

Type 2 isopentenyl diphosphate isomerase from a thermoacidophilic archaeon *Sulfolobus shibatae*

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Although isopentenyl diphosphate–dimethylallyl diphosphate isomerase is thought to be essential for archaea because they use the mevalonate pathway, its corresponding activity has not been detected in any archaea. A novel type of the enzyme, which has no sequence similarity to the known, well-studied type of enzymes, was recently reported in some bacterial strains. In this study, we describe the cloning of a gene of a homologue of the novel bacterial isomerase from a thermoacidophilic archaeon *Sulfolobus shibatae*. The gene was heterologously expressed in *Escherichia coli*, and the recombinant enzyme was purified and characterized. The thermostable archaeal enzyme is tetrameric, and requires

NAD(P)H and Mg^{2+} for activity, similar to its bacterial homologues. Using its apoenzyme, we were able to confirm that the archaeal enzyme is strictly dependent on FMN. Moreover, we provide evidence to show that the enzyme also has NADH dehydrogenase activity although it catalyzes the isomerase reaction without consuming any detectable amount of NADH.

Keywords: isopentenyl diphosphate–dimethylallyl diphosphate isomerase; isoprenoid; archaea; flavoprotein; NADH dehydrogenase.

Isoprenoid compounds are the most diverse family of metabolites found in nature. They are necessary for all living organisms because they are functional parts of important compounds, including vitamins, hormones, respiratory quinones, and archaeal membranes [1]. The majority of isoprenoid compounds are synthesized from linear prenyl diphosphates, which are formed via the consecutive condensation of isopentenyl diphosphate (IPP), the active isoprene C₅-unit, to its highly electrophilic isomer dimethylallyl diphosphate (DMAPP).

Isopentenyl diphosphate–dimethylallyl diphosphate isomerase (IPP isomerase; EC 5.3.3.2) catalyzes the interconversion of IPP and DMAPP and is a key enzyme in the biosynthesis of isoprenoids [2]. Based on studies using eukaryotes, IPP has been shown to be synthesized from acetyl-CoA via the well-known mevalonate pathway and is further converted to DMAPP by IPP isomerase [3]. On the other hand, many bacteria, green algae, and chloroplasts of higher plants have recently been shown to use a different isoprenoid biosynthetic pathway, which is referred to as the

nonmevalonate pathway [4,5]. It has been reported that IPP and DMAPP are synthesized separately in *Escherichia coli* and that the IPP isomerase gene was not essential for this organism [6]. *Synechocystis* sp. strain PCC6803, which also utilizes the nonmevalonate pathway for the biosynthesis of isoprenoids, was shown to be deficient in IPP isomerase activity [7]. In short, IPP isomerase is necessary for the biosynthesis of isoprenoid compounds via the mevalonate pathway, and unnecessary for that of the nonmevalonate pathway. However, despite the sole utilization of the mevalonate pathway for isoprenoid biosynthesis, many archaea and some bacteria lack homologues of IPP isomerase genes in their genome sequences [8].

Kaneda *et al.* recently cloned the gene *fni* from *Streptomyces* sp. strain CL190, which possesses genes of the mevalonate pathway as a cluster in addition to those of the nonmevalonate pathway [9]. The *fni* gene was located in the cluster of mevalonate pathway genes [10]. They demonstrated that *fni* encodes a totally new type of IPP isomerase designated as type 2 IPP isomerase. This new enzyme characteristically requires redox coenzymes, i.e. both FMN and NAD(P)H, for activity while the known IPP isomerase (type 1 IPP isomerase) has no cofactor requirement except for divalent metal ions. As the result of a homology search, it was found that *fni* homologues are present in the whole-genome sequences of many organisms, including archaea and some bacteria.

Although IPP isomerase is thought to be essential for archaea because they use the mevalonate pathway, the activity of IPP isomerase has not been detected in any archaea to date. The existence of homologues of *fni* in their genomes strongly suggests that archaea possess type 2 IPP isomerases. To investigate this issue further, we cloned a gene of the homologue of *fni* from the thermoacidophilic archaeon *Sulfolobus shibatae*. The gene was expressed in *E. coli*, and the recombinant enzyme was purified and

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Abbreviations: DMAPP, dimethylallyl diphosphate; GGPP, geranylgeranyl diphosphate; IPP, isopentenyl diphosphate.

Database: The nucleotide sequences reported in this paper are available from the DDBJ/GenBank™/EMBL Data Bank under the accession numbers AB118244 and AB118245.

Enzyme: isopentenyl diphosphate–dimethylallyl diphosphate isomerase (IPP) isomerase (EC 5.3.3.2).

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confirmed to have IPP isomerase activity. Moreover, the enzyme was used in a detailed study of the unique properties of type 2 IPP isomerase, especially its requirement for the redox coenzymes.

Materials and methods

Materials

[1-¹⁴C]IPP was purchased from Amersham Biosciences. Dimethylallyl diphosphate was donated by Drs K. Ogura and T. Koyama, Tohoku University. All other chemicals were of analytical grade.

General procedures

Restriction enzyme digestion, transformation, hybridization, and other standard molecular biology techniques were carried out as described by Sambrook and Russell [11].

Cloning of the gene encoding archaeal IPP isomerase

We previously cloned the plasmid pGGPS1 containing ORF3 [12], which is homologous with *fni*, from a *Sulfolobus acidocaldarius* genomic library. On the basis of the nucleotide sequence of pGGPS1, the ORF3 was amplified using the PCR primers 5'-TAAATCATGATAACGGGCATGACTGG-3' and 5'-TTAAGGGATCCATATTCTTCTTTTCTAAC-3'. The genome of *S. acidocaldarius*, as the template, and KOD DNA polymerase (TOYOBO) were used for the reaction. The amplified fragment was subcloned into pUC119 to yield the plasmid ORF3-pUC119. We used a 952 bp *SacI/XbaI* fragment containing nearly full-length ORF3, which was derived from ORF3-pUC119, as a probe for colony hybridization and subsequently isolated eight positive clones from $\approx 32\,000$ colonies of the *S. shibatae* genomic library. The plasmid g43-2, from one of the positive clones, was sequenced and found to contain an open reading frame of 1107 bp (termed *idi* herein). The *idi* gene was amplified using the PCR primers 5'-TAAGAGGTAGGCCATATGCC-3' and 5'-CTTAATTCGTCAGGATCCTTATTCTCTC-3', which include newly introduced *NdeI* and *BamHI* restriction sites (underlined), respectively. The plasmid g43-2, as the template, and KOD DNA polymerase were used for the reaction. The amplified fragment was cleaved with *NdeI* and *BamHI* and then ligated into the *NdeI-BamHI* sites of the pET-15b vector (Novagen). The resulting plasmid was designated as pET-*idi*.

Expression and purification of recombinant enzyme

E. coli BL21(DE3) transformed with pET-*idi* was cultivated in 3 L of M9YG broth supplemented with ampicillin (50 mg·L⁻¹). When the D_{600} of the culture reached 0.5, 0.1 mM (final concentration) isopropyl thio- β -D-galactoside was added to the medium. After an additional overnight cultivation, the cells were harvested and disrupted by sonication in Start buffer composed of 20 mM phosphate buffer (pH 7.4), 0.5 M NaCl, and 10 mM imidazole. The homogenate was centrifuged at 20 000 *g* for 20 min, and the supernatant was recovered as a crude extract. The crude

extract was heated at 55 °C for 60 min, and the denatured proteins were removed by centrifugation at 20 000 *g* for 20 min. The supernatant was applied to a HisTrap column (Amersham Biosciences) previously equilibrated with the Start buffer. The resin was washed with the Start buffer, and the protein bound to the resin was then eluted with 20 mM phosphate buffer (pH 7.4), containing 0.5 M NaCl and 0.5 M imidazole. Active fractions were dialyzed overnight at 4 °C against buffer A composed of 10 mM Tris/Cl buffer (pH 7.7), 1 mM EDTA, and 10 mM 2-mercaptoethanol, and then used for characterization. The level of purification was determined by SDS/PAGE (15% polyacrylamide). To determine the subunit structure of *S. shibatae* IPP isomerase, the purified enzyme was loaded onto a Superdex 200 HR 10/30 gel-filtration column (Amersham Biosciences) and eluted with buffer A. The molecular mass of the enzyme was calculated based on a correlation curve made by means of a Gel Filtration HMW Calibration Kit (Amersham Biosciences).

Preparation of apoenzyme

The purified *idi* product was dialyzed against 10 mM acetate buffer (pH 4.5) containing 1 mM EDTA, 10 mM 2-mercaptoethanol, 2 M KBr for 96 h at 4 °C. During the dialysis, activated charcoal (4 g·L⁻¹) was added to absorb the released FMN. After the complete loss of FMN absorbance, the dialysis buffer was changed to buffer A, and the dialysis continued overnight. The resulting apoenzyme was used in a reconstitution experiment. Absorption spectra of the purified *idi* product and the apoenzyme were recorded on a visible spectrophotometer (SpectraMax 340PC³⁸⁴; Molecular Devices).

Assay for IPP isomerase

The assay system is based on the acid-lability of DMAPP when exposed to acid [13]. The standard assay mixture contained, in a final volume of 50 μ L, 5 nmol of [1-¹⁴C]IPP (0.19 GBq·mmol⁻¹), 0.25 μ mol of MgCl₂, 2 μ mol of malate/NaOH buffer (pH 6.0), 25 nmol of NADH, 0.5 nmol of FMN, and a suitable amount of enzyme. This mixture was incubated at 60 °C for 10 min, and the reaction was terminated by adding 0.2 mL of 25% concentrated HCl in MeOH and 0.5 mL of H₂O saturated with NaCl, followed by incubation at 37 °C for 10 min. This mixture was then extracted twice with 0.5 mL pentane. The pentane extracts were added, and the radioactivity was measured. To determine the dissociation constant for FMN, various concentrations of FMN were previously added to the assay mixture using the apoenzyme and lacking NADH, and the mixtures were placed on ice for 30 min and then used for enzyme assay by adding NADH. All kinetic parameters were calculated using the ENZYMEKINETICS software program (Trinity Software) using the nonlinear-regression method.

Detection of DMAPP production

The conversion of IPP to DMAPP catalyzed by IDI was detected using the same reaction mixture as that used in the IPP isomerase assay, except for the addition of a suitable

amount of purified *Sulfolobus acidocaldarius* geranylgeranyl diphosphate (GGPP) synthase into the mixture [12]. This mixture was incubated at 60 °C for 10 min, and the reaction was stopped by chilling the mixture in an ice bath. The mixture was shaken with 600 μ L of 1-butanol saturated with H₂O. The butanol layer, extracting GGPP, was washed with water saturated with NaCl, and the radioactivity in 100 μ L of the butanol layer was measured.

NADH dehydrogenase assay

The standard assay mixture for the NADH dehydrogenase activity of *S. shibatae* IDI contained, in a final volume of 100 μ L, 10 nmol of IPP, 0.5 μ mol of $MgCl_2$, 10 nmol of NADH, 5 μ mol of a malate/NaOH buffer (pH 6.0), and 14.4 pmol (as monomer) of the purified enzyme. To minimize the interference by the absorption of FMN, FMN was not added to the mixture, except for that bound to IDI. This mixture was incubated at 60 $^{\circ}C$ for 20 min, and the reaction was stopped by adding 100 μ L of water saturated with NaCl. The absorbance of NADH at 340 nm was measured with a visible spectrophotometer.

Measurement of the oxygen peroxide production

The assay mixture contained, in a final volume of 100 μL , a variable concentration of NADH, 5 μmol of a succinate/NaOH buffer (pH 6.0), and 28.8 pmol of the purified enzyme. This mixture was incubated at 37 $^{\circ}\text{C}$ for 20 min, and 100 μL of chromogenic assay solution, containing 1 μmol of phenol, 1.25 μmol of 4-aminoantipyrine, and 650 mU of horseradish peroxidase, was then added. The absorbance of quinoneimine dye at 505 nm and that of NADH at 340 nm were measured immediately after the solution was mixed.

Results

Cloning and heterologous expression of the gene encoding archaeal IPP isomerase

In our previous studies, we cloned the GGPP synthase gene (*gds*) from a thermoacidophilic archaeon *S. acidocaldarius* with some ORFs in the proximity of *gds* [12]. One of the ORFs, designated ORF3 (accession number: AB118245), is located next to *gds*, and both genes are thought to exist in an operon. A homology search revealed that ORF3 has a high sequence similarity with *fni*, the type 2 IPP isomerase gene from *Streptomyces* sp. strain CL190. Thus we attempted to express the ORF in *E. coli* to determine whether the gene also encodes the new type IPP isomerase. However, the recombinant expression of ORF3 in *E. coli* was unsuccessful: we were not able to detect thermostable IPP isomerase activity in the crude extract of the transformed *E. coli*. Therefore we isolated a homologue of *fni* from *S. shibatae*, a relative of *S. acidocaldarius*. By colony hybridization using ORF3 as the probe, we cloned a plasmid g43-2, which contains the homologue of *fni*, from a genomic library of *S. shibatae*. The homologue, named *idi* (accession number: AB118244), is 1107 bp in length and encodes a 368 amino acid protein, which shows a 62% identity with the enzyme encoded by ORF3 (Fig. 1). The partial sequence of a gene homologous with *gds* also exists immediately downstream of *idi*, suggesting that the genes form an operon whose structure is similar to that of *S. acidocaldarius*. The *idi* gene was amplified by PCR using the plasmid g43-2 as a template and subcloned into an expression vector, pET-15b. *E. coli* strain BL21(DE3) was then transformed with the construct pET-*idi*. As the result of an assay using the crude extract of the transformant, thermostable and NADH-dependent IPP isomerase activity was detected. Because the

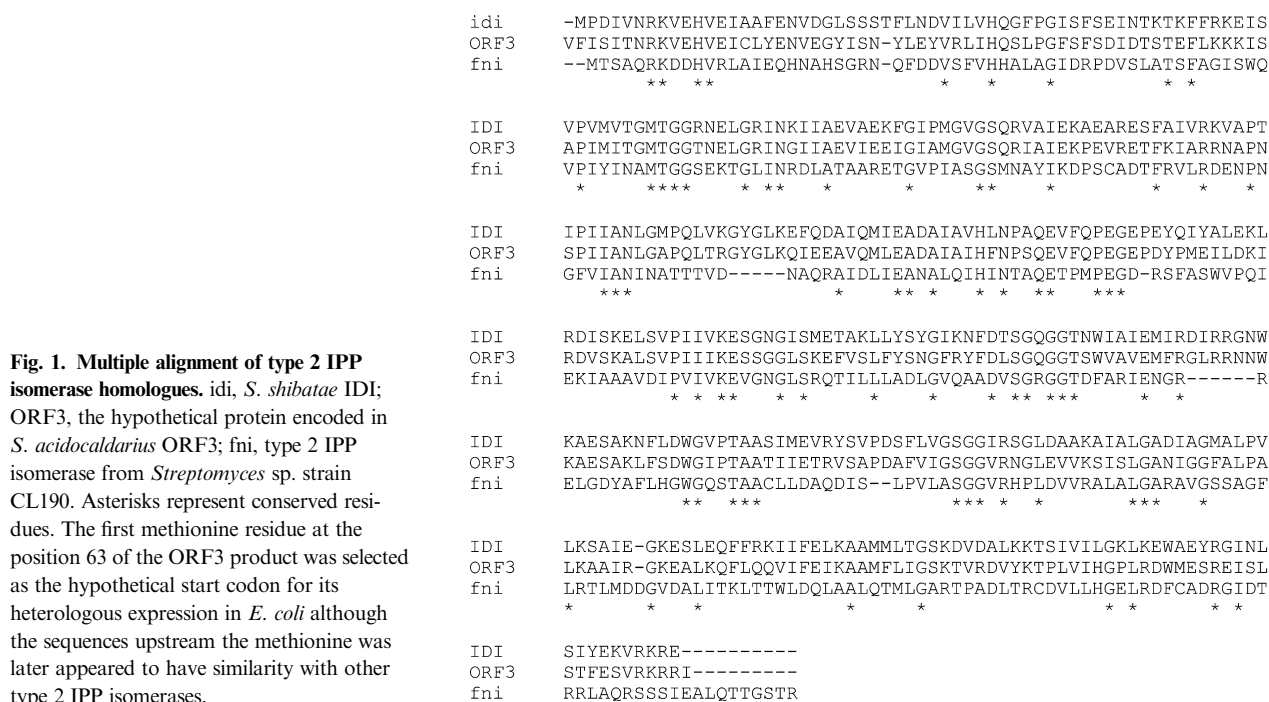


Fig. 1. Multiple alignment of type 2 IPP isomerase homologues. idi, *S. shibatae* IDI; ORF3, the hypothetical protein encoded in *S. acidocaldarius* ORF3; fni, type 2 IPP isomerase from *Streptomyces* sp. strain CL190. Asterisks represent conserved residues. The first methionine residue at the position 63 of the ORF3 product was selected as the hypothetical start codon for its heterologous expression in *E. coli* although the sequences upstream the methionine was later appeared to have similarity with other type 2 IPP isomerases.

endogenous activities of IPP isomerase and prenyltransferases of host cells were not detected under our standard conditions, the *idi* gene was considered to encode IPP isomerase, designated as IDI.

Purification and characterization of the recombinant enzyme

The crude extract was subjected to a heat-treatment, and the supernatant fraction from the centrifugation after the heat-treatment was applied to a Ni^{2+} -chelating column. Because a histidine-tag was attached to the amino terminus of IDI, the recombinant enzyme was efficiently and selectively trapped by the column. After elution of the enzyme from the column, it ran as a single band in an SDS/PAGE analysis (data not shown). The molecular mass of the enzyme was estimated to be ≈ 40 kDa based on the SDS/PAGE analysis, which is consistent with the molecular mass calculated from the amino acid sequence including the His-tag, 42 590. The molecular mass determined by gel filtration column chromatography was 180 kDa, suggesting that IDI forms a tetramer like the *fni* product. The ability of IDI to synthesize DMAPP was confirmed by adding a purified GGPP synthase of *S. acidocaldarius* to the standard IPP isomerase assay mixture and by detecting the formation of a C_{20} product. GGPP synthase is known to catalyze the consecutive condensations of IPP with DMAPP [2]. As a result, GGPP was produced in the reaction mixture containing both purified IDI and *S. acidocaldarius* GGPP synthase, but not in those containing only IDI or GGPP synthase (data not shown). The pH and temperature optima for the enzyme were 6.0 and 80 °C, respectively. The enzyme was stable after incubation at 60 °C for 1 h, and 83% of the activity remained after heat treatment at 70 °C for 1 h. Like the product of *fni* from *Streptomyces* sp. strain CL190, IDI required Mg^{2+} and NAD(P)H for activity. In addition, the use of NAD^+ instead of NADH led to a complete loss of activity. The dissociation constants of IDI for Mg^{2+} , NADPH, and NADH were 0.31 ± 0.05 mM, 23.8 ± 3.9 μM , and 90.4 ± 7.6 μM , respectively. Those results indicate that IDI prefers NADPH to NADH, like the *fni* product. However, unlike the *fni* product, the addition of flavin coenzymes had no significant effect on its activity. The K_m and k_{cat} values of the IDI for IPP at 60 °C were determined to be 63 μM and 0.2 s^{-1} , respectively. Those values are similar to those of IPP isomerases from *Streptomyces* sp. strain CL190 ($K_m = 450$ μM , $k_{\text{cat}} = 0.7$ s^{-1}) and *Staphylococcus aureus* ($K_m = 19$ μM , $k_{\text{cat}} = 1.3$ s^{-1}).

Binding of flavin coenzymes to IDI

As the color of the purified IDI solution was yellow, we measured the absorption spectrum of the enzyme. The characteristic spectrum, which has a peak near 450 nm, clearly indicated that IDI also contains a flavin coenzyme (Fig. 2). Interestingly, the amount of FMN per monomer subunit of IDI was calculated to be 0.9 $\text{mol}\cdot\text{mol}^{-1}$ by comparing the absorption of IDI at 450 nm with the extinction coefficient of free FMN ($\epsilon = 12.2$ $\text{mm}^{-1}\cdot\text{cm}^{-1}$ at 450 nm), while the *fni* product has been reported to bind 0.35 – 0.4 mol of FMN per mole of monomer. Considering the fact that the addition of FMN did not significantly

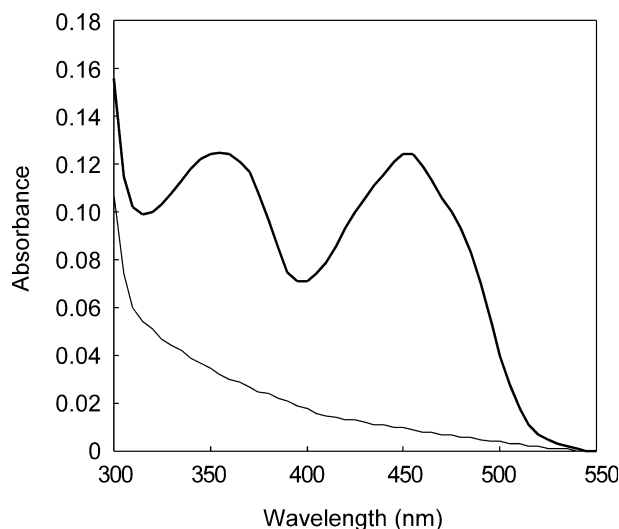


Fig. 2. Absorption spectra of IDI and its apoenzyme. Broad line, purified IDI; thin line, apo-IDI.

Table 1. FMN-dependence of IDI and its apoenzyme. The assay mixtures contained 5 nmol of $[1\text{-}^{14}\text{C}]\text{IPP}$ (0.19 $\text{GBq}\cdot\text{mmol}^{-1}$), 0.25 μmol of MgCl_2 , 2 μmol of malate/NaOH buffer (pH 6.0), 25 nmol of NADH, the indicated amounts of FMN, and a suitable amount of purified IDI or apo-IDI, in a final volume of 50 μL . The acid-labile radioactivity extracted with pentane in the experiment using IDI and 5 μM FMN was defined as 100%.

	FMN (μM)	Relative activity (%)
IDI	0	92
	5	100
apo-IDI	0	0
	1	64
	5	98
	10	103

increase the activity of IDI, the flavin-binding sites of IDI are thought to be nearly fully saturated. Thus, we postulate that the enzyme has one flavin-binding site per monomer. To investigate the role of the flavin coenzyme, we prepared the apoenzyme of IDI by dialyzing the solution of purified IDI against 2 M KBr. The absorption spectrum of the apoenzyme no longer showed a peak around 450 nm, suggesting that the flavin coenzyme was removed from IDI (Fig. 2). Moreover, we showed that the apoenzyme completely lost its activity and that the activity could be recovered to the same level of nontreated IDI when 5 μM FMN was added to the assay mixture (Table 1). The dissociation constants of the apoenzyme for various flavin compounds show that IDI has the highest affinity for FMN (Table 2).

NADH dehydrogenase activity of IDI

Recently, the crystal structure of *Bacillus subtilis* type 2 IPP isomerase was reported by Steinbacher *et al.* [17]. The structure appeared to be a TIM barrel, which is the common structure of flavoproteins, but the roles of FMN

Table 2. Dissociation constants of IDI for flavin coenzymes. To determine the dissociation constants for the flavin coenzymes, various concentrations of the coenzymes were previously added to the assay mixture using the apoenzyme and without NADH, and the mixtures were placed on ice for 30 min and then used for enzyme assay by adding NADH. The kinetic parameters were calculated using the nonlinear-regression method.

	K_d (μM)
FMN	0.319 ± 0.026
FAD	4.50 ± 1.60
Riboflavin	No binding

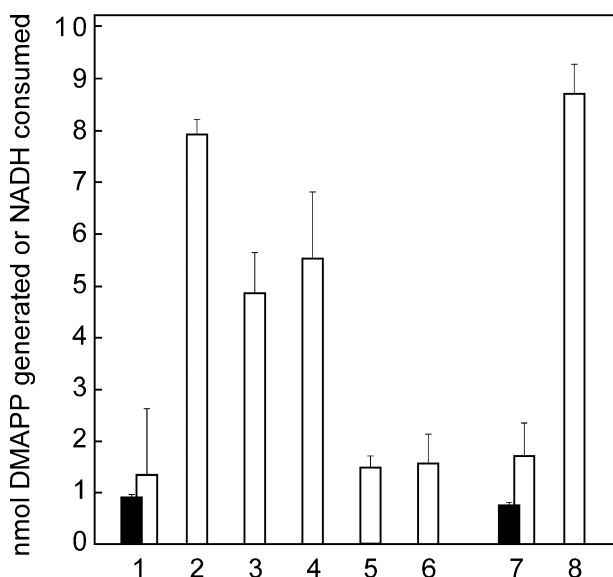


Fig. 3. NADH consumption and DMAPP production of IDI. DMAPP production (closed bars) and NADH consumption (open bars) were measured independently. Condition 1, standard reaction; condition 2, reaction in the absence of Mg^{2+} and IPP; condition 3, reaction without Mg^{2+} ; condition 4, reaction without IPP; condition 5, incubation without enzyme; condition 6, reaction without enzyme and IPP; conditions 7 and 8, the same with conditions 1 and 4, respectively, except for changing the buffer from malate/NaOH to succinate/NaOH. All measurements were repeated three times.

and NAD(P)H in the enzyme reaction could not be elucidated by the structural study. As many flavoproteins have NAD(P)H dehydrogenase activity, we next attempted to determine whether NADH is oxidized in the enzyme reaction of IDI. We observed the change in the absorption of NADH at 340 nm in the reaction mixture for the IDI assay, including IPP, NADH, Mg^{2+} , purified IDI, and malate/NaOH buffer (Fig. 3). The absorption at 340 nm appeared to decrease after the incubation, which corresponds to the oxidation of 1.25 nmol of NADH (condition 1, open bar). In the same reaction time, about 1 nmol of DMAPP was found to be produced (condition 1, closed bar). However, when IPP and Mg^{2+} were excluded from the reaction mixture to measure the activity just as NADH dehydrogenase, about 8 nmol of NADH was shown to be consumed (condition 2). The K_m of the enzyme for NADH

in the NADH dehydrogenase reaction (without IPP) was $87.4 \pm 5.8 \mu\text{M}$. This value is in good agreement with the K_m for NADH obtained in the isomerase reaction of IDI, $90.4 \mu\text{M}$. This fact confirmed that IDI actually acts as NADH dehydrogenase. Moreover, IPP and Mg^{2+} , the substrate and cofactor of the isomerase reaction, were suspected to even inhibit the redox reaction. To confirm this hypothesis, the effects of the components on the NADH dehydrogenase activity of IDI were studied in detail. As a consequence, IPP and Mg^{2+} were shown to have independent inhibitory effects (conditions 3 and 4, respectively). It should be noted, however, that Mg^{2+} promoted the consumption of NADH in the absence of the enzyme (condition 5), while the addition of IPP to the mixture had no effect (condition 6). These data suggest that origin of the consumption of NADH observed under condition 1 could be from the effect of Mg^{2+} , and not from the catalytic activity of the enzyme, and that the addition of both IPP and Mg^{2+} completely inhibits the oxidation of NADH.

The electron acceptor in IDI reaction

Our next interest was the acceptor of electrons in the NADH dehydrogenase reaction. We first replaced the malate buffer in the reaction mixture with succinate buffer because of doubts as to whether malate might act as an electron acceptor. When the buffer was exchanged, however, the NADH dehydrogenase activity of IDI did not decrease, but even increased (Fig. 3, condition 8). Interestingly, IPP isomerase activity decreased slightly when the succinate buffer was used (condition 7, closed bar). Thus we assumed molecular oxygen to be the electron acceptor because many redox-catalyzing flavoproteins are able to use it and produce hydrogen peroxide. To confirm this hypothesis, we measured the production of hydrogen peroxide using horseradish peroxidase and a chromogenic substrate. As a result, a considerable, but not stoichiometric amount of hydrogen peroxide was shown to be produced accompanying the oxidation of NADH (Fig. 4). Although the

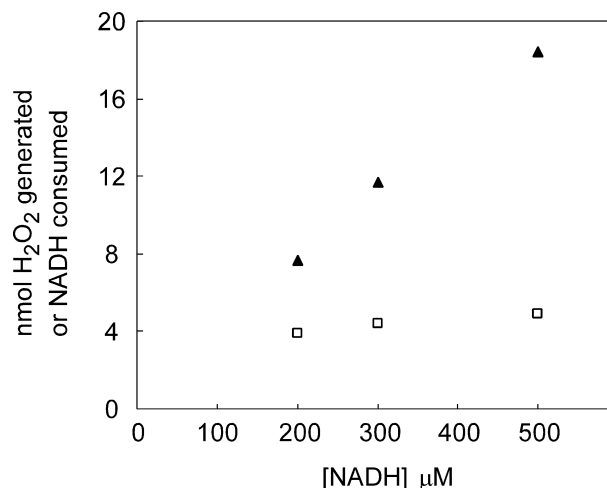


Fig. 4. Generation of hydrogen peroxide during the NADH dehydrogenase reaction of IDI. Hydrogen peroxide formation ($\square$) and NADH consumption ($\blacktriangle$) of IDI were measured at various concentrations of NADH.

amount of hydrogen peroxide production did not completely agree with that of NADH consumption, this result strongly suggests that molecular oxygen in the reaction mixture acts as the electron acceptor.

Discussion

In this paper, we demonstrate that the recombinant *S. shibatae* IDI, which is encoded by the archaeal homologue of the type 2 IPP isomerase gene *fni*, has thermostable IPP isomerase activity. It is the first report of an IPP isomerase from archaea, a class of organisms that have been expected to require the enzyme. IDI appears to be an NAD(P)H-dependent flavoprotein, like the known bacterial type 2 IPP isomerases [9], and the pH and temperature optima for IDI are in good agreement with the previously defined properties of some isoprenoid biosynthetic enzymes from *Sulfolobus* sp. [12,14,15]. The existence of the IPP isomerase gene in the proximity of the GGPP synthase gene in *Sulfolobus* sp. reflects its importance in the biosynthesis of ether-linked membrane lipids, which are the most characteristic and essential isoprenoid compounds in archaea [12].

In a previous study of bacterial type 2 IPP isomerase from *Streptomyces* sp. strain CL190, the enzyme was reported to be FMN and NAD(P)H-dependent [9]. However, there were no experimental data on the flavin dependence of the enzyme using a flavin-free system. On this occasion, we successfully prepared the apoprotein of IDI and provide proof that IPP isomerase activity of IDI is completely dependent on flavin coenzymes, particularly FMN. In addition, as apo-IDI could be completely reconstituted by free-FMN even after a lengthy treatment under acidic conditions, it is likely that it contains very stable folding. Although the dissociation constant for FMN of the bacterial type 2 IPP isomerase from *Streptomyces* sp. strain CL190 has not been determined, IDI is thought to have a higher affinity for flavin coenzymes than the bacterial enzyme because IDI, in contrast to the bacterial molecule, hardly lost flavin during the usual purification steps and thus was not efficiently activated by the addition of FMN. These findings suggest that the dissociation constant for FMN is small, probably in the nanomolar range, like most flavoproteins. However, the K_d value of the apoenzyme was determined to be 0.3 μM . This discrepancy might be explained by the conjecture that the binding of FMN was slow relative to the times used in our assays. The K_d values given in Table 2 would represent only apparent values.

We found, for the first time, that the IDI has NADH dehydrogenase activity and that hydrogen peroxide was produced during the NADH dehydrogenase reaction of IDI although the amount of hydrogen peroxide produced did not precisely agree with that of NADH consumed. However, we assume that hydrogen peroxide would be produced stoichiometrically and that the hydrogen peroxide production was not precisely evaluated because of its partial degradation before the assay or some problems with the assay condition. On the other hand, we also showed that IPP and Mg^{2+} inhibit the NADH dehydrogenase activity of IDI. This synergistic effect indicates that Mg^{2+} might be involved in the binding of the diphosphate group of IPP directly, as has been suggested for (all-*E*) prenyl diphos-

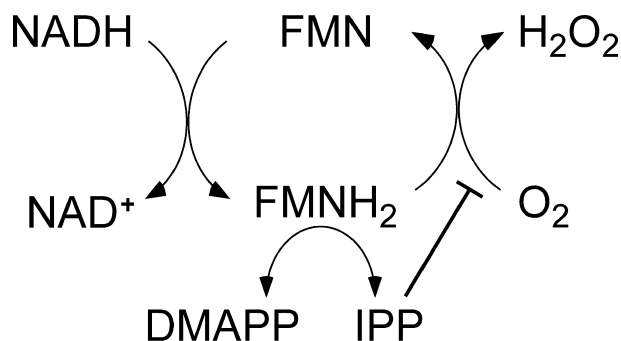


Fig. 5. Hypothetical catalytic cycle of the type 2 IPP isomerase.

phate synthases [2]. The above findings imply that IPP competes with molecular oxygen, the putative electron acceptor in the dehydrogenase reaction. What, then, is the role of NAD(P)H in the isomerization catalyzed by IDI while the interconversion of IPP and DMAPP involves no net redox change? Based on the catalytic mechanisms of other NAD(P)H-dependent flavoenzymes such as chorismate synthase, Bornemann suggested that NAD(P)H is used to reduce FMN and that the reduced form of FMN is essential for the activity of type 2 IPP isomerase [16]. From our results, the isomerase reaction appeared to proceed without consuming NAD(P)H. Thus we hypothesize the reaction mechanism of IDI, in which the reduced state of FMN (FMNH₂) plays an important role in the catalysis of the isomerization (Fig. 5). The binding of IPP (probably DMAPP as well) would inhibit the approach of molecular oxygen to the active site of the enzyme, which would involve FMNH₂. Without the substrates, molecular oxygen accepts electrons from FMNH₂, and the catalytic cycle of NAD(P)H dehydrogenase reaction proceeds. Indeed, the formation of FMNH₂ has not been detected in this study. We are currently in the process of examining the redox cycle of IDI in more detail, as it may contribute to our understanding of the roles of coenzymes on the catalytic reaction of type 2 IPP isomerase.

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