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Bacillus subtilis ypgA Gene Is *fni*, a Nonessential Gene Encoding Type 2 Isopentenyl Diphosphate Isomerase

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We previously identified the *fni* gene of *Streptomyces* sp. strain CL190 as type 2 isopentenyl diphosphate (IPP) isomerase, which needs both FMN and NADPH for enzyme activity. An *fni* gene homolog, *ypgA*, was detected in the database of the *Bacillus subtilis* genome. However, the *ypgA* product was about 140 amino acids shorter in the N-terminal than the *Streptomyces fni* gene product. A database search found three new putative start codons in 129, 225, and 411 bases upstream of the original start codon of the *ypgA* gene. The longest gene product, which was named *ypgA3*, showed the most significant homology to the *Streptomyces fni* gene product. The *ypgA3* gene was expressed with an N-terminal His-tag in *Escherichia coli* and the purified soluble protein was characterized in detail. The *ypgA3* protein converted IPP to its isomer dimethylallyl diphosphate in the presence of both FMN and NADPH. The enzyme also catalyzed the reverse reaction in the presence of both the cofactors. Disruption of the *ypgA3* gene was not lethal to *B. subtilis*. These results indicate that *Bacillus ypgA3* gene is *fni*, a nonessential gene encoding type 2 IPP isomerase.

Key words: isoprenoid; biosynthesis; *Bacillus subtilis*; isomerase; 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway

All terpenoids are synthesized by consecutive condensations of a five-carbon-basic unit, isopentenyl diphosphate (IPP) to its isomer, dimethylallyl diphosphate (DMAPP).[1] IPP isomerase (EC 5.3.3.2) catalyzes an important reaction in the biosynthesis of terpenoids by converting IPP to DMAPP (Fig. 1).[2] Many IPP isomerase genes, which are encoded by *idi* genes, have been cloned from various organisms such as humans,[3] *Saccharomyces cerevisiae*,[4] *Escherichia coli*,[5] and *Rhodobacter capsulatus*.[6] This enzyme needs only a divalent cation such as Mg^{2+} for its enzyme activity. However, we previously identified an open reading frame of *Streptomyces* sp. strain CL190 as a new type of IPP isomerase that strictly needs both FMN and NADPH for enzyme activity.[7] The amino acid sequence of this new IPP isomerase, designated as type 2 IPP isomerase, shares no similarity to those of the *idi* gene products. The *fni* gene homologs encoding type 2 IPP isomerase are found in the gene cluster of the mevalonate pathway not only from *Streptomyces* sp. strain CL190, but also from *Borrelia burgdorferi*, and Gram-positive cocci such as *Staphylococcus aureus* and *Enterococcus faecalis*. This fact implied that the *fni* gene is specific for the mevalonate pathway.

Two distinct biosynthetic pathways for IPP and DMAPP are known (Fig. 1).[8–12] One is the well-known mevalonate pathway (Fig. 1A), which operates in eukaryotes, archaebacteria, Gram-positive cocci, and cytosols of higher plants. The other is a recently discovered pathway, the 2-*C*-methyl-D-erythritol 4-phosphate (MEP) pathway (formerly called the non-mevalonate pathway) (Fig. 1B), which is used by most eubacteria, green algae, and the chloroplasts of higher plants. In *E. coli* with the MEP pathway, IPP isomerase is not essential, because IPP and DMAPP are biosynthesized independently (Fig. 1B).

Bacillus subtilis has all the genes of the MEP pathway and therefore uses this pathway for the biosynthesis of IPP and DMAPP.[13] In addition to these genes, an *fni* gene homolog, *ypgA*, was detected in the database of the *B. subtilis* genome.[14] However, it was ambiguous that the *ypgA* gene product actually had IPP isomerase activity, because the amino acid sequence of the gene product was about 140 amino acids shorter in the N-terminal than the *fni* gene products from *Streptomyces* sp. strain CL190 and *S. aureus*.

In these studies, we found an actual start codon for the *ypgA* gene. In this paper, we describe the first unambiguous assignment of the *ypgA* gene product as type 2 IPP isomerase and the enzymatic properties of the *ypgA* gene products overexpressed in *E. coli*.

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Abbreviations: IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; MEP, 2-*C*-methyl-D-erythritol 4-phosphate

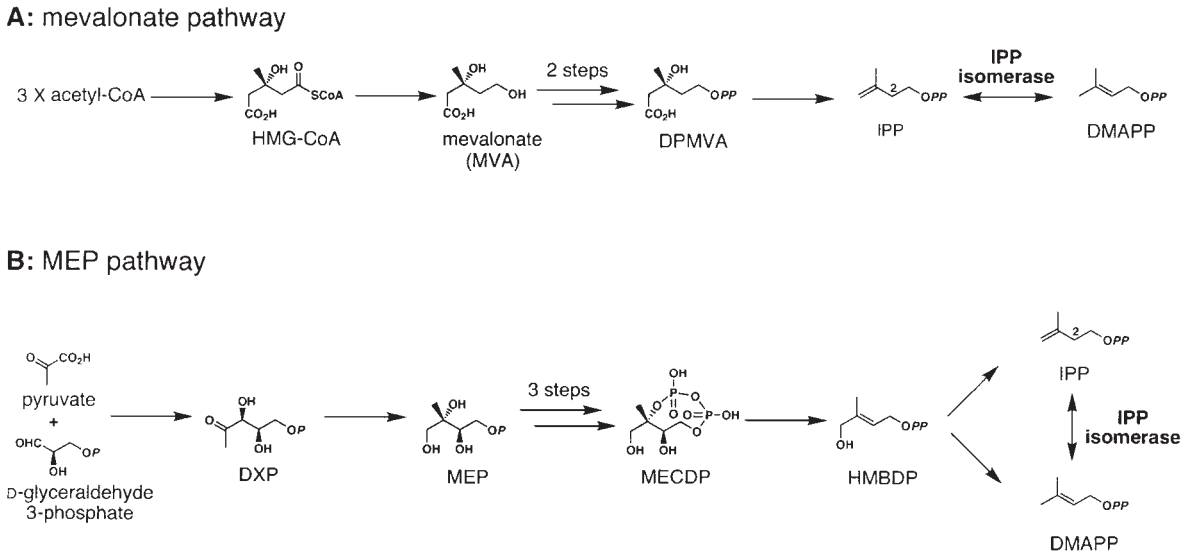

Fig. 1. Mevalonate and MEP Pathways for IPP and DMAPP Biosyntheses.

In the MEP pathway, both IPP and DMAPP are formed from HMBDP by the action of the *lytB* gene product. HMG-CoA, 3-hydroxy-3-methylglutaryl-CoA; DPMVA, diphosphomevalonate; IPP, isopentenyl diphosphate; DMAPP, dimethylallyl diphosphate; DXP, 1-deoxy-D-xylulose 5-phosphate; MEP, 2-*C*-methyl-D-erythritol 4-phosphate; MECDP, 2-*C*-methyl-D-erythritol 2,4-diphosphate; HMBDP, *E*-4-hydroxy-3-methyl-2-butenyl diphosphate.

Materials and Methods

Strains and vectors. B. subtilis 168 strain (wild-type) and the *ypgA* gene-disruptant were kindly provided by Prof. Ogasawara of the Nara Institute of Science and Technology. This disruptant has the erythromycin-resistance gene in the *ypgA* gene. pQE30, an expression vector in *E. coli*, was purchased from Qiagen.

Growth conditions for B. subtilis. Fresh *B. subtilis* cells were suspended in LB medium and the optical density at 655 nm of this cell suspension was adjusted to 10^{-3}. One hundred μl of the cell suspension was incubated in a well of a 96-well microplate at 37°C with shaking (700 rpm/min). The growth of the cell was monitored at 655 nm with a Benchmark Microplate Reader (BIORAD) adjusted at 37°C.

DNA sequence analysis. The DNA sequence was analyzed by the dideoxynucleotide chain-termination method[15] with an automated sequencer (model 4000L, Li-cor) and the protocol of the supplier. The FASTA program[16] did homology searches with protein databases of the DNA Data Bank of Japan (DDBJ) and the National Center for Biotechnology Information (NCBI). A GENETYX program ver. 11 (Software Development, Tokyo, Japan) was used to search for putative start codons and to align amino acid sequences.

Construction of the Plasmid for Overexpression of ypgA3 in E. coli. On the basis of the nucleotide sequence of *ypgA3* from *B. subtilis* 168, two oligonucleotide primers, 5′-GGGGATCC ACTCGAGCAGAACGAAAAAGAC-3′ (5′ of *ypgA3*) and 5′-GGGGATCC TCGCACACTATAGCTTGATGTATTG-3′ (3′ of *ypgA3*), including a *Bam*HI restriction site (underlined) were synthesized (Sigma) and used together with total DNA from *B. subtilis* 168 to amplify *ypgA3*. By using Taq DNA polymerase (Roche Molecular Biochemicals) and the protocol recommended by the supplier, a 1.1-kb fragment was amplified. The PCR fragment was digested with *Bam*HI and cloned into the multi-cloning site of the expression vector pQE30 to give pQEYpgA3.

Expression and Purification of the Recombinant ypgA3 Product. E. coli M15 containing pREP4 [*neo*, *lacI*] (Qiagen) was used as a host for the expression of *ypgA3*. M15 (pREP4, pQEYpgA3) was cultured at 18°C in one liter of LB medium containing 25 μg/ml kanamycin (Nacalai Tesque, Kyoto) and 200 μg/ml ampicillin (Sigma) for 6 h with the addition of 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) when an optical density at 660 nm of 0.8 was reached. Cells were harvested by centrifugation and resuspended in Buffer A, composed of 100 ml of 0.1 M Tris-HCl (pH 8.0) and 5 mM DTT. After sonication, the lysate was centrifuged at 10,000 × g for 20 min and the supernatant was retained. The crude extract was put on a Ni-nitrilotriacetic acid agarose column (Qiagen) previously equilibrated with Buffer A. The resin was washed with 50 mM imidazole in Buffer A and then the protein bound to the resin was eluted with 200 mM imidazole in Buffer A.

Assay for type 2 IPP isomerase. An assay system for type 2 IPP isomerase was based on the acid lability of DMAPP. This assay mixture consisted of 0.1 M HEPES buffer (pH 7.0), 5 mM $MgCl_2$, 1 mM DTT, 10 μM

[1-^{14}C]IPP (2.0 GBq/mmol; American Radiolabeled Chemicals, USA), 40 μM IPP, 10 μM FMN and 1 mM NADPH in a final volume of 50 μl. The reaction was started by adding enzyme to the assay system followed by incubation routinely at 37°C for 1 min. Further manipulations were done as described before.[7)]

pH-Dependent Activity of IPP isomerase. The IPP isomerase assay was done as described above. Assay solutions consisted of 0.1 M MES at pH 5.0–6.5, 0.1 M HEPES at pH 6.5–7.5 or 0.1 M Tris-HCl at pH 7.0–9.5, 5 mM $MgCl_2$, 10 μM FMN, 5 mM NADPH and 1 mM DTT.

Nucleotide sequence accession number. The nucleotide sequence of the *fni* gene of *B. subtilis* has been deposited in the DDBJ/EMBL/GenBank nucleotide sequence databases with the accession number AB047343.

Results

Identification of the fni gene homolog in the B. subtilis genome

To identify *fni* gene homologs in the *B. subtilis* genome, homology searches that use the sequence of the *fni* gene product from *Streptomyces* sp. strain CL190 as a query sequence was done using databases of DDBJ and NCBI. This search identified a *ypgA* gene product (accession no. L47648) with unknown function.[14)] Figure 2 shows the multiple alignment of the amino acid sequences of the *ypgA* gene product and the FNI homologs from *Streptomyces* and *Staphylococcus*. The *Bacillus* gene product showed significant homology to these FNI homologs. However, the *ypgA* product was about 140 amino acids shorter in the N-terminal than the FNI homologs. This finding led us to assume that there is another start codon upstream of the original one. As expected, a database search found three new putative start codons in 129, 225, and 411 nucleotides upstream of the original start codon of the *ypgA* gene (Fig. 2). The corresponding genes were named *ypgA1*, *ypgA2*, and *ypgA3*, respectively. The longest gene product, YPGA3, showed most significant homology to the *Streptomyces fni* gene product, suggesting that the *ypgA3* gene actually encodes *Bacillus* FNI (Fig. 2). The stop codon for *ypfD* gene flanking the *ypgA* gene was found 15 nucleotides upstream of the start codon for the *ypgA3*, also supporting the above suggestion.

Expression and Purification of the recombinant ypgA3 product

To discover the *in vitro* function of the *ypgA3* product, the *ypgA3* was expressed with an N-terminal His-tag in *E. coli* using the T7 promoter system and the product was purified to homogeneity as a soluble protein. After purification, the concentrated protein solution was yellow, as was the *fni* protein from *Streptomyces* sp. strain CL190, indicating that the *ypgA3* product is also a flavoprotein. SDS-PAGE showed a subunit molecular mass of 41 kDa. The apparent molecular mass of the *ypgA3* product was estimated to be 135 kDa by Superdex 200 gel filtration. These data indicate that the *ypgA3* product is most likely to be a tetramer. When we attempted to overexpress the *ypgA*, *ypgA1*, and *ypgA2* genes in *E. coli* by the same method as that for the *ypgA3* gene, we were able to obtain insoluble proteins but not soluble proteins.

FMN/NADPH-dependent IPP isomerase activity of the recombinant ypgA3 product

We next investigated whether the recombinant *ypgA3* product catalyzes conversion of IPP and DMAPP in the presence of FMN and NADPH. As expected, the *ypgA3* product showed IPP isomerase activity in the presence of both the cofactors, confirming that the gene actually encodes type 2 IPP isomerase (Table 1). The activity was maximal when 10 μM FMN and 1 mM NADPH were added. The activity was slightly below maximal when 10 μM FMN and 1 mM NADH was used. However, replacement of FMN with FAD resulted in the loss of IPP isomerase activity. Without addition of FMN or NADPH, no activity was detected, demonstrating that IPP isomerization catalyzed by the *ypgA3* product was strictly dependent on both FMN and NADPH. This enzyme required Mg^{2+}, Mn^{2+}, or Ca^{2+}. The activity was maximal with Mg^{2+}, but lower with Mn^{2+} and Ca^{2+}. No effects of other divalent cations such as Co^{2+}, Cu^{2+}, and Zn^{2+} at 5 mM were detected. Addition of 5 mM EDTA resulted in almost complete loss of the enzyme activity.

The IPP isomerase encoded by *ypgA3* was most active in HEPES buffer. The optimum activity of the enzyme occurred at pH 6.5–7.0. The effects of temperature on the enzyme activity were investigated over the range of 15 to 55°C, with the maximum activity being observed at approximately 37°C. Table 2 summarizes enzymatic properties of type 2 IPP isomerases from *B. subtilis*, *Streptomyces* sp. strain CL190,[7)] and *S. aureus*.[7)]

Phenotype of the Bacillus-ypgA3-disruptant

To investigate *in vivo* function of the *ypgA3* gene, a *Bacillus-ypgA3*-disruptant was cultivated in liquid LB medium. The growth curve revealed that the disruption of the gene had no apparent effects on growth. Fosmidomycin (3.1 μg/ml), a specific inhibitor of DXP reductoisomerase of the MEP pathway,[17)] completely inhibited the growths of both *B. subtilis* 168 and the *ypgA3*-disruptant, showing that enough IPP and DMAPP to support growth were not supplied from LB medium. These results demonstrate that *ypgA3* is a nonessential gene for *B. subtilis* growth.

Discussion

In this study, we have demonstrated that the *B.*

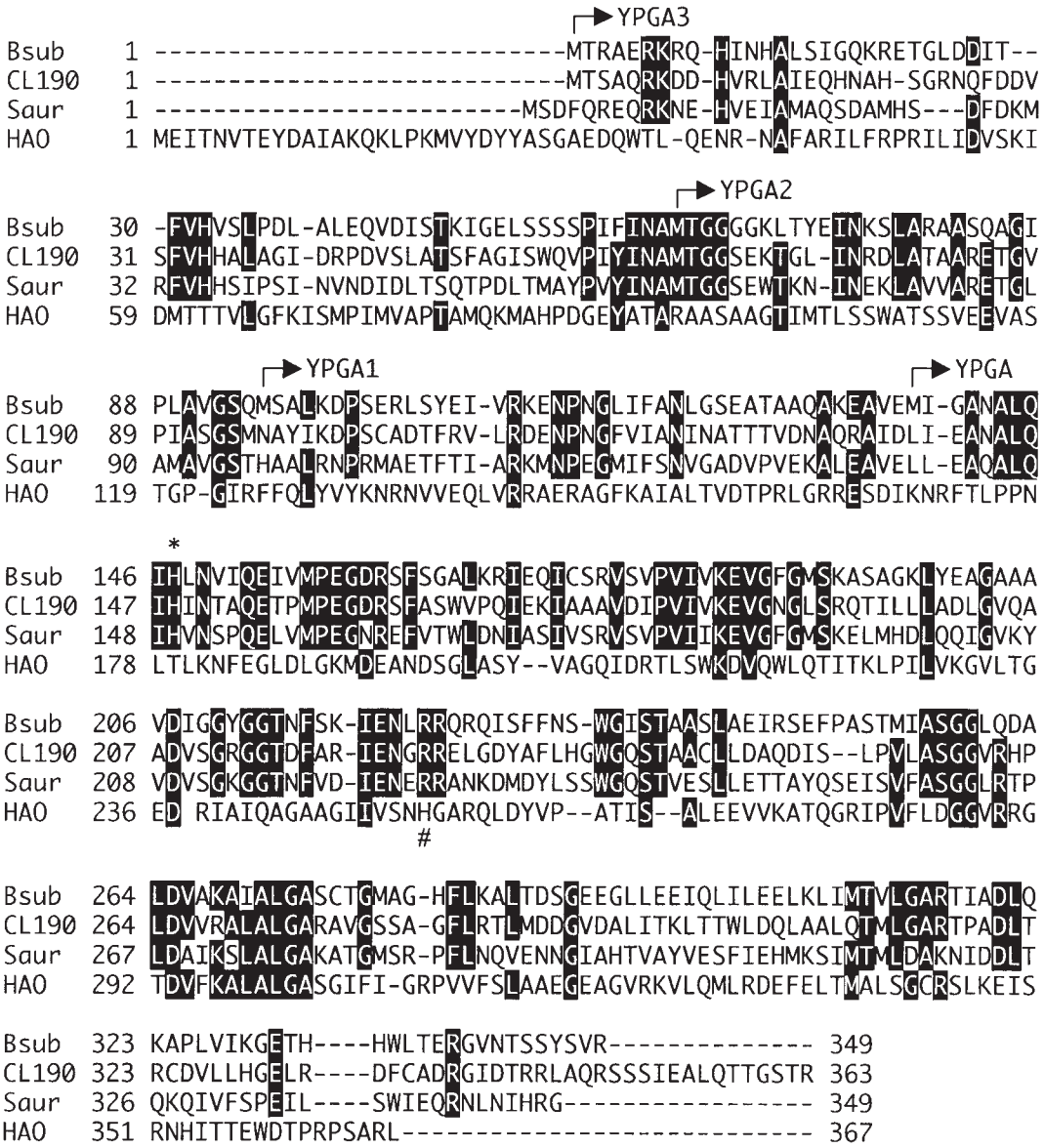

Fig. 2. Amino Acid Alignment of Type 2 IPP Isomerases and (*S*)-2-Hydroxy-acid Oxidase.

The putative first methionines of YPGA are marked by arrows. The type 2 IPP isomerases from three origins were aligned with (*S*)-2-hydroxy-acid oxidase from *A. thaliana* using the GENETYX program. Amino acid residues conserved among 3 or 4 proteins are boxed. Dashes indicate gaps introduced for the optimization of the alignment. The highly conserved His residues are marked by an asterisk and the His254 in the active site of (*S*)-2-hydroxy-acid oxidase is marked by a sharp. Bsub, *B. subtilis* (accession no. AB047343); CL190, *Streptomyces* sp. strain CL190 (accession no. AB046667); Saur, *S aureus* (accession no. AB047344); HAO, *A. thaliana* (*S*)-2-hydroxy-acid oxidase (accession no. Q9LRR9).

subtilis ypgA3 gene is a nonessential gene encoding type 2 IPP isomerase and compared the kinetic parameters of the *B. subtilis* enzyme to those of *Streptomyces* and *Staphylococcus* homologs. *B. subtilis* enzyme was also a flavoprotein and had FMN- and NAD(P)H-dependent IPP isomerase activity. The enzymatic properties of the *B. subtilis* enzyme were almost the same as those of the *Streptomyces* enzyme (Table 2). However, the K_m value for IPP of the *B. subtilis* enzyme was about 30-fold higher than that of the *S. aureus* enzyme. This higher value might be explained by the non-essentiality of type 2 IPP isomerase for *B. subtilis* growth. On the other hand, type 2 IPP isomerase is essential for the growth of *S. aureus*. Therefore, the K_m value of type 2 IPP isomerase of *S. aureus* could be lower than those of IPP isomerase from *Streptomyces* sp. strain CL190 and *B. subtilis*, both of which use the MEP pathway for growth.

It should be noted that two types of IPP isomerases are widespread in microorganisms.[12] To the best of our knowledge, all archaebacteria, which use the mevalonate pathway, have type 2 IPP isomerase. In eubacteria, Gram-positive cocci such as *Staphylococcus*, *Streptococcus*, and *Enterococcus*, which also use the mevalonate pathway, have type 2 enzyme. *E. coli* with the MEP pathway has type 1 IPP isomerase, as do all eukaryotes. Interestingly, *B. subtilis* and *Synechocystis* sp. strain PCC6803 using the MEP pathway have type 2 IPP isomerases, which are nonessential for their growth.

Table 1. FMN and NAD(P)H Dependence of the Activity of the *Bacillus* IPP Isomerase

Additions to reaction buffer[a]		
FMN or FAD, μM	NAD(P)H, mM	relative activity, %
FMN	NADPH	
0	0	ND
0	1	2.7
10	0	4.1
10	1	100
	NADH	
10	1	97.8
FAD	NADPH	
10	1	ND

[a] The basal reaction buffer consisted of 0.1 M HEPES (pH 7.0), 5.0 mM $MgCl_2$, 1 mM DTT, 5 μg enzyme. ND, not detected.

Table 2. Comparison of Enzymatic Properties of Type 2 IPP Isomerases

	B. subtilis	CL190*	*S. aureus**
K_m for IPP (μM)	670	450	19
V_{max} (mmol/min/mg of protein)	400	490	270
Optimum temperature (°C)	37	35	NA
Optimum pH	6.5–7.0	7.0	NA
Divalent cations	Mg^{2+}	Mg^{2+}	Mg^{2+}
Molecular mass			
SDS-PAGE (kDa)	40	42	41
Superdex-200 (kDa)	135	135	155
Multimeric form	tetramer	tetramer	tetramer

*These data were cited from reference number 7.
NA, not available.

Expression analysis with DNA microarray of *B. subtilis* has revealed that the *ypgA* gene is actually expressed in the exponential growth period (Kobayashi, K., personal communication), demonstrating that *B. subtilis* uses the *ypgA* gene to supply DMAPP from IPP. On the other hand, it has been demonstrated that *Synechocystis* IPP isomerase had no function *in vivo*.[18,19] Therefore, *B. subtilis* represents the first example of a microorganism that uses both the MEP pathway and type 2 IPP isomerase for the biosynthesis of the isoprene units, IPP and DMAPP.

It is also very interesting that two types of the enzymes showing no sequence homology catalyze the same reaction. How does nature evolve these two types of IPP isomerases? What are the roles of FMN and NADPH in the reaction catalyzed by type 2 IPP isomerase? These are the next questions to be answered.

In order to gain insight into these questions, homology searches that use the sequence of *Streptomyces* FNI as a query were done using the FASTA program and the SWISS-PROT database. Interestingly, this search identified (*S*)-2-hydroxy-acid oxidase (accession no. Q9LRR9) from *Arabidopsis thaliana* (22.1% identity in 339 amino acids overlap).[20] This enzyme is a flavoprotein and belongs to the FMN-dependent α-hydroxy acid dehydrogenases family. Multiple amino acid alignment (Fig. 2) identified two conserved regions, ^{258}GG(L/V)(R/Q) and ^{265}D(V/A)X(K/R)(A/S)(I/L)-ALGA in the *B. subtilis* FNI. These conserved regions could in part associate with FMN binding in the enzyme molecule, because both the enzymes strictly need FMN as the common cofactor. In addition, His254 in (*S*)-2-hydroxy-acid oxidase has been proposed to remove the α-proton of the substrate as the first step in catalysis (Fig. 2).[21] In analogy with this enzyme, the highly conserved His147 in *B. subtilis* IPP isomerase might be involved in the removal of 2-H of the IPP molecule (Fig. 2).

During preparation of this paper, Steinbacher *et al.* reported the crystal structure of type 2 IPP isomerase from *B. subtilis*.[22] The structural analysis revealed that the phosphate function of FMN was hydrogen bonded to the amide of Gly259 of the glycine-rich motif ^{258}GG-(L/V)(R/Q) mentioned above and to the amides of Ala280 and Gly28. Also, His147 in the crystal structure is located near the FMN molecule, suggesting that this residue could play an important role in catalysis, as discussed above. This crystal structure will help to clarify the mechanism of the unusual isomerase reaction.

Acknowledgments

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