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Temperature-dependent modulation of farnesyl diphosphate/geranylgeranyl diphosphate synthase from hyperthermophilic archaea ☆

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Abstract

Enzyme characteristics of *trans*-prenyl diphosphate synthase (*Tk*-IdsA) from *Thermococcus kodakaraensis*, which catalyzes the consecutive *trans*-condensation of isopentenyl diphosphate (C_5) units with allylic diphosphate, were examined. Product analysis revealed that *Tk*-IdsA is a bifunctional enzyme, farnesyl diphosphate (FPP, C_{15})/geranylgeranyl diphosphate (GGPP, C_{20}) synthase, and mainly yields both C_{15} and C_{20}. The FPP/GGPP product ratio increases with the rise of the reaction temperature. The kinetic parameters obtained at 70 and 90 °C demonstrated that the rise of the temperature elevates the k_0 value for the C_{10} allylic substrate to more than those for the C_5 and C_{15} allylic substrates. These data suggest that *Tk*-IdsA contributes to adjust the membrane composition to the cell growth temperature by modulating its substrate and product specificities. Mutation study indicated that the aromatic side chain of Tyr-81 acts as a steric hindrance to terminate the chain elongation and defines the final product length.

Keywords: Farnesyl diphosphate synthase; Geranylgeranyl diphosphate synthase; Archaea; Hyperthermophiles; *Thermococcus kodakaraensis*; *trans*-prenyl diphosphate synthase; Thermostable enzyme

One of the most characteristic features of archaea is the molecular architecture of their membrane lipids, which seems to permit their survival under severe conditions, e.g., at high temperatures or extreme pHs [1,2]. Instead of the fatty acid esters found in other organisms, archaeal core membrane lipids (archaeols) consist of diethers containing two C_{20} isopranoid (phytanyl) chains attached to glycerol. In most thermophilic members of archaea and some mesophilic archaea, the C_{20} hydrocarbon chains are covalently linked in the center of the bilayer to form macrocyclic tetraethers (caldarchaeols) containing C_{40} isopranoid (biphytanyl) units. The isopranoid moieties undergo further modifications by cyclizations in extreme and moderate thermophiles. It is

☆ *Abbreviations:* GGPP, geranylgeranyl diphosphate; FPP, farnesyl diphosphate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; GPP, geranyl diphosphate; TLC, thin-layer chromatography; *Tk-ids*A, a gene for *T. kodakaraensis* short-chain isoprenyl diphosphate synthase; PCR, polymerase chain reaction; *Tk*-IdsA, *T. kodakaraensis* short-chain isoprenyl diphosphate synthase; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CD, circular dichroism; Gdn-HCl, guanidine hydrochloride; Mops, 3-(*N*-morpholine)propanesulfonic acid; FARM, first aspartate-rich motif. SARM, second aspartate-rich motif.

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known that some thermophiles, such as *Sulfolobus solfataricus*, increase the degree of cyclizations as the growth temperature increases [2]. As for thermophilic methanogen, it has been reported that archaeols decline and caldarchaeols, macrocyclic archaeol polar lipids, increase as the temperature rises in *Methanococcus jannaschii* [3]. On the other hand, it has been also reported that the ratio of caldarchaeols to archaeols decreases with the growth time in *Methanothermobacter thermautotrophicus* (formerly *Methanobacterium thermoautotrophicum*) [4].

In addition to their core membrane lipids, archaea contain various C_{15}, C_{20}, and higher isoprenoid compounds, such as squalene, its derivatives [5], carotenoids [1], prenylquinones [2], and prenylated proteins [6]. These isoprenoid compounds are derived from linear *trans*-prenyl diphosphates with various chain lengths. GGPP (C_{20}) is a precursor for the core membrane lipids and carotenoids, and FPP (C_{15}) is a precursor for squalene and its derivatives. These linear compounds are synthesized by a specific enzyme *trans*-prenyl diphosphate synthase, which catalyzes the consecutive condensation of IPP (C_5) with allylic diphosphate in the *trans*-configuration as shown in Fig. 1 [7]. So far, several genes encoding *trans*-prenyl diphosphate synthases have been isolated from archaea and characterized [8–12]. However, the relationship between the viability of thermophilic archaea at high temperature and the regulation of their membrane biosynthesis by these enzymes still remains to be elucidated.

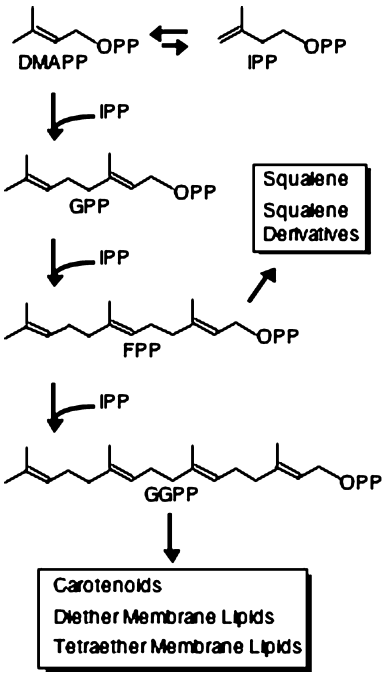

Fig. 1. Isoprenoid biosynthesis in archaea. Membrane components are derived from linear C_{15}–C_{20} isoprenoid compounds synthesized by a specific enzyme catalyzing the consecutive *trans*-condensation of IPP with allylic diphosphate. OPP, diphosphate moiety.

Thermococcus kodakaraensis KOD1 isolated from a solfatara (102 °C, pH 5.2) in Kodakara Island, Kagoshima, Japan, is a sulfur-reducing hyperthermophilic archaeon that shows optimum growth at 90–95 °C [13,14]. The genome analysis of *T. kodakaraensis* is preliminarily completed. The size of its circular genome is 2,088,737 bp, in which both eucaryal and bacterial orthologs are found [15]. In order to obtain the knowledge about the membrane biosynthesis in this hyperthermophile and find a clue to the mechanism of its adaptation to high temperature, we isolated an ortholog of *trans*-prenyl diphosphate synthases from *T. kodakaraensis* by utilizing the genome sequence database and investigated its thermodynamic and enzymatic characteristics at various temperatures. In addition, mutation study was performed to obtain further information about chain length regulation of *trans*-prenyl diphosphate synthase.

Materials and methods

Materials. Thermococcus kodakaraensis KOD1 was cultivated as described previously [14], and its genomic DNA was prepared according to the method previously reported [16]. *Escherichia coli* BL21-CodonPlus(DE3)-RP (Stratagene), *E. coli* BL21(DE3)pLysS (Stratagene), and a pET-8c vector (Novagen) were used for the expression of the recombinant protein. [4-^{14}C]IPP was purchased from NEN Life Science Products. Nonlabeled IPP, DMAPP, GPP, (all-*trans*)-FPP, (all-*trans*)-GGPP, and acid phosphatase from potato were products of Sigma. A pre-coated reverse-phase TLC plate (RP-18) was from Merck.

Gene isolation. An orthology search from the sequence database of the *T. kodakaraensis* genome was performed using the amino acid sequence of *Sulfolobus acidocaldarius* GGPP synthase as a query [10]. An open reading frame for the ortholog, termed *Tk-ids*A, was amplified by PCR with the *T. kodakaraensis* genome as the template and the specific primers, Tk-idsA-*Nco*I-F (5′-GTGCTACCATGGGAAAGT ACGATGAGCT-3′) and Tk-idsA-*Bam*HI-R (5′-CGCGGATCCAG CACAAACTAACGATGGAA-3′). The newly introduced *Nco*I and *Bam*HI sites are underlined. The PCR product was digested with *Nco*I and *Bam*HI, and then ligated into the *Nco*I–*Bam*HI sites of the pET-8c vector, yielding the expression plasmid pET8-Tk-idsA. We confirmed that non-specific mutation had not been introduced during PCR by DNA sequencing.

Expression and purification. Escherichia coli BL21-CodonPlus(DE3)-RP was transformed with pET8-Tk-idsA, and the transformant was grown at 37 °C in 3 L of an NZCYM medium (1% NZ amine, 0.5% yeast extract, 0.5% NaCl, 0.1% casamino acids, and 0.2% $MgSO_4 \cdot 7H_2O$, pH 7.0) supplemented with ampicillin (50 mg/L) until the optical density at 600 nm reached 0.4. Subsequently, isopropyl-β-D-thiogalactopyranoside was added to a final concentration of 1 mM, followed by further cultivation for 5 h at 37 °C. Cells were harvested by centrifugation (8500*g*, 10 min) and then disrupted by sonication in buffer 1 (50 mM sodium phosphate, 10 mM 2-mercaptoethanol, and 50 mM NaCl, pH 6.0). After centrifugation (11,000*g*, 30 min), the supernatant was heated at 90 °C for 10 min, and denatured proteins were excluded by centrifugation (22,000*g*, 30 min). The thermostable proteins were precipitated by the addition of $(NH_4)_2SO_4$ to 75% saturation. After centrifugation (22,000*g*, 30 min), the pellet was dissolved in buffer 1 and dialyzed against buffer 1. The dialyzed solution was heated at 90 °C for 10 min and then centrifuged at 22,000*g* for 30 min.

The supernatant was applied to an SP Sepharose Fast Flow column (Amersham Biosciences) equilibrated with buffer 1. The column was washed with buffer 1, and the flow-through fraction was recovered and then directly loaded onto a Q Sepharose Fast Flow column (Amersham Biosciences) equilibrated with buffer 1. The column was washed with buffer 1 and eluted with a stepwise gradient of 150–250 mM NaCl in buffer 2 (50 mM sodium phosphate, 10 mM 2-mercaptoethanol, pH 6.0). The fractions of the *Tk-ids*A gene product (*Tk*-IdsA) were subjected to gel filtration performed with a Superdex 200 HR 10/30 column (Amersham Biosciences) at 0.4 ml/min with buffer 2 containing 150 mM NaCl. The homogeneity of *Tk*-IdsA was confirmed by SDS–PAGE with silver staining [17]. Broad range molecular weight marker was purchased from New England Biolabs (Catalog No. P7702S). Protein concentrations were measured by the method of Bradford with bovine serum albumin as a standard [18].

Gdn-HCl titration and thermodynamic analysis with CD spectra. Denaturation of *Tk*-IdsA was induced by various concentrations of Gdn-HCl at various temperatures, and its unfolding profiles were measured with CD ellipticity at 222 nm on a JASCO J-820 automatic spectropolarimeter (Japan Spectroscopic Company). For the equilibrium measurement, 0.1 mg/ml *Tk*-IdsA solutions containing 0–7 M Gdn-HCl in 50 mM sodium phosphate (pH 6.0) were prepared in a total volume of 500 μl. They were incubated for three days at a temperature ranging from 40 to 90 °C and then applied to the CD analysis at the same temperature as that used for the incubation. The raw data were analyzed by a two-state folding model as follows. An apparent equilibrium constant (K_{app}) during the unfolding process at a certain temperature was determined according to Eq. (1),

$$K_{app} = f_U/f_N = (y_N - y)/(y - y_U), \quad (1)$$

where f_N and f_U are the fractions of the folded and unfolded states, and y_N, y_U, and y represent the signals of the folded, unfolded, and measured intensities, respectively. The conformational free-energy change (ΔG) of the unfolding process is related to the apparent equilibrium constant by $\Delta G = -RT\ln K_{app}$. A two-state unfolding model can assume a linear dependence between ΔG and the denaturant concentration ([Gdn-HCl]), as shown in Eq. (2),

$$\Delta G = \Delta G_0 - m[\text{Gdn-HCl}], \quad (2)$$

where ΔG_0 means ΔG in the denaturant-free state and m represents the slope of the linear line between [Gdn-HCl] and ΔG.

The ΔG_0 values at the different temperatures were yielded as outlined above, and the temperature dependence of the ΔG_0 value was calculated as reported previously [19]. The ΔG value is expressed as a function of the melting temperature (T_m), the change of enthalpy due to protein unfolding at T_m (ΔH_m), and the heat capacity change accompanying protein unfolding (ΔC_p) according to Gibbs–Helmholtz equation.

$$\Delta G = \Delta H_m(1 - T/T_m) - \Delta C_p[(T_m - T) + T\ln(T/T_m)]. \quad (3)$$

Using Eq. (3), a calculation program (IGOR PRO 4.0, WaveMatrics, Oregon, USA) was instructed to fit T_m, ΔH_m, and ΔC_p.

Measurement of enzymatic activity and product analysis. The enzymatic activity was measured by determination of the amount of [4-^{14}C]IPP incorporated into butanol-extractable polyprenyl diphosphates. The standard assay was performed in a total volume of 200 μl containing a 100 mM Mops buffer, pH 8.0, 5 mM $MgCl_2$, 50 μM [4-^{14}C]IPP (37 GBq mol^{-1}), a 50 μM allylic substrate (DMAPP, GPP, FPP, or GGPP), and 1–2 ng of the purified enzyme. The reaction was carried out at 70 °C for 10 min or at 90 °C for 5 min and terminated by adding 200 μl H_2O saturated with NaCl before 10% of the substrates were consumed. We confirmed that the addition of NaCl-saturated H_2O completely terminates this enzymatic reaction by the control experiment (data not shown). The reaction products were then extracted with 1 ml of 1-butanol saturated with NaCl-saturated H_2O, and the radioactivity in the 1-butanol extract was measured by the dpm mode with an LS 6500 Multi-Purpose Scintillation Counter (Beckman Coulter) and a Clear-sol I scintillation cocktail (Nacalai tesque).

For kinetic studies, the concentration of allylic substrate (DMAPP/GPP/FPP) or [4-^{14}C]IPP was varied, while its counter-substrate of [4-^{14}C]IPP or DMAPP/GPP/FPP was kept at saturating concentration. Kinetic parameters and their standard errors were estimated by non-linear regression analysis using Enzyme Kinetics software version 1.5 (Trinity Software).

To analyze the products, the extracted prenyl diphosphates were hydrolyzed to the corresponding alcohols with potato acid phosphatase according to the method reported previously [20]. The alcohols were extracted with *n*-pentane and analyzed by TLC on a reverse-phase RP-18 plate with a solvent system of acetone-H_2O (9:1). The positions of authentic standards were visualized with iodine vapor, and the absolute radioactivities of the spots were detected with a Bio-image analyzer BAS1500 (Fuji). The product distributions were determined on the basis of the molar ratios of the products that were obtained by division of the absolute radioactivity of each spot by the number of IPPs incorporated into the corresponding prenyl alcohol.

Construction and analysis of mutated enzyme. Site-directed mutagenesis was carried out by the PCR method. In order to amplify the 5′-partial mutated *Tk-ids*A gene, in which tyrosine at the position 81 was replaced with serine and alanine, PCR was carried out with the *T. kodakaraensis* chromosomal DNA as the template, the forward primer of Tk-idsA-*Nco*I-F, and the reverse primer of Tk-idsA(Y81S)-*Sac*I-R (5′-CCT GAG CTCGTC CAT GTC CAT TAT ATC GTC GTG AAC GAG GGA G**CT** GTT GTG GAT GAA CTC-3′) and Tk-idsA(Y81A)-*Sac*I-R (5′-CCT GAG CTCGTC CAT GTC CAT TAT ATC GTC GTG AAC GAG GGA G**GC** GTT GTG GAT GAA CTC-3′). A mismatch is indicated by a bold letter, and a *Sac*I site is underlined. The PCR product was digested with *Nco*I and *Sac*I, and then ligated into the pET8-Tk-idsA vector obtained from the digestion of *Nco*I and *Sac*I to construct the plasmids designated pET8-Tk-idsA(Y81S) and pET8-Tk-idsA(Y81A) for expression of the single-mutated enzyme, *Tk*-IdsA(Y81S) and *Tk*-IdsA(Y81A), respectively. Plasmid construction was performed using *E. coli* JM109 as a host. The mutagenesis was confirmed by DNA sequencing.

E. coli BL21(DE3)pLysS was transformed with pET8-Tk-idsA(Y81S), and then *Tk*-IdsA(Y81S) was prepared from the transformant according to the same procedure as the wild-type enzyme. Measurement of the enzymatic activity and the product analysis of the mutated enzyme were also conducted in the same way as for the wild-type enzyme. Plasmid pET8-Tk-idsA(Y81A) could not be introduced into *E. coli* BL21(DE3)pLysS cells probably due to its product toxicity. *Tk*-IdsA(Y81A) was hence expressed by cell-free protein production system, Rapid Translation System (Roche), and prepared by the recommended procedure. The solution was heated at 90 °C for 10 min and then centrifuged at 22,000*g* for 30 min to remove *E. coli* derived protein contaminants. The supernatant was obtained and used as an enzyme sample.

Sequence accession number. The nucleotide sequence and deduced amino acid sequence of *Tk-ids*A are available under Accession No. AB109219.

Results and discussion

Isolation of Tk-idsA

We searched the sequence database of the *T. kodakaraensis* genome for genes encoding *trans*-prenyl diphosphate synthases using the *S. acidocaldarius* GGPP synthase amino acid sequence as a query [10]. The search resulted in the identification of one orthologous

gene. The deduced amino acid sequence encoded by the gene consists of 343 amino acids and contains several conserved regions through *trans*-prenyl diphosphate synthases, including the FARM and the SARM, which are regarded as the substrate-binding domains [7]. Moreover, we found that the residue at the fifth position before the FARM is tyrosine (Tyr-81) as shown in Fig. 2. It is characteristic of short-chain *trans*-prenyl diphosphate synthase, such as GGPP synthase and FPP synthase, to have a bulky amino acid at the fifth position before the FARM [21–23]. Thus, we presumed that the candidate gene encodes a short-chain *trans*-prenyl diphosphate synthase and named the gene *Tk-idsA* (*T. kodakaraensis* short-chain isoprenyl diphosphate synthase). The *Tk-ids*A protein (*Tk*-IdsA) shows similarity with other archaeal *trans*-prenyl diphosphate synthases so far published: 45% identity with FPP/GGPP synthase from *Methanothermobacter marburgensis* str. Marburg (formerly *Methanobacterium thermoautotrophicum* str. Marburg) (Accession No. Q53479) [8], 46% identity with GGPP synthase from *Archaeoglobus fulgidus* (Accession No. F69535) [12], 39% identity with GGPP synthase from *S. acidocaldarius* (Accession No. BAA43200) [10], 36% identity with farnesylgeranyl diphosphate synthase from *Aeropyrum pernix* (Accession No. BAA88983) [11], and 24% identity with hexaprenyl diphosphate synthase from *Sulfolobus solfataricus* (Accession No. AAK42496) [9].

Purification of Tk-IdsA

For functional analysis, *Tk*-IdsA was heterologously expressed in *E. coli* cells and purified to homogeneity by a combination of two rounds of heat treatment and three chromatographic steps (Fig. 3). The major active peak was observed corresponding to the molecular mass of 85 kDa by gel filtration chromatography on a Superdex 200 HR column. Considering that the calculated molecular mass of *Tk*-IdsA was 38,326 Da and that short-chain prenyl diphosphate synthases generally dimerize as an active form [7], we judged that the native *Tk*-IdsA is a dimer. We performed further investigations using the purified protein.

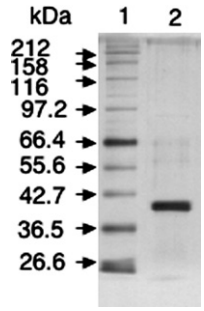

Fig. 3. SDS–PAGE with silver staining of purified recombinant protein. Lane 1, molecular mass marker (myosin, 212,000; maltose-binding protein fused with β-galactosidase, 158,194; β-galactosidase, 116,351; phosphorylase b, 97184; serum albumin, 66,409; glutamic dehydrogenase, 55,561; maltose-binding protein, 42,710; lactate dehydrogenase, 36,487; and triosephosphate isomerase, 26,625); lane 2, *Tk*-IdsA.

Thermostability of Tk-IdsA

The thermostability of *Tk*-IdsA was examined by CD analysis. Far-UV CD spectra (206–260 nm) of *Tk*-IdsA at the temperature ranging from 20 to 90 °C are shown in Fig. 4A. The spectrum at 20 °C showed minima at 208

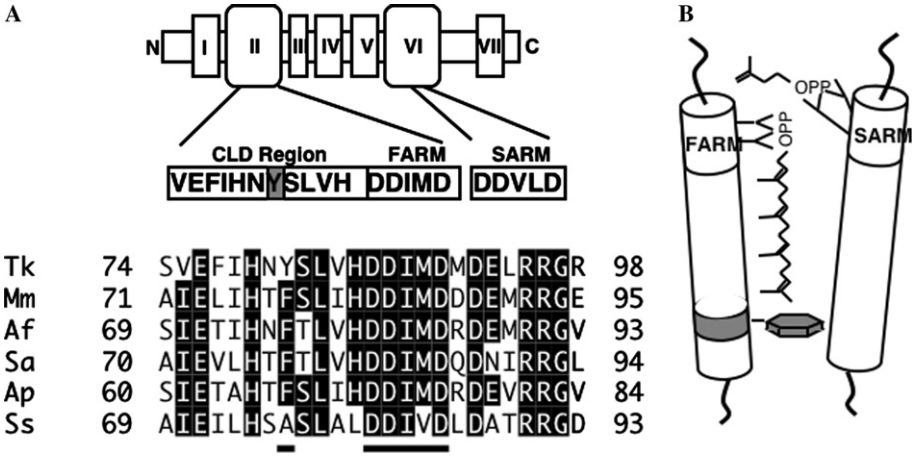

Fig. 2. Alignment of amino acid sequence around the FARM of *Tk*-IdsA with those of other archaeal *trans*-prenyl diphosphate synthases. (A) Structural feature and sequence alignment around the FARM. The chain-length determination (CLD) regions of the enzymes are shown, and the residue essential for product specificity and the FARM are underlined in the lower panel. Conserved residues are shown within filled boxes. Tk, *Tk*-IdsA; Mm, *M. marburgensis* str. Marburg FPP/GGPP synthase (Accession No. Q53479); Af, *A. fulgidus* GGPP synthase (Accession No. F69535); Sa, *S. acidocaldarius* GGPP synthase (Accession No. BAA43200); Ap, *A. pernix* farnesylgeranyl diphosphate synthase (Accession No. BAA88983); and Ss, *S. solfataricus* hexaprenyl diphosphate synthase (Accession No. AAK42496). (B) Structural model for chain-length determination. Tyr-81 is highlighted by gray color.

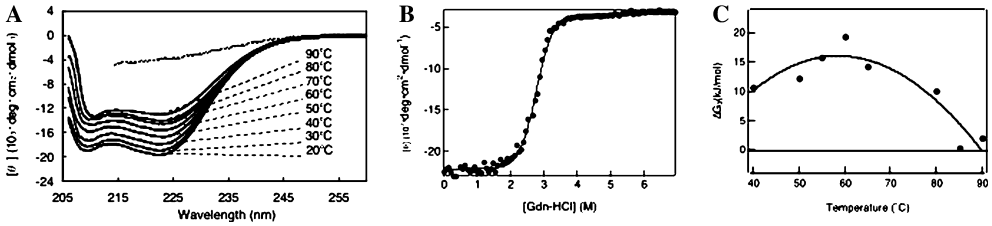

Fig. 4. CD spectra analysis of *Tk*-IdsA. (A) Far-UV CD spectra collected at various temperatures in 50 mM sodium phosphate (pH 6.0). Dot line indicates the spectra of fully unfolded form of *Tk*-IdsA at 80 °C. Unfolding was performed by incubation in 6 M Gdn-HCl at 80 °C for three days. Spectra data of short wavelength (<215 nm) were removed due to undesirable noise. (B) Gdn-HCl titration curve of *Tk*-IdsA with in 50 mM sodium phosphate (pH 6.0) at 65 °C. Line indicates the two-state theoretical curve of *Tk*-IdsA. (C) Dependence of the ΔG_0 on temperature. The ΔG_0 values were determined by the two-state folding model, to that the data of the Gdn-HCl titration at various temperatures were applied as described in Materials and methods. A fitting quadratic curve of ΔG_0 values against temperatures indicated that the $\Delta G_{0\,\max}$ value was 16 kJ mol^{-1} at 60 °C at the vertex and the T_m value was 91.0 °C at the horizontal intersection.

and 222 nm, a typical pattern of an α-helical protein. Based on the intensity of ellipticity ([θ]) at 208 nm, the α-helical content of *Tk*-IdsA was estimated at 47%. As the temperature increased, the decline of the two unique peaks was observed, indicating that the conformation of the protein was changed with the temperature. The thermal denaturation curve was obtained by plotting the change in the [θ] value at 222 nm. The [θ] value at 222 nm increased linearly with the temperature but did not reach a constant value even at 90 °C. *Tk*-IdsA was still not fully unfolded even at 90 °C. Because the thermal unfolding of the protein was not fully reversible at this condition, we employed the chemical denaturation method for obtaining the thermodynamic parameters that characterize the thermal unfolding. Gdn-HCl was used as a chemical denaturant. Denaturation of *Tk*-IdsA was performed by incubating in 6 M Gdn-HCl at 40–80 °C. At these conditions, the far-UV CD intensity decreased and depicted a typical unfolded spectrum in three days. Thus, we incubated *Tk*-IdsA for three days in 0–7 M Gdn-HCl at temperatures ranging from 40 to 90 °C for thermodynamic analysis. Spectra at wavelengths shorter than 215 nm were not clearly monitored due to undesirable noises. Thus, we used the [θ] value at 222 nm for the detection of the unfolding profiles of *Tk*-IdsA. The denaturation profiles of *Tk*-IdsA at various temperatures were in good agreement with the two-state folding model. Typical denaturation curve at 65 °C is shown in Fig. 4B. The ΔG_0 values, which represent the conformational free-energy change in the denaturant-free state, were then determined at various temperatures and are plotted as shown in Fig. 4C. A plot of these ΔG_0 values against temperatures was fitted to a quadratic curve, and the maximum free-energy change ($\Delta G_{0\max}$) of *Tk*-IdsA was calculated to be 16 kJ mol^{-1} at 60 °C. This fitting curve also gave the thermodynamic parameters. The melting temperature (T_m) of *Tk*-IdsA was 91.0 °C at the horizontal intersection. The change of enthalpy (ΔH_m) was 307.6 kJ mol^{-1}, and the heat capacity change (ΔC_p) was 8.5 kJ mol^{-1} K^{-1}. The T_m value indicates that *Tk*-IdsA is extremely thermostable, which is quite reasonable in view of an optimal growth temperature (90–95 °C) of *T. kodakaraensis*. It is noteworthy that *Tk*-IdsA showed the highest thermostability among the prenyl diphosphate synthases so far reported. How can *Tk*-IdsA maintain its native structure at high temperature? Equilibrium thermodynamics for protein folding requires the measurement of ΔG_0 between a native state and an unfolded state as a function of temperature. Based on theoretical thermodynamics, three different models, i.e., high stability, flattened, and shifted models, have been proposed to explain the strategy for thermostabilization [19,24]. In the first case, the high stability model is due to the higher conformational stability ($\Delta G_{0\,\max}$). This directly relates to the increase in both $\Delta G_{0\,\max}$ and T_m values. In this case, which mainly contributes to an increase in enthalpy (ΔH_m), a large hydrophobic core and favorable interactions between side-chains are the main contributors to the increase in thermostability. Although thermostable proteins may be thought to commonly possess high thermodynamic stability ($\Delta G_{0\,\max}$), in some cases, a protein shows higher T_m even though $\Delta G_{0\,\max}$ decreases [25]. Consequently, the curve profile shows a flattened version. This is the second case of thermostabilization that is highly related to a decrease in ΔC_p. The third case is a shifted model that is a simple curve shift to higher temperature. The thermostability of *Tk*-IdsA is explained by combination of the second (flattened) and the third (shifted) cases. The values of ΔC_p and $\Delta G_{0\,\max}$ of *Tk*-IdsA were relatively low and temperature for $\Delta G_{0\,\max}$ was shifted to higher temperature in comparison with other studied thermostable proteins. In general, the thermostable protein possesses a high content of secondary structural elements and low content of hydrophobic protein surface [19,26]. *Tk*-IdsA also exhibited these features. Based on the CD analysis, its α-helical content was estimated to be 47%. The structural and thermodynamic analyses suggested that the low content of expanded loops and/or surface hydrophobic residues of *Tk*-IdsA resulted in its high thermodynamic stability.

Enzymatic activity of Tk-IdsA

Tk-IdsA was incubated with 50 μM DMAPP and 50 μM [4-^{14}C]IPP, and condensation activity was monitored. When the incubation was carried out at 70 °C for 10 min, maximum activity was achieved under the condition of pH 8.0 (100 mM Mops buffer) and 5 mM $MgCl_2$. Triton X-100 did not affect the enzymatic activity, unlike the case of *S. acidocaldarius* GGPP synthase [27]. *Tk*-IdsA showed maximum activity at 90 °C. Even after being heated at 90 °C for 1 h, *Tk*-IdsA still retained 82% of the activity. In addition to its extreme thermostability, *Tk*-IdsA also exhibited a significantly high activity, and which was over 50-fold higher than that of FPP/GGPP synthase from *M. marburgensis* str. Marburg [28].

Four kinds of allylic diphosphates at 50 μM were used as a primer substrate with 50 μM [4-^{14}C]IPP under the optimum condition at 70 or 90 °C. As a result, *Tk*-IdsA utilized DMAPP, GPP, and FPP as a primer substrate but not GGPP (Table 1). Although the activities for DMAPP and GPP were comparable, the activity for FPP was lower than those for DMAPP and GPP. The reaction products were dephosphorylated and then analyzed by reverse-phase TLC (Fig. 5A). When DMAPP or GPP was used as the primer substrate, farnesol and geranylgeraniol were mainly detected by TLC analysis, indicating that *Tk*-IdsA gave FPP and GGPP as the main products. On the other hand, when the reaction was carried out with FPP as the primer substrate, only GGPP was formed. Hence, we concluded that *Tk*-IdsA is FPP/GGPP synthase, a bifunctional enzyme, which has the functions of both FPP synthase (EC 2.5.1.10) and GGPP synthase (EC 2.5.1.29). Interestingly, it seems that the product ratio of FPP to GGPP increased as the reaction temperature increased (Fig. 5A and Table 2). In the reaction with DMAPP as the primer substrate, the FPP/GGPP product ratio obtained at 70 °C was 0.98, whereas that obtained at 90 °C was 1.6. A similar tendency was observed when GPP was used as the primer substrate.

It has been reported that *M. marburgensis* str. Marburg carries the bifunctional enzyme similar to *Tk*-IdsA [8,28]. *T. kodakaraensis* and *M. marburgensis* str. Marburg are classified into the same phylum of archaea, *Euryarchaeota* [29]. It is also reported that, in *M.*

Table 1
Allylic substrate specificity of *Tk*-IdsA

Temperature (°C)[a]	Specific activity (nmol min^{-1} μg^{-1})[b]	Relative activity (%)[c]			
		DMAPP[a]	GPP	FPP	GGPP
70	34 ± 3	100 ± 8	121 ± 3	5.8 ± 3.3	<0.5
90	47 ± 5	100 ± 10	142 ± 12	4.7 ± 0.7	<0.5

[a] Enzymatic reactions were carried out with 50 μM [4-^{14}C]IPP and 50 μM of the indicated allylic substrate at the indicated temperature.
[b] Specific activities obtained from the reaction with DMAPP were shown. The unit was defined as the nmol amount of IPP converted into products per min by 1 μg of the enzyme.
[c] Relative activities were determined based on the specific activity with DMAPP at the each temperature. Each of the activities is represented as mean value ± SD ($n = 3$).

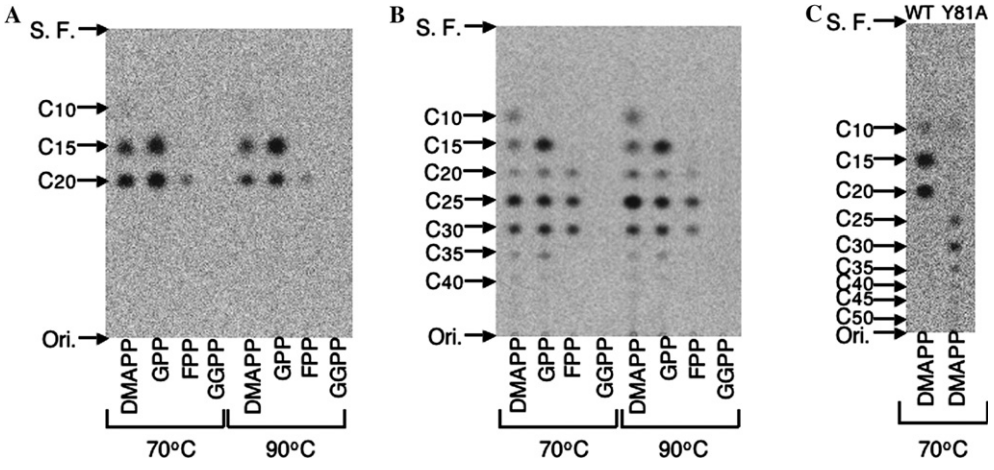

Fig. 5. TLC autoradiochromatograms of prenyl alcohols obtained by enzymatic hydrolysis. (A) Product distribution by *Tk*-IdsA. (B) Product distribution by *Tk*-IdsA(Y81S). The incubation of [4-^{14}C]IPP plus DMAPP, GPP, FPP, or GGPP was performed at the indicated temperatures. (C) Product distribution by in vitro synthesized *Tk*-IdsA and *Tk*-IdsA(Y81A). The incubation of [4-^{14}C]IPP plus DMAPP was performed at 70 °C. The resultant products were analyzed by reverse-phase RP-18 TLC, as described in the Materials and methods. Ori., origin; S. F., solvent front.

Table 2
Product distribution of *Tk*-IdsA

Temperature (°C)[a]	Allylic substrate[a]	Product distribution (%)[b]			C_{15}/C_{20} ratio
		C_{10}	C_{15}	C_{20}	
70	DMAPP	15	42	43	0.98
	GPP	—	62	38	1.7
	FPP	—	—	100	—
90	DMAPP	20	49	31	1.6
	GPP	—	82	18	4.5
	FPP	—	—	100	—

[a] Enzymatic reactions were carried out with 50 μM [4-^{14}C]IPP and 50 μM of the indicated allylic substrate at the indicated temperature.
[b] The products were hydrolyzed and the resultant alcohols were separated by reverse-phase TLC, as described in Materials and methods. The amount of each prenyl alcohol was determined by dividing the absolute radioactivity by the number of IPPs incorporated into the product. Each assay was repeated three times and mean was represented.

Table 3
Kinetic parameters of *Tk*-IdsA

Temperature (°C)[a]	Substrate	Counter-substrate[b]	K_m (μM)	k_0 (s^{-1})[c]
70	DMAPP	100 μM IPP	9.5 ± 1.5	57 ± 3
	GPP	100 μM IPP	2.2 ± 0.2	104 ± 2
	FPP	100 μM IPP	1.7 ± 0.5	59 ± 7
	IPP	50 μM DMAPP	23 ± 2	75 ± 3
	IPP	25 μM GPP	22 ± 2	120 ± 3
	IPP	3 μM FPP	9.4 ± 1.0	49 ± 2
90	DMAPP	150 μM IPP	13 ± 3	75 ± 6
	GPP	100 μM IPP	3.0 ± 0.3	231 ± 8
	FPP	100 μM IPP	0.81 ± 0.2	73 ± 7
	IPP	50 μM DMAPP	79 ± 11	110 ± 8
	IPP	25 μM GPP	31 ± 3	279 ± 9
	IPP	3 μM FPP	16 ± 2	77 ± 3

[a] The kinetic parameters of *Tk*-IdsA were determined at the indicated temperature, and the parameters and their SE were calculated with non-linear regression method as described in Materials and methods.
[b] The concentrations of the counter-substrates were set at saturating levels, as indicated.
[c] The k_0 value was defined by the unit of nmol of IPP converted into products per s by 1 nmol of the dimer enzyme.

marburgensis str. Marburg, the neutral lipids are mostly a mixture of squalene and its derivatives synthesized from FPP [5], whereas the core membrane lipids consist of C_{20} (phytanyl) and C_{40} (biphytanyl) isopranoid chains derived from GGPP [2]. Thus, based on an analogy between *T. kodakaraensis* and *M. marburgensis* str. Marburg, we assumed that FPP and GGPP produced by *Tk*-IdsA would be utilized for the biosynthesis of these membrane components in *T. kodakaraensis*. Our results shown here suggest that, as the growth temperature rises, the production ratio of FPP to GGPP in the cell increases, leading to the enhanced production of squalene (derivatives) and the reduced production of the core membrane lipids. Because of their relatively rigid structure, the introduction of squalene (derivatives) into the cell membrane would stabilize the membrane architecture under a high-temperature condition. *T. kodakaraensis* might adapt to the extreme environment by such a modulation of the membrane components. In order to obtain further information, kinetic analyses were performed.

Kinetic analysis of Tk-IdsA

The kinetic parameters of *Tk*-IdsA were determined with [4-^{14}C]IPP and DMAPP, GPP, or FPP as substrates at the reaction temperature of 70 or 90 °C. The results are summarized in Table 3. When the reaction was carried out at 70 °C, the K_m values for the allylic substrates decreased with the extension of the chain-length of the allylic substrate. A similar decrease in the K_m values for the allylic substrates was observed at 90 °C. The elevation of the reaction temperature raised all of the k_0 values. In particular, the rise of the k_0 value for GPP was prominent. When the reaction was conducted at 70 °C, *Tk*-IdsA showed a 1.8-fold higher k_0 value for GPP than those for DMAPP and FPP. In the reaction at 90 °C, the k_0 value for GPP was 3.1- and 3.2-fold higher than those for DMAPP and FPP. These data indicate that the relative activity of *Tk*-IdsA for GPP became more remarkable as the reaction temperature rose. The comparison of the data obtained at 70 °C and those at 90 °C also revealed that the K_m values for the C_5 substrate, such as DMAPP and IPP, increased with the rise of the reaction temperature, whereas the K_m values for the longer substrate, GPP and FPP, did not vary. This result suggests that the hydrophobic interaction between the enzyme and the hydrocarbon of the longer substrate, which contributes to substrate binding, was not affected by temperature. In the present study, we used the same batch of purified enzymes throughout all of the kinetic analysis and confirmed that the activity did not change during repeated kinetic analyses. Thus, we considered that the differences between the k_0 values were significant.

The analysis of the products given at 70 and 90 °C revealed that the FPP/GGPP ratio increases with a rise in the reaction temperature (Fig. 5A and Table 2). In addition, the kinetic study performed at 70 and 90 °C showed that the k_0 value for GPP is higher than those for the other allylic substrates and that the degree of the prominence of the k_0 value for GPP becomes more remarkable as the reaction temperature increases (Table 3). On the basis of these data, we hypothesized that FPP is accumulated as the intermediate product because of the faster turnover from GPP to FPP than that from DMAPP to GPP and that from FPP to GGPP.

Table 4
Product distribution of *Tk*-IdsA(Y81S)

Temperature (°C)[a]	Allylic substrate[a]	Product distribution (%)[b]							C_{15}/C_{20} ratio	C_{25}/C_{30} ratio
		C_{10}	C_{15}	C_{20}	C_{25}	C_{30}	C_{35}	C_{40}		
70	DMAPP	30	23	7.8	24	12	2.4	3	2.9	2.0
	GPP	—	66	8.7	15	8.4	1.7	6	7.5	1.8
	FPP	—	—	39	40	21	ND[c]	ND	—	1.9
90	DMAPP	24	18	8.7	40	7.2	1.3	0.8	2.0	5.6
	GPP	—	70	8.1	14	6.2	1.0	0.5	8.3	2.3
	FPP	—	—	40	45	15	ND	ND	—	3.0

[a] Enzymatic reactions were carried out with 50 μM [4-^{14}C]IPP and 50 μM of the indicated allylic substrate at the indicated temperature.
[b] The products were hydrolyzed and the resultant alcohols were separated by reverse-phase TLC, as described in Materials and methods. The amount of each prenyl alcohol was determined by dividing the absolute radioactivity by the number of IPPs incorporated into the product. Each assay was repeated three times and mean was represented.
[c] ND, not detected.

According to this hypothesis, the FPP/GGPP ratio should be regulated by the modulation of the relative activity for GPP dependent on the temperature. These data suggest that *Tk*-IdsA contributes to adjust the membrane composition to the cell growth temperature by modulating its substrate and product specificities. As mentioned above, the T_m value of *Tk*-IdsA was 91.0 °C, therefore, when *Tk*-IdsA was incubated at 90 °C, the temperature near the T_m value, it was assumed that the structure of the enzyme might fluctuate vigorously enough to cause changes in the allylic substrate specificity and the product specificity. The analyses on membrane composition from *T. kodakaraensis* cells cultivated at 70 and 90 °C are in progress.

Essential amino acid for chain-length determination

Previous studies on the mechanism that determines the chain length of the final product have revealed that, in archaeal GGPP synthase, the aromatic residue at the fifth position before the FARM acts as a steric hindrance to terminate the chain elongation and defines the final product length as C_{20} [7,21,23]. By analogy with this, we presumed that the Tyr-81 at the fifth position before the FARM of *Tk*-IdsA determines the final product length of C_{20}. We constructed a single-mutated enzyme, *Tk*-IdsA(Y81S) and (Y81A), in which Tyr-81 is replaced by serine and alanine, respectively. Mutant *Tk*-IdsA(Y81S) was obtained as recombinant form from *E. coli* BL21(DE3)pLysS cells harboring pET8-Tk-idsA(Y81S). However, plasmid pET8-Tk-idsA(Y81A) could not be introduced into *E. coli* BL21(DE3)pLysS cells probably due to its toxicity. Serine contains aliphatic hydroxyl group and it can be thought of as a hydroxylated version of alanine. The hydroxyl group on serine makes it more hydrophilic and reactive than Alanine. An elimination of the bulky side-chain and elevating hydrophilicity at the position could allow the enzyme to produce longer chain product ($>C_{20}$). Such long chain hydrophobic product would have toxic interaction with cytoplasmic or membrane components in *E. coli* cells. In the present study, we could obtain *Tk*-IdsA(Y81A) mutant by using in vitro expression system. Further study on *Tk*-IdsA(Y81A) was conducted with the enzyme extract obtained by the in vitro system.

The reaction products of *Tk*-IdsA(Y81S) and (Y81A) were analyzed according to the method applied to the products of the wild-type enzyme (Figs. 5B and C and Table 4). When the enzymatic reaction of *Tk*-IdsA(Y81S) was carried out at 70 °C with DMAPP, GPP, or FPP as the primer substrate, the mutated enzyme gave the C_{25} product as the main product in the long-chain region ($>C_{20}$). Small amounts of the C_{35} and C_{40} products were also detected when DMAPP or GPP was used as the primer substrate. While, the C_{30} product was the main product of *Tk*-IdsA(Y81A), and more long-chain products (C_{35}, C_{40}) were detected. This result clearly indicates that Tyr-81 is essential for determining the chain length of a final product in the sense that it blocks the chain elongation and that elimination of the bulky side-chain at the position allows the mutated enzyme to catalyze the condensation of IPP beyond the C_{20}. The main product in the long-chain region was still C_{25} when the enzymatic reaction was conducted at 90 °C with *Tk*-IdsA(Y81S). However, *Tk*-IdsA(Y81S) increased the C_{25}/C_{30} product ratio as the reaction temperature increased (Fig. 5B and Table 4). In the reaction with DMAPP as the primer substrate, the C_{25}/C_{30} product ratio obtained at 70 °C was 2.0, whereas that obtained at 90 °C was 5.6. A similar tendency was observed when GPP or FPP was used as the primer substrate. This property seems analogous to that of the wild-type enzyme, i.e., to increase the C_{15}/C_{20} product ratio with a rise in the reaction temperature (Fig. 5A and Table 2). These results suggest that *Tk*-IdsA(Y81S) also retains the function of temperature-dependent modulation of the product specificity and that the mutation at the position 81 is not crucial for this function.

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