white lysozyme stock solution (10 mg/ml, Amresco). After incubation at 37°C for 30 min, CoQ_{10} was extracted from the cell lysate with 900 μl of a hexane/propanol (5:3) mixture and then with 500 μl of hexane. Following each extraction, the hexane organic phase containing CoQ_{10} was separated from the aqueous phase by centrifugation at 14,000 rpm and then collected into a new tube. The hexane extract was evaporated to dryness via overnight incubation in a vacuum chamber at room temperature. The remaining pellet was dissolved in 500 μl of absolute ethanol (Fisher Scientific, HPLC grade), from which 10 μl was injected into a HPLC machine (Shimadzu 10A system) equipped with a Symmetry® C18 column (Waters). The chromatography was run at room temperature using an isocratic solvent mixture of ethanol and methanol (70:30 v/v, Fisher Scientific, HPLC grade) as a mobile phase at a flow rate of 1 ml/min. A UV detector was used at 275 nm for the detection and quantification of CoQ_{10}. Authentic standards, CoQ_9 and CoQ_{10} (Sigma), were used to distinguish the corresponding peaks in the HPLC chromatograms of the experimental samples. A standard curve was made for quantification of CoQ_{10} by making serial dilutions of a CoQ_{10} standard stock solution (1 mg/ml). A reproducible high correlation (r^2>0.999) between CoQ_{10} concentrations and peak areas was obtained.

Prenyl diphosphate synthase assay

The enzyme activity was measured in a 200-μl reaction mixture containing 100 mM 4-2-hydroxyethyl-1-piperazineethanesulfonic acid (pH 7.5), 5 mM $MgCl_2$, 50 mM KCl, 0.3% Triton X-100, a given concentration of a priming substrate, and 17.14 μM [1-^{14}C] IPP. After the addition of 1.14 μg (30 pmol) of recombinant DPPS enzyme, reactions were run for 10 min at 37°C. Finally, the reactions were stopped by the addition of 400 μl of 20 mM EDTA. The products were then extracted with 600 μl of water-saturated 1-butanol (Pan et al. 2002), and aliquots from both aqueous and butanol phases were taken for liquid scintillation spectrometry. Experimental data were fitted to the following equation: $rate = (V_{max} \times [allylic\ diphosphate])/(K_M + [allylic\ diphosphate])$, where V_{max} is the maximum rate and K_M is the Michaelis–Menten constant equal to the concentration of allylic diphosphate at $V_{max}/2$. To determine the optimum concentration of $MgCl_2$ and Triton X-100, as well as to study the effect of dithiothreitol (DTT) and KCl (50 mM) on the reaction rate, 100 μM FPP was used as the priming substrate; otherwise, reactions were run under the same conditions as outlined above.

Analysis of reaction products

The chain length of radioactive prenyl diphosphates was determined by TLC. Reaction mixtures containing 60 μM [1-^{14}C] IPP and 100 μM GPP, FPP, or GGPP were incubated at 30°C for 1 h. The reactions were then stopped by the addition of 20 mM EDTA; the radiolabeled polyprenyl diphosphates were extracted with 1-butanol. The butanol phase was transferred to a clean tube and evaporated to dryness. The polyprenyl diphosphates were converted to corresponding polyprenols by the addition of 250 μl of phosphatase solution containing 50 mM sodium acetate (pH 4.7), 6 U/ml potato acidic phosphatase, 20% propanol, and 0.1% Triton X-100 (Koyama et al. 1985). After 8 h of incubation at 37°C, the polyprenols were extracted with 600 μl of n-hexane. Following centrifugation, the hexane layer was transferred to a new tube and evaporated to dryness. The resulting pellet was dissolved in 20 μl of hexane and spotted on a reverse-phase TLC plate. The TLC plate was developed using acetone/water (19:1) as a mobile phase (Shimizu et al. 1998). Finally, the plate with radiolabeled products was exposed to a film and the radioactivity distribution was determined by autoradiography.

Results

Cloning of the DPPS ORF (*rsdds*) from *R. sphaeroides*

A homology search of *R. sphaeroides* genome for the DPPS gene (*dds*) resulted in an ORF showing high homology with known *dds* genes. The alignment of the deduced amino acid sequence of the ORF with several other (*all-E*) prenyl diphosphate synthases from *A. tumefaciens* (Atdds, National Center for Biotechnology Information accession no. AY224190), *G. suboxydans* (Gsdds, accession no. AB006850), *P. denitrificans* (Pddds, accession no. E34002), *Paracoccus zeaxanthinifaciens* (Pzdds, accession no. AJ431695), and *S. pombe* (Spdds, accession no. D84311) revealed conserved sequences, including two aspartate-rich motifs. The deduced amino acid sequence of the ORF showed similarity with that of others in the following order: Pzdds (81%), Pddds (76%), Gsdds (59%), Atdds (56%), and Spdds (24%).

To verify that the ORF encodes a functional DPPS, the ORF was amplified from the *R. sphaeroides* genome and inserted into pTrc99A to make pTrsdds. The nucleotide sequence of the insert was confirmed by sequencing. This plasmid was used for the transformation of *E. coli* DH5α, resulting in *E. coli* PTR. The HPLC analysis of CoQs produced in the recombinant *E. coli* PTR indicated the production of CoQ_{10} in addition to CoQ_8 (Fig. 2c). Notably, wild-type *E. coli* does not produce any CoQ_{10} but rather produces CoQ_8 as the main ubiquinone (Okada et al. 1998a,b). This result together with the information obtained from the sequence analysis was used as a basis for determining that the ORF from *R. sphaeroides* encodes a functional DPPS (Rsdds). Accordingly, the ORF is composed of 999 nucleotides encoding a protein that is 333 amino acids long. The sequence of the gene was deposited to GenBank under the accession number AY847292.

Comparative study of in vivo catalytic activity and product specificity of Rsdds

Among the bacterial species described above, *A. tumefaciens* contains a DPPS enzyme that has the least similarity with its counterpart from *R. sphaeroides*. A comparison was made between the enzymes in terms of their stringency for CoQ_{10} production and in vivo catalytic activities. For this purpose, the *ddsA* gene (*atdds*) was isolated from genomic DNA of *A. tumefaciens* and inserted into pTrc99A, as was done previously for the *dds* gene of *R. sphaeroides*. The resulting plasmid, pTatdds, was utilized for transformation of *E. coli* DH5α to make *E. coli* PTA. Both *E. coli* PTR and *E. coli* PTA were cultured for CoQ_{10} production under the same culture conditions. *E. coli* PTR produced CoQ_{10} as the major CoQ besides a negligible amount of CoQ_9 and normally produced CoQ_8 (Fig. 2c). In contrast, *E. coli* PTA produced higher amounts of CoQ_9 up to about 12% of CoQ_{10} content (Fig. 2b).

To confirm the higher product specificity of Rsdds for the decaprenyl diphosphate synthesis, cell free homogenates of both *E. coli* PTR and *E. coli* PTA were used as crude enzymes for in vitro conversion of [1-^{14}C] IPP to radiolabeled polyprenyl diphosphates. The resulting products were separated on a TLC plate and visualized by autoradiography. As shown by the images inset in Fig. 2, the formation of solanesyl diphosphate as a side chain for CoQ_9 biosynthesis was significantly higher in the case of Atdds.

The CoQ_{10} production in *E. coli* PTR was 408±35 μg/gDCW and could be elevated to 845±33 μg/gDCW via induction using 0.1 mM IPTG as an inducer of P_{lac} promoter in pTrsdds. In *E. coli* PTA, the CoQ_{10} production was 637±15 μg/gDCW. The induction of P_{lac} promoter in pTatdds with 0.1 mM IPTG adversely affected the CoQ_{10} production, which decreased to 356±30 μg/gDCW (Fig. 3).

Overexpression and purification of Rsdds

To characterize the DPPS of *R. sphaeroides* (Rsdds), the enzyme was tagged with histidine (6× His) at the N-terminus and purified via metal affinity chromatography. As indicated by SDS-PAGE, a highly purified fraction of

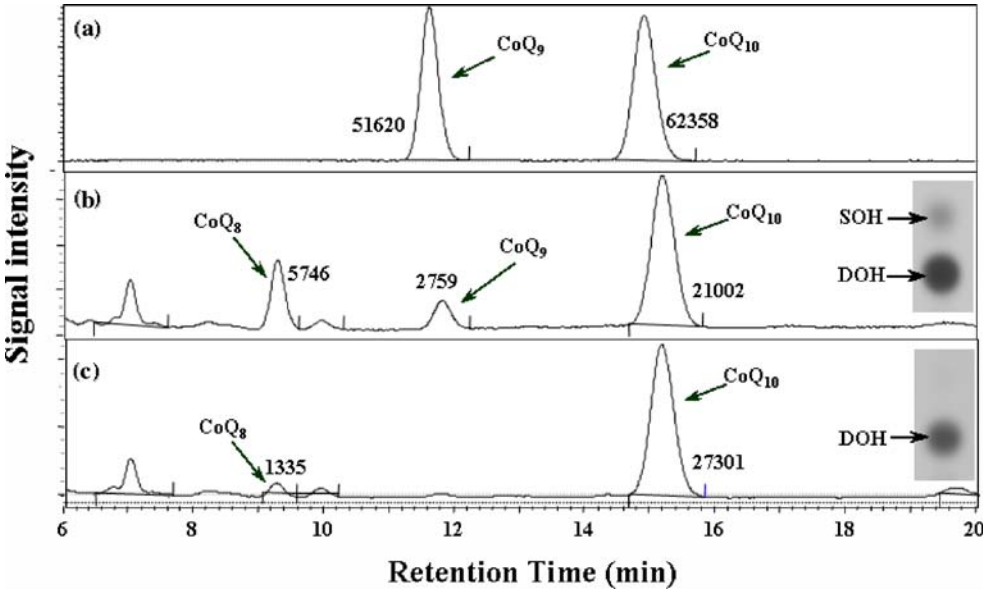

Fig. 2 HPLC chromatograms of: *A* standard CoQ_9 and CoQ_{10}, *B* ubiquinones extracted from a recombinant *E. coli* harboring DPPS of *A. tumefaciens* (*atdds*), and *C* ubiquinones extracted from a recombinant *E. coli* harboring DPPS of *R. sphaeroides* (*rsdds*). The recombinant strains produce high amounts of CoQ_{10} and some CoQ_9 in addition to naturally occurring CoQ_8. The amount of CoQ_9 produced by Rsdds was quite less than that of Atdds. As shown by the *inset images*, using cell-free homogenate of each recombinant strain so as to the in vitro biosynthesis of decaprenyl diphosphate, it was confirmed that the relative octaprenyl diphosphate synthesis by Rsdds was substantially less than that of Atdds

enzyme was obtained by eluting the column with two volumes of 200 mM imidazol (Fig. 4). The mobility of denatured His-tagged fusion Rsdds suggested a mass that was in agreement with the theoretically predicted value of 38.1 kDa (Fig. 4).

Effects of additives on the activity of Rsdds

The in vitro activity of Rsdds was confirmed by using [1-^{14}C] IPP and FPP as substrates for making polyprenyl diphosphates. It has previously been noted that for optimum in vitro activity of polyprenyl diphosphate synthases, some ancillary components, including Mg^{2+}, Triton X-100, and DTT, should be added to the reaction mixture (Koyama et al. 1985; Meganathan 2001). $MgCl_2$ was included to final concentrations of 1, 3, 5, 7, and 10 mM in the reaction mixture with 0.3% Triton X-100 (Fig. 5). As was expected from the properties of many enzymes with phosphorylated substrates, $MgCl_2$ was found to be essential for Rsdds enzymatic activity; in the absence of $MgCl_2$, no activity was measured. The highest enzyme activity was obtained with 5 mM $MgCl_2$ (Fig. 5a).

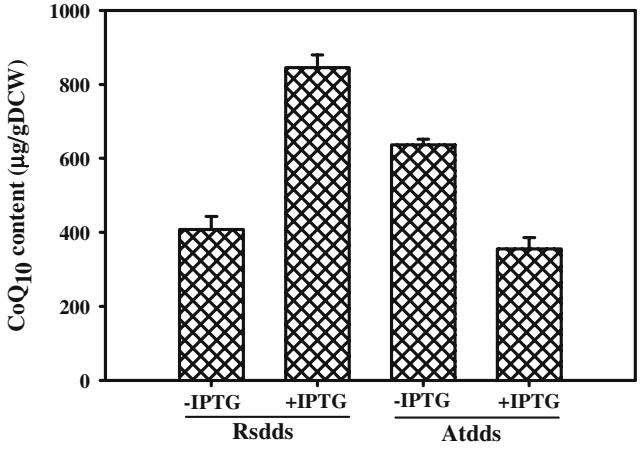

Fig. 3 Comparison between the in vivo catalytic activities of the DPPSs of *R. sphaeroides* (Rsdds) and *A. tumefaciens* (Atdds). *E. coli* DH5α transformed with either pTrsdds (*E. coli* PTR) or pTatdds (*E. coli* PTA) was cultured in trypticase–glucose–yeast extract medium. The catalytic activities of the enzymes were determined in terms of the CoQ_{10} production under uninduced and induced conditions

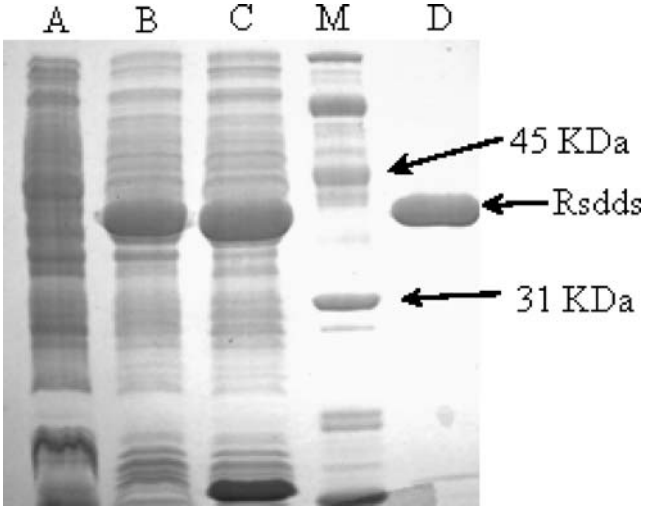

Fig. 4 SDS-PAGE of protein samples taken at various stages of Rsdds purification. *A* cell-free homogenate of *E. coli* PQR before induction, *B* cell-free homogenate of *E. coli* PQR after induction with 0.2 mM IPTG, *C* supernatant obtained after sonication and centrifugation of the induced cells, *M* marker, and *D* purified His-tagged Rsdds

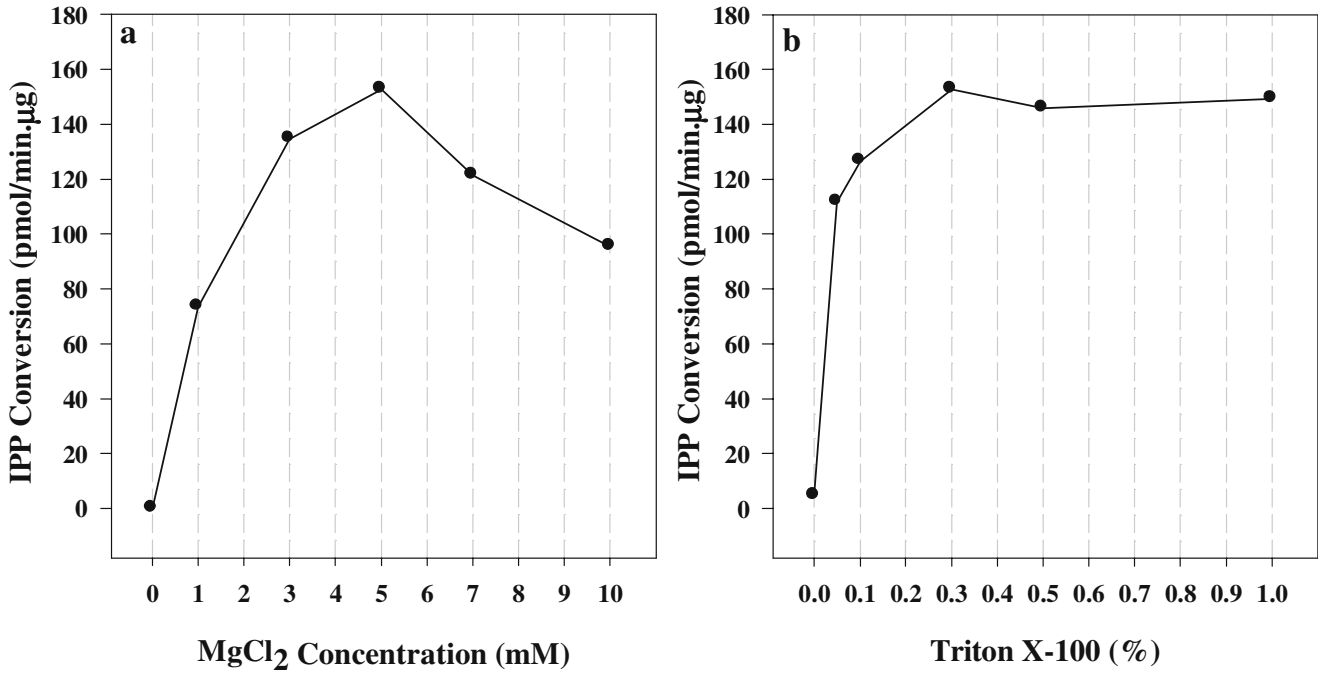

Fig. 5 Effects of $MgCl_2$ and Triton X-100 on the rate of the Rsdds-catalyzed reaction. **a** Various concentrations of $MgCl_2$ were tested with 0.3% Triton X-100. **b** Various concentrations of Triton X-100 were tested with 5 mM $MgCl_2$. Otherwise, the reactions were run under the same conditions as described in "Materials and methods"

The effect of Triton X-100 on Rsdds activity was studied at 0.05, 0.1, 0.3, 0.5, and 1% concentrations using the optimum $MgCl_2$ concentration of 5 mM. The addition of Triton X-100, even at the lowest tested concentration (0.05%), resulted in a significant increase in enzyme activity. Triton X-100 at 0.3% provided the maximum activity (Fig. 5b). Higher concentrations of Triton X-100 did not significantly affect the activity. DTT at final concentrations of 0, 1, 2, 4, and 6 mM was studied with 0.3% Triton X-100 and 5 mM $MgCl_2$. However, DTT had no significant effect on enzyme activity. To determine the effect of KCl on the enzyme activity, KCL was removed from the reaction mixture, resulting in a decrease of activity from 150 to 103 pmol/min.

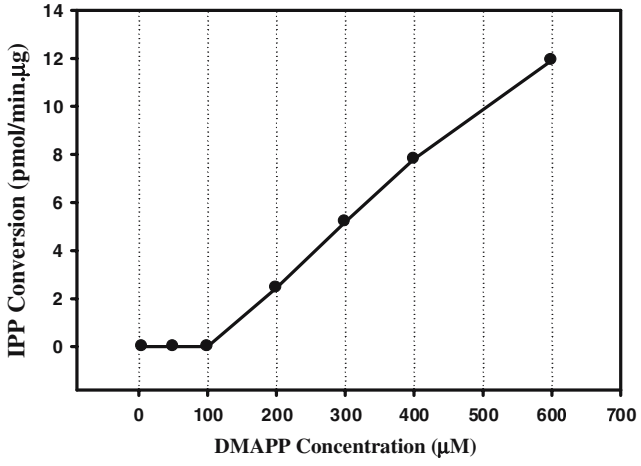

Fig. 6 The effect of DMAPP concentration on the rate of the Rsdds-catalyzed reaction. Various concentrations of DMAPP were used with a fixed concentration of IPP. Otherwise, the reactions were conducted under the same conditions as descried in "Materials and methods"

Kinetic characterization of Rsdds

The effect of priming substrate concentration on the reaction rate was studied by using various substrate concentrations, including DMAPP, GPP, FPP, and GGPP, with a fixed concentration of 17.14 mM [1-^{14}C] IPP. The reaction conditions were otherwise the same as before. Results showed that DMAPP was not a suitable substrate for Rsdds (Fig. 6). At 5, 50, and 100 mM concentrations of DMAPP, no significant radioactivity was measured in butanol phase. DMAPP at 200 mM resulted in a small activity that slowly but linearly increased as the substrate concentration was increased to 300, 400, and 600 mM. In the case of FPP, GPP, and GGPP, Michaelis constants were calculated to be 13.1, 18.5, and 11.5 µM, respectively (Fig. 7b–d; Table 2). Compared with other priming substrates examined, FPP resulted in the highest values for the reaction rate and k_{cat}/K_M, i.e., 264.6 pmol/min and 0.67, respectively. The reaction kinetics for GPP and GGPP were similar under the described reaction conditions. The kinetics constants of the enzyme reactions are summarized in Table 2.

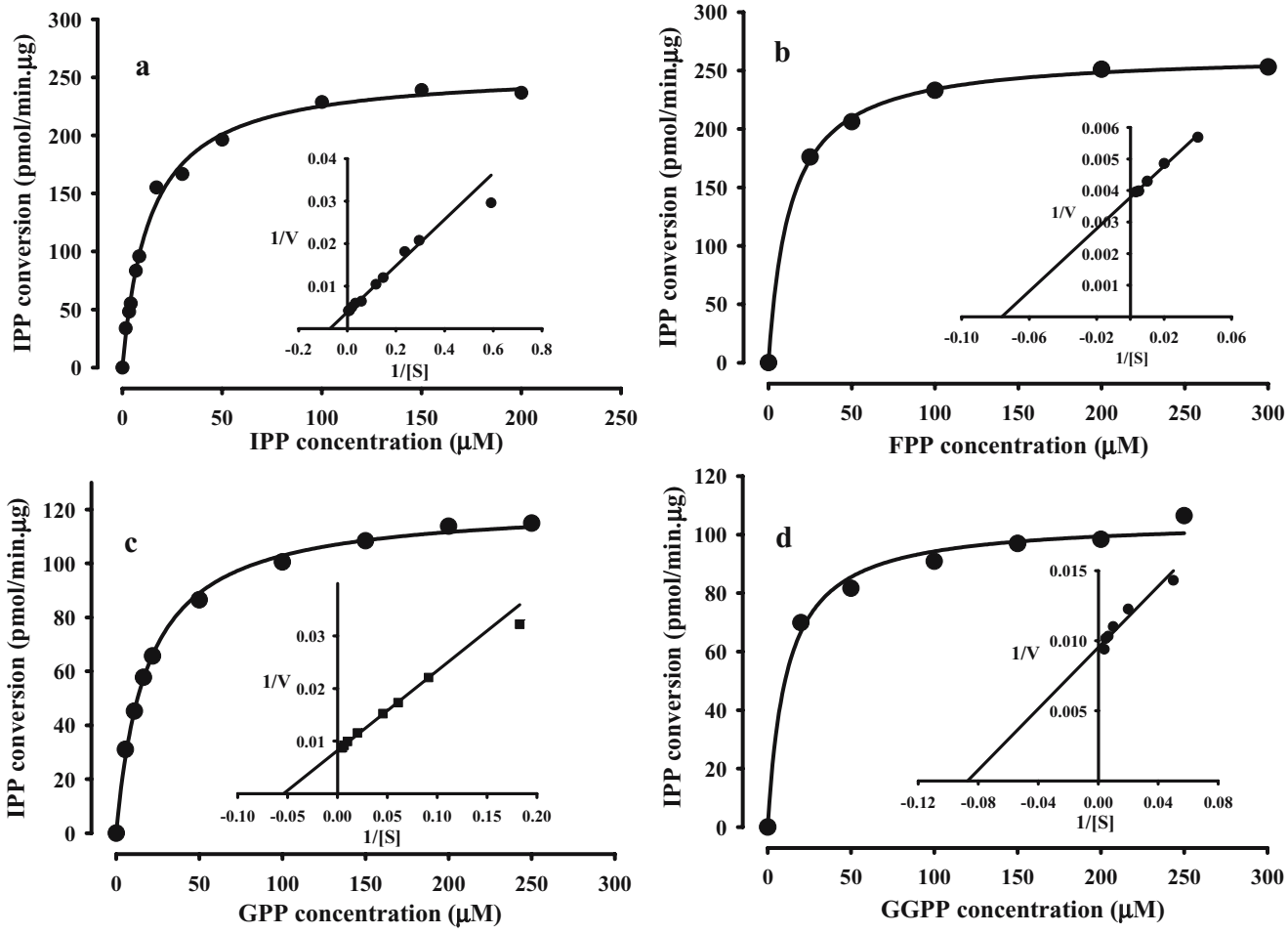

Fig. 7 Effect of substrate concentration on the rate of Rsdds-catalyzed reaction. **a** Various concentrations of [1-^{14}C] IPP were used with 100 mM FPP as a priming substrate. **b** Various concentrations of FPP were used with 17 mM [1-^{1}4C] IPP. **c** Various concentrations of GPP were used with 17 mM [1-^{14}C] IPP. **d** Various concentrations of GGPP were used with 17 mM [1-^{14}C] IPP. Otherwise, the reactions were conducted under the same conditions as descried in "Materials and methods"

To study the effect of IPP concentration on the rate of product formation, various concentrations of [1-^{14}C] IPP were added to reaction mixtures containing FPP at a fixed concentration of 100 mM (Fig. 7a). The reaction rate followed a typical Michaelis–Menten trend, with a maximum conversion rate of 256.5 (pmol/min). The kinetics constants of K_M and k_{cat} were 13.8 µM and 8.8 1/min, respectively.

The analysis of the reaction products via TLC revealed that decaprenyl diphosphate was the major product and

Table 2 Calculated kinetic parameters of Rsdds

Parameter	Substrate			
	IPP[a]	GPP[b]	FPP[b]	GGPP[b]
V_{max} (pmol/min)[c]	256.5±22	122±13	264.6±47	105±28
K_M (µM)[c]	13.8±4.6	18.5±3.5	13.1±6.3	11.5±5.1
k_{cat} (1/min)	8.6	4	8.8	3.5
k_{cat}/K_M	0.62	0.21	0.67	0.3

[a]Various concentrations of IPP were used with a fixed concentration of FPP as a priming substrate to determine the kinetic parameters
[b]A fixed concentration of IPP was used with various concentrations of GPP, FPP, or GGPP as a priming substrate to determine the kinetic parameters
[c]Mean value of three separate experiments±standard deviation

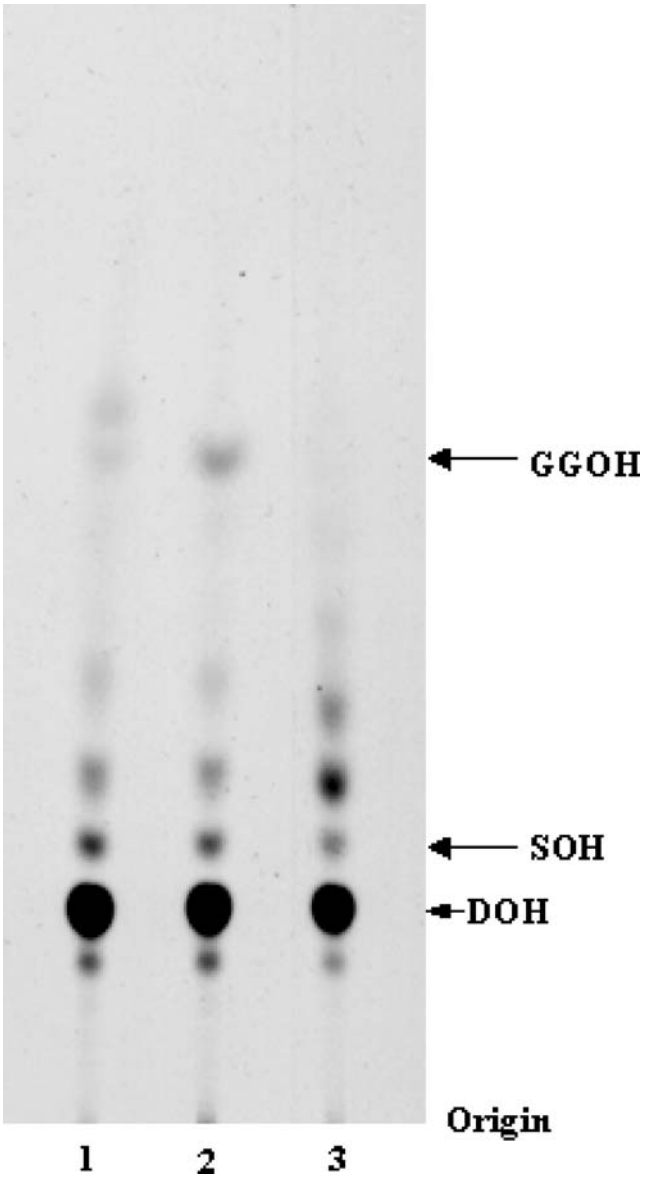

Fig. 8 Thin-layer chromatogram of the radiolabeled products of the Rsdds-catalyzed reactions. The alcohols obtained by enzymatic dephosphorylation of the products formed by incubation of [1-^{14}C] IPP and GPP (*1*), ω,*E,E*-FPP (*2*), and ω,*E,E,E*-GGPP were analyzed by reverse-phase TLC. *Arrows* indicate the positions of the authentic geranylgeraniol (*GGOH*) and solanesol (*SOH*). The *arrowhead* indicates the position of synthesized decaprenol (*DOH*)

other prenyl diphosphates comprised only minor components (Fig. 8). The general pattern of product formation was almost the same for GPP, FPP, and GGPP as priming substrates. However, as expected, GPP resulted in the detection of some FPP and GGPP, in addition to longer components, whereas FPP as a priming substrate resulted in prenyl diphosphates longer than GGPP. When GGPP was used as priming substrate, only medium and long chain prenyl diphosphates were detected on the TLC plate.

Discussion

Given the beneficial role of CoQ_{10} in human health, as well as its applications in medicine and cosmetics industries, the biosynthesis pathway and biotechnological production of CoQ_{10} have been the subjects of numerous studies (Meganathan 2001; Johnson et al. 2005; Yoshida et al. 1998; Sakato et al. 1992; Park et al. 2004). *Rhodobacter sphaeroides* is a member of α-proteobacteria, which are characterized by the production of CoQ with a side chain composed of ten isopren units. This type of CoQ is identical to that produced in humans (CoQ_{10}).

In this study, an ORF, showing high homology with already known *dds*A genes, was identified in the genome of *R. sphaeroides*. The functional expression of the ORF in *E. coli* as a heterologous host resulted in the CoQ_{10} formation in addition to the normally produced CoQ_8. These observations provided convincing evidence for determining the ORF as encoding for a DPPS (Ogura and Koyama 1998). The in vivo catalytic activity of the enzyme (Rsdds) was further compared with that of *A. tumefaciens* (Atdds) in *E. coli.* The CoQ_{10} production and the relative amount of CoQ_9 formation were respectively used as criteria for the evaluation of in vivo catalytic activity and product specificity of the enzymes. Accordingly, it was shown that the catalytic activity of Rsdds was less than that of Atdds; however, its product specificity was higher. The induction of pTrsdds in *E. coli* PTR resulted in a two times increase in CoQ_{10} production, whereas the induction of pTatdds in *E. coli* PTA decreased the CoQ_{10} production. This observation may be accounted for by the assumption that the in vivo catalytic activity of Rsdds in *E. coli* is less than that of Atdds. If so, the increase of the Rsdds dosage should compensate for its lower catalytic activity, as was experimentally confirmed. It can be assumed that because of the high catalytic activity of Atdds, the induction of pTatdds may place some metabolic burden on the cells, such as temporary and abrupt shortage of IPP. This in turn leads to the downregulation of CoQ_{10} biosynthesis.

It has previously been shown that the slow release of product is the primary rate-limiting step for the catalytic activity of prenyl diphosphate synthases (Pan et al. 2000). The slow release was ascribed to the hydrophobic nature of product. Nonionic detergent Triton X-100 greatly improves the in vitro catalytic activity of the enzyme. Triton X-100 not only improves the release of product from the enzyme at the end of chain formation, but also may play a role in increasing the solubility of hydrophobic priming substrates. It is believed that under in vivo conditions inside the cells, an accessory protein molecule fulfills this function (Ohnuma et al. 1991). However, elaborated kinetics studies have demonstrated that the IPP condensation rate for a single-turnover reaction is 100 times larger than the rate for

steady state reactions. Triton X-100 may improve the steady state reaction rate to some extent (Pan et al. 2002). A comparison between the deduced amino acid sequence of Rsdds with that of Atdds shows that Rsdds is more hydrophobic than Atdds (42.2 vs 38.3%). However, the extent to which the higher hydrophobicity may affect the catalytic activity of the enzyme has yet to be elicited. Nevertheless, the higher product specificity of Rsdds for CoQ_{10} production, which results in very lower CoQ_9 formation, is an indication that product is more strictly attached to the enzyme. It has previously been shown that the product specificity of DPPS enzymes for decaprenyl diphosphate synthesis varies among enzymes isolated from various organisms (Takahashi et al. 2003; Saiki et al. 2003; Collins and Jones 1981).

To study the in vitro kinetics of Rsdds, the enzyme was purified using metal affinity chromatography, resulting in highly purified enzyme preparations. Using DMAPP, GPP, FPP, and GGPP composed of 5, 10, 15, and 20 carbons, respectively, it was shown that the enzyme could exploit all the substrates for consecutive additions of IPP units. However, DMAPP was not an appropriate substrate, resulting in a very low catalytic activity of Rsdds. It is likely that the relatively hydrophilic nature of the small substrate is the cause of the low enzyme activity. Under our in vitro conditions, FPP proved to be the most efficient priming substrate among the assessed substrates, yielding the highest V_{max} and k_{cat} values. The same result had previously been reported for DPPS enzyme of *G. suboxydans* (Okada et al. 1998a,b). Like other prenyl diphosphate synthases, Mg^{2+} was required for the enzyme activity. It is believed that allylic diphosphates bound through Mg^{2+} to the first two Asp residues in the DDxxD motif, with the hydrocarbon tails of all the ligands growing down the hydrophobic pocket (Meganathan 2001).

The high product specificity of Rsdds for decaprenyl diphosphate synthesis was also confirmed by a TLC analysis of the reaction products. Under in vitro conditions, the major product of the Rsdds reaction was the decaprenyl diphosphate. This observation was in agreement with the data obtained via HPLC analysis of CoQ_{10} production in the recombinant *E. coli* harboring heterologous *rsdds* gene.

Although the DPPSs have previously been isolated from a number of microbial strains, there are few reports on their kinetics and none on the comparison between their CoQ_{10} production efficiencies. To the best of our knowledge, this is the first report on making a comparison between CoQ_{10} production efficiencies, as well as product specificities of two enzymes with the least sequence similarity among all published bacterial DPPS enzymes. This study is important with respect to the essential role of the DPPS in the biosynthesis of CoQ_{10} and the global interest in biotechnological production of CoQ_{10}.

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