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Inhibition of type 2 isopentenyl diphosphate isomerase from *Methanocaldococcus jannaschii* by a mechanism-based inhibitor of type 1 isopentenyl diphosphate isomerase

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Abstract—Type 2 isopentenyl diphosphate:dimethylallyl diphosphate isomerase (IDI-2, EC 5.3.3.2) is a flavoprotein, which requires FMN, NADPH, and Mg^{2+} for the activity to convert isopentenyl diphosphate to dimethylallyl diphosphate. For investigation of the reaction mechanism of IDI-2, 3,4-epoxy-3-methylbutyl diphosphate (EIPP), a mechanism-based inhibitor of type 1 IDI (IDI-1), was treated with the overexpressed IDI-2 (MjIDI) from methanogenic archaeon *Methanocaldococcus jannaschii*. EIPP showed the time- and concentration-dependent inhibition (K_I; 56.5 mM, k_{inact}; 0.10 s^{-1}, k_{inact}/K_I; 1.76 s^{-1} M^{-1}) and the UV–vis spectrum of MjIDI after treatment with EIPP was apparently different from that of the untreated MjIDI. These results indicated that EIPP modified FMN through a covalent bond in the active site of MjIDI. The formed EIPP–FMN complex was separated from the reaction mixture and the spectrometric analysis of the complex suggested that the reduced form of FMN bound to EIPP at the N5 position. These results may suggest that the IDI-2 reaction is similar to IDI-1, which proceeds via carbocation-type intermediate.

1. Introduction

Isoprenoid compounds are ubiquitous in all living species and diverse in biological function. More than 30,000 naturally occurring isoprenoid derivatives are known, including such important classes of bioactive compounds as carotenoids, and plant isoprenoids. All of these isoprenoids are synthesized from key C_5 units, isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP). IPP isomerizes to DMAPP and also serves as the elongating C_5 donor in terpenoid biosynthesis. Isopentenyl diphosphate isomerase (IDI, EC 5.3.3.2) is responsible for the isomerization of the carbon–carbon double bond of IPP to create the potent electrophilic DMAPP as shown in Figure 1.[1]

IDI is known to be categorized into two distinct types which show no sequence homology to each other.[2] Type 1 IDI (IDI-1) is known to be utilized by most of eukaryotes, some bacteria, and a part of halophilic archaea,

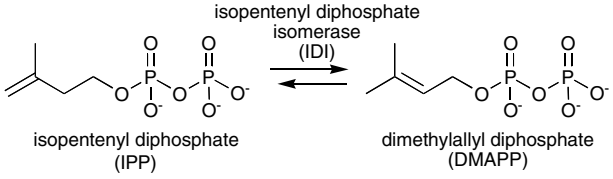

Figure 1. Isopentenyl diphosphate isomerase (IDI) reaction.

and type 2 IDI (IDI-2) has recently been reported to be produced by bacteria such as *Streptomyces*,[2] *Bacillus*,[3] *Cyanobacteria*,[4] trypanosomatid, and archaea.[5–8]

IDI-2 is a flavoprotein, which requires FMN, NADPH, and Mg^{2+} for the activity to convert IPP to DMAPP. Many flavoproteins are known to possess NAD(P)H:FMN oxidoreductase activity.[2] In fact, it was reported that the recombinant IDI-2 derived from *Sulfolobus shibatae* had FMN/NADPH oxidoreductase activity and that the reduced form of FMN was essential for the IDI-2 activity.[9] Thus, the role of NADPH is thought to be a reducing agent of FMN in IDI-2 reaction since it can be replaced by an alternate reducing agent such as dithionite. However, this enzyme catalyzes the isomerization reaction with no net redox change.

Keywords: Type 2 isopentenyl diphosphate isomerase; Reaction mechanism; Archaea; Inhibitor.
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Because Gram-positive pathogens such as *Staphylococcus* and *Enterococcus* utilize IDI-2, this enzyme is the potent target for new antibacterial drugs specific to these pathogens.[10] But, the exact catalytic mechanism of IDI-2 is still unknown, while that of IDI-1 enzyme, which requires only divalent metal cations for activity, has been understood in detail. In this paper, we describe an inhibition analysis of IDI-2 derived from methanogenic archaeon *Methanocaldococcus jannaschii* as an example of IDI-2 with a mechanism-based inhibitor of IDI-1, 3,4-epoxy-3-methylbutyl diphosphate (EIPP).

2. Results and discussion

The putative IDI-2 gene, MJ0862, from *M. jannaschii* was heterologously expressed in *Escherichia coli*. The purified enzyme showed yellow color, which revealed that the purified enzyme included FMN. The absorption at 447 nm of the purified enzyme indicated that FMN bound to MjIDI at 0.8–0.9:1 ratio. The purified enzyme catalyzed conversion from IPP to DMAPP in the presence of NADH and Mg^{2+} ion, which indicated that the MJ0862 gene in *M. jannaschii* encoded IDI-2. The properties of the purified enzyme under the optimal conditions are summarized in Table 1.

Concerning the reaction mechanism of IDI-2, one potential role for FMN is that the reduced form of FMN could reduce the substrate to give a saturated intermediate, which might be subsequently oxidized to yield the isomerized product.[11] Alternatively, Nishino and co-workers proposed that the reduced form of FMN could transfer only a single electron to generate the tertiary free radical intermediate, which could transfer its electron back to the flavin with the concomitant removal of a proton from the 2-position.[9] In both cases, the redox-state of FMN must be changed during the enzyme reaction. However, no significant change in UV–vis region was observed by a stopped-flow experiment of the MjIDI reaction in our laboratory (data not shown), which suggested that the isomerization proceeded with no transient redox role in catalysis.

For further mechanistic studies of IDI-2, we analyzed the enzyme reaction in the presence of an inhibitor. EIPP is known as a mechanism-based inhibitor of IDI-1. The reaction mechanism of EIPP with IDI-1 in *E. coli* involves the protonation of EIPP inhibitor by the cysteine-67 residue in the active site to form a tertiary carbocation intermediate.[12,13] By a nucleophilic attack of the cysteine-67 residue into the tertiary carbocation intermediate in the active site, IDI-1 is irreversibly inhibited by making a covalent bond as shown in Figure 2. Although the exact reaction mechanism of IDI-2 is not fully understood, if a carbocationic intermediate is involved in the reaction of IDI-2 as in the case of IDI-1, EIPP would inhibit the enzyme reaction and accept an attack of a near-by nucleophilic group of the IDI-2 active center to form a covalent bond. If this is not the case, EIPP may only act as a competitive inhibitor. Based on this hypothesis, we pursued the inhibition analysis of IDI-2 with EIPP.

Table 1. Biochemical properties of MjIDI

Optimal pH	Optimal temp.	K_m (IPP)	k_{cat}	k_{cat}/K_m
7.0–7.2	85–95 °C	15.3 mM	191 s^{-1}	1.25×10^4 s^{-1} M^{-1}

Racemic EIPP was chemically synthesized by the method of Muehlbacher and Poulter[14] and the synthesized EIPP was subjected to the reaction with the purified IDI-2 (MjIDI) derived from *M. jannaschii*. The IDI-2 activity decreased in a time- and concentration-dependent manner by EIPP as in the case of IDI-1 as shown in Figure 2. These results strongly suggested that the IDI-2 reaction proceeded via carbocation-type intermediate as in the case of IDI-1. The inhibition constants for inactivation were determined by Kitz and Wilson plot[15] (K_I = 56.5 mM, k_{inact} = 0.10 s^{-1}, k_{inact}/K_I; 1.76 s^{-1} M^{-1}). The k_{inact} value for EIPP suggested rather slow inactivation in comparison with catalytic activity, k_{cat} for the substrate IPP (Fig. 3).

To confirm whether a covalent bond between EIPP and an amino acid residue of MjIDI was formed or not, ESI-MS analysis of MjIDI after treatment with EIPP was performed. Molecular mass of the EIPP treated MjIDI was found to be 39887 ± 10 Da, which was similar to the native protein (39897 ± 10 Da). These results indicated that an amino acid residue of MjIDI did not make a covalent bond with EIPP.

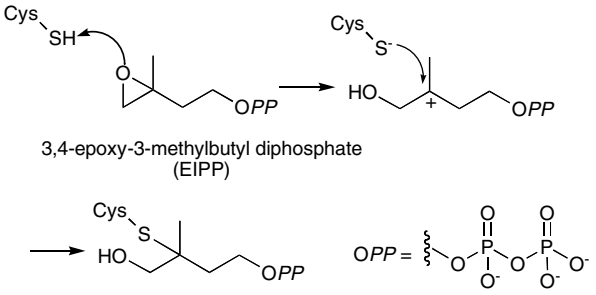

Figure 2. Inhibition mechanism of EIPP to IDI-1.

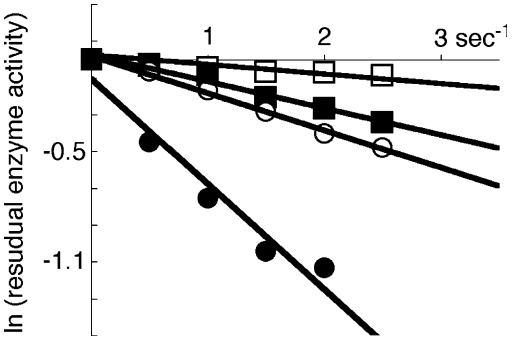

Figure 3. Time- and concentration-dependent inactivation of MjIDI by EIPP. EIPP concentration: open square, 1 mM; closed square, 2.5 mM; open circle, 5 mM; closed circle, 10 mM.

For further studies of the inhibition mechanism of EIPP, the UV–vis spectrum of the EIPP treated MjIDI was analyzed. As was reported by Nishino et al.,[9] when an enzyme mixture of MjIDI and IPP in the presence of dithionite was exposed to air, the reduced form of FMN was rapidly oxidized to FMN by oxygen and the characteristic absorption of FMN at ca. 450 nm was regenerated. In contrast, the characteristic absorption of FMN was not observed in the case of the EIPP treated MjIDI as shown in Figure 4. This spectral difference demonstrated that the reduced form of FMN in MjIDI was modified by EIPP by a covalent bond.

Several attempts for separation of the complex from MjIDI have not succeeded because of instability of this complex. But, denaturing by guanidine–HCl was found to release the EIPP–FMN complex from the enzyme with minimum decomposition. Thus, the denatured protein was removed by ultrafiltration and the filtrate was analyzed by HPLC. New two peaks were detected at about 10 min compared with the control experiment (Fig. 5A). Both peaks showed the same broad absorption at 380 nm as shown in Figure 5B, which is characteristic for N5-substituted FMN.[16] The ESI-MS analysis of these two peaks showed the same mass spectra at m/z = 741.1 and 756.7 as shown in Figure 5C. These ion peaks corresponded to the expected molecular masses of $[M+Na–H_2O]^+$, $[M+K–H_2O]^+$ for the EIPP–FMN complex, respectively. The appearance of two peaks was probably due to diastereoisomers depending on the stereogenic centers of the EIPP–FMN complex.

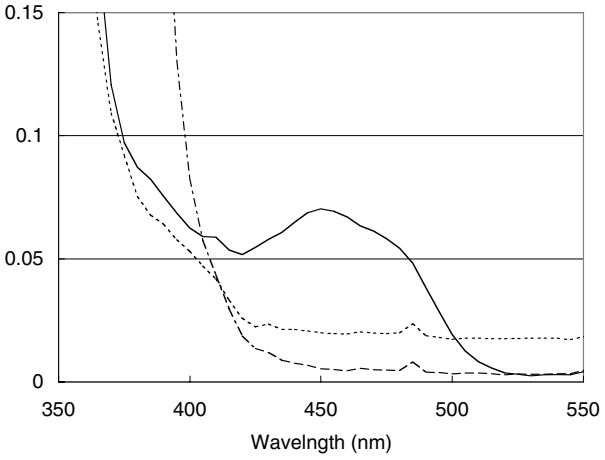

Figure 4. UV–vis spectra of MjIDI. The purified MjIDI was treated with dithionite (dash-dotted line). After enzyme reaction, the mixture was exposed to air (solid line). The purified MjIDI was treated with EIPP in the presence of dithionite, and then the mixture was exposed to air (dotted line).

Thus, the inhibition mechanism of EIPP was suggested as follows. First, an amino acid residue in the active site activates epoxide moiety of EIPP inhibitor by the protonation. Then, the reduced form of FMN nucleophilically attacks the activated epoxide by the S_N2-like mechanism. The formed EIPP–FMN complex is easily oxidized and hydrolyzed when the complex is exposed to air to give the N5-substituted FMN as shown in Figure 6. Since the inhibition mechanism of EIPP against IDI-2 was quite similar to that of IDI-1, the present study may suggest that the IDI-2 reaction is similar to IDI-1, which proceeds via carbocation-type intermedi-

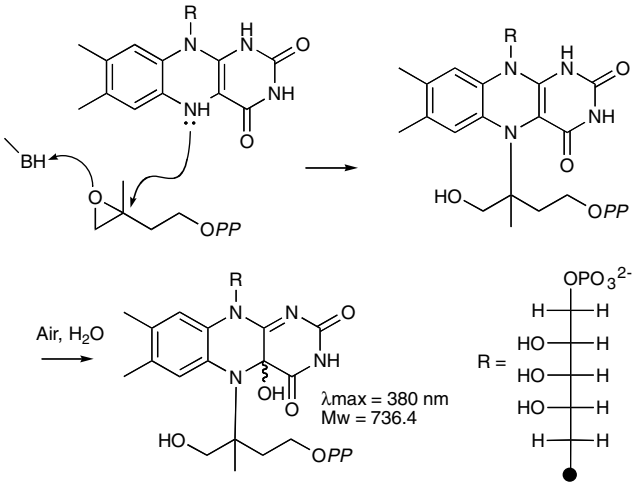

Figure 6. Inhibition mechanism of EIPP to MjIDI.

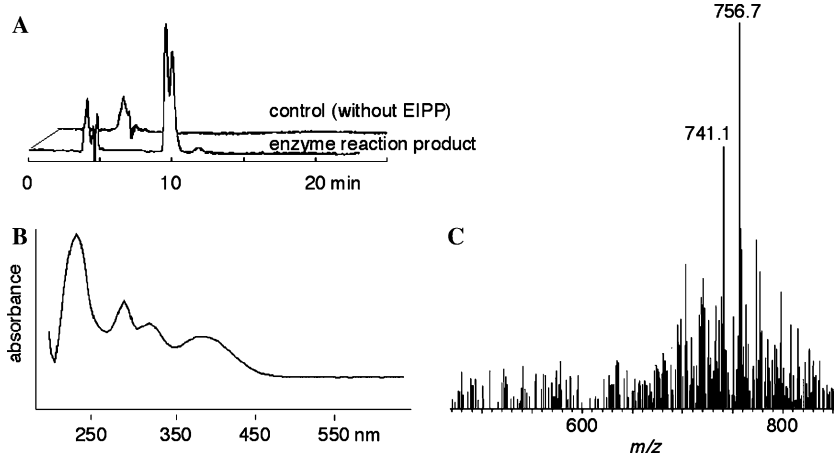

Figure 5. Analysis of the reaction product of IDI-2 with EIPP. (A) HPLC analysis of the enzyme reaction product with EIPP. (B) UV–vis spectrum of the two peaks eluted at 10 min. (C) ESI-MS spectrum of the two peaks.

ate. During the isomerization reaction, the reduced flavin may stabilize the carbocation intermediate. In the IDI-1 reaction, the tryptophan residue in the active site is thought to stabilize the carbocation intermediate through a quadrupole–charge interaction.[13] Clearly, further studies will be necessary to clarify the detailed enzyme mechanism in the apparently simple isomerization reaction performed by this newly characterized family of flavoproteins.

3. Experimental

3.1. Organisms

Methanocaldococcus jannaschii JCM10045^T (= DSM 2661) was obtained from the Japan Collection of Microorganism, RIKEN, and was cultured in a JCM medium 232 at 80 °C under anaerobic conditions. *E. coli* JM109 and BL21(DE3) were cultured in LB medium or on LB-agar containing 50 μg/ml of ampicillin or 30 μg/ml of kanamycin, when necessary.

3.2. Reagents and materials

DNA sequencing was carried out with a LONG READIR 4200 (Li-Cor) according to the manufacturer's protocol. PCR was performed by a GeneAmp PCR System 9700 (Amersham Bioscience, USA) using AmpliTaq Gold DNA polymerase (Perkin-Elmer, USA). Oligo DNAs for PCR primer were synthesized in Sigma Genosys Japan. Purification of plasmids was carried out with a GFX Micro Plasmid Prep Kit (Amersham Bioscience, USA). Restriction enzymes and modification enzymes were purchased from TaKaRa (Japan). Alkaline phosphatase was purchased from Sigma (USA). Genome preparations were carried out with Dr. GENTLE (TaKaRa, Japan). IPP and EIPP were chemically synthesized by the method of Davisson et al.[17] and Muehlbacher and Poulter,[14] respectively. All other reagents were of the highest grade commercially available.

3.3. Expression of MJ0862 in *E. coli*

DNA manipulations were performed as described in the literature.[18] To obtain MJ0862 for heterologous expression, IPP-f (GAACATATGGTTAATAATAGAAATGAGAT) and IPP-r (TCCCAGAGATTAAAATAAAACAGAATTCTT) were designed as primers. *Nde*I site (solid underline) and *Eco*RI site (dashed underline) were added in IPP-f and IPP-r, respectively. PCR (95 °C for 10 min, and 95 °C for 1 min, 45 °C for 1 min, 72 °C for 2 min in total 30 cycles, and then 72 °C for 10 min) was performed using the *M. jannaschii* chromosome as a template. PCR products were once subcloned into pT7 blue-T vector, and the sequence of it was confirmed. *Nde*I–*Eco*RI fragment of the resulting plasmid was subcloned into the corresponding site of pET30b, to yield pETIPP. *E. coli* BL21(DE3) carrying pETIPP was cultivated in 1.2 L of LB medium containing 30 μg/mL of kanamycin at 37 °C until OD_{600} reached 0.6. After the culture was cooled on ice, isopropyl β-D-thiogalactopyranoside was added to a final concentration of 0.1 mM. After 24 h of additional cultivation at 20 °C, cells were harvested by centrifugation (10,000*g*, 10 min) and stocked at −30 °C until use.

3.4. Purification of the expressed protein

The frozen cells (5 g) were thawed, homogenized in 0.1 M potassium phosphate buffer (pH 7.0) containing 5 mM $MgCl_2$ and 1 mM dithiothreitol (DTT), and disrupted by a sonic oscillator (Sonifier Type-250, Branson, USA) for a total period of 20 min on ice bath. Unbroken cells were removed by centrifugation (10,000*g*, 10 min) to obtain a cell-free extract. The extract thus obtained was then heat-treated at 80 °C for 30 min under argon atmosphere and centrifuged at 10,000*g* for 10 min. The resulting supernatant was concentrated by ultrafiltration and then subjected to a column of Hi Load 26/60 Superdex 200 pg equilibrated with 100 mM potassium phosphate buffer (pH 7.0) by FPLC system (Amersham Bioscience, USA).

3.5. Enzyme assay

The standard reaction mixture contained 1 μM enzyme, 2 mM IPP, 10 μM FMN, 5 mM NADPH (or 5 mM sodium dithionite), and 0.1 M of potassium phosphate buffer (pH 7.0) in a total volume of 0.1 mL under argon atmosphere. For estimation of the optimal pH for activity, potassium phosphate buffer (pH 6.4–7.6) was used. The reaction was initiated by addition of IPP, and the mixture was incubated at 85 °C. To the solution, 0.1 mL of 0.1 M Tris–HCl buffer (pH 9.5) and alkaline phosphatase (1 U) were added to remove diphosphate group, and the mixture was incubated at 37 °C for 12 h. The reaction products were extracted with diethyl ether and analyzed by gas chromatography (GC-14B, Shimadzu, Japan).

3.6. LC/MS analysis of the EIPP treated MjIDI

A solution (total volume 100 μL) containing 25 μM MjIDI, 10 mM EIPP, 5 mM dithionite, and the reaction buffer was incubated at 85 °C for 5 min. The reaction mixture was loaded on to a Senshu Pak PEGASIL-300 C4P column (1.0 mm∅ × 15 cm, Senshu Scientific, Tokyo), which was connected on-line to an electrospray ionization (ESI)-mass spectrometer (Finnigan LCQ). The column was first washed with acetonitrile/water (1:9) containing 0.1% HCOOH and 0.01% TFA for 10 min at a flow rate of 100 μl /min. Elution was further carried out with a mixture of acetonitrile/water (9:1) containing 0.1% HCOOH and 0.01% TFA. The elution was monitored with a UV detector at 280 nm, and mass spectra were simultaneously scanned for a range from 300 to 2000 amu. The molecular mass was estimated by deconvolution with Bio-Works 1.0 software (ThermoQuest).

3.7. Time- and concentration-dependent inhibition assay

For an inactivation assay, a solution (30 μL) of MjIDI (0.1 mM) and 5 mM dithionite containing varying amounts of EIPP (1–10 mM) in 0.1 M of potassium

phosphate buffer (pH 7.0) was preincubated at 85 °C for 2 min. The reaction mixture was diluted to 100-fold with the same buffer. An assay mixture (total 50 μL) containing the diluted preincubation solution (10 μL) was incubated at 85 °C for 1 min. The enzyme reaction was terminated by addition of 0.1 mL of 0.1 M Tris–HCl buffer (pH 9.5). One unit of alkaline phosphatase was added and the mixture was incubated at 37 °C for 12 h. The reaction products were extracted with diethyl ether and analyzed by gas chromatography. The half-time ($t_{1/2}$) for inactivation at each inhibitor concentration was plotted against 1/[EIPP], referred to as a Kitz and Wilson plot,[15] and K_I and k_{inact} values were obtained by the following equation: $t_{1/2} = 0.69/k_{inact} + 0.69/k_{inact}\ K_I/[I]$ ([I], the inhibitor concentration; k_{inact}, the rate of inactivation; K_I, the concentration of the inhibitor that produces half the maximal rate of inactivation).

3.8. Separation and analysis of EIPP–FMN complex

A solution containing 0.1 mM MjIDI, 2 mM EIPP, and 10 mM dithionite was incubated at 37 °C under anaerobic conditions. The reaction mixture was diluted with 3 M guanidine–HCl and incubated at 4 °C for 12 h. After ultrafiltration with Amicon YM-10 filter, a 10 μL aliquot of the supernatant was injected into an HPLC system (L-7000 series, Hitachi) using a PDA detector and a Shiseido Capcell Pak Column 4.6∅ × 250 mm (Shiseido Fine Chemicals). The column was washed with 20 mM ammonium acetate (pH 5.0) for 10 min, and then the complexes were eluted by a gradient to 50% acetonitrile in 20 mM ammonium acetate over a period of 30 min.

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