Studies on the Biosynthesis of Fosfomycin

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Several antibiotics containing a unique C-P bond have been reported to occur in nature. These include bialaphos¹⁾, fosfomycin (FM)²⁾, FR-33289³⁾, phosphazinomycin⁴⁾, phosalacine⁵⁾ and fosfonochlorin⁶⁾. Fosfomycin [(-)-*cis*-1,2-epoxypropyl phosphonic acid], the smallest member of this class, was discovered in 1968 as a secondary metabolite of several *Streptomyces* species and later has proven to be produced by Gram-negative bacteria, *Pseudomonas syringae*⁷⁾ and *Pseudomonas viridiflava*⁸⁾. This compound, acting as a phosphoenolpyruvate analog, irreversibly inhibits phosphoenolpyruvate UDP-*N*-acetyl-glucosamine-3-*O*-enolpyruvyltransferase (enolpyruvyltransferase), an enzyme catalyzing the first step of peptideglycan biosynthesis. Since it has almost no toxicity in humans and exhibits broad antibacterial spectrum covering many pathogenic and opportunistic Gram-positive and Gram-negative bacteria, it is widely used in therapy. The structure of FM arouses considerable interest, since it has a stable epoxide and a direct carbon-phosphorus (C-P) bond; both of which are necessary for its antimicrobial activity.

We have been studying C-P bond formation mechanism of bialaphos¹⁾, a commercially important secondary metabolite produced by *Streptomyces hygroscopicus*, and have revealed that the first reaction of the biosynthesis was intramolecular rearrangement of phosphoenolpyruvate to form C-P bond of phosphonopyruvic acid (PnPy) catalyzed by phosphoenolpyruvate phosphomutase⁹⁾. Imai *et al.* reported that 2-hydroxyethylphosphonic acid and 2-aminoethylphosphonic acid, early intermediates of

bialaphos biosynthesis, were also converted to FM by a blocked mutant of *Streptomyces wedmorensis* named NP-7¹⁰⁾. This finding suggests that the initial reactions of biosynthesis of FM including C-P bond formation are identical to those of bialaphos. Here we describe the elucidation of biosynthetic pathway of FM and cloning the biosynthetic genes.

1. Biosynthetic pathway of fosfomycin

As described above, the first reaction of bialaphos biosynthesis, the C-P bond formation catalyzed by PEP phosphomutase, is supposed to be common to all C-P compounds producing organisms. PEP phosphomutase activity was also detected in a cell free extract of FM high producer *S. wedmorensis* 144-91 at a far weaker level¹¹⁾ than that of BA-producing *S. hygroscopicus*. It was thus confirmed that C-P bond of FM was formed by PEP phosphomutase reaction (Fig. 1, (1)) in a similar manner as for bialaphos although enzymatic properties or stability of *S. wedmorensis* PEP phosphomutase assumed to be different from that of *S. hygroscopicus*.

Since the equilibrium of PEP phosphomutase is far favor of the reverse reaction (cleavage of C-P bond)⁹⁾, the reaction product (PnPy) must be removed from the reaction system by the step 2 enzyme presumably catalyzing decarboxylation of phosphonopyruvic acid (Fig. 1, (2)). Nothing is, however, known about step 2 reaction mechanism because step 2 mutants have not been obtained from bialaphos producing organism. Although PEP phosphomutase gene of *S. hygroscopicus* could be expressed in mutant NP-7, it could not restore FM productiv-

Fig. 1. Biosynthetic pathway of fosfomycin. PEP=phosphoenolpyruvate, PnPy=phosphonopyruvic acid, PnAA=phosphonoacetaldehyde, AEP=2-aminoethylphosphonic acid, HEP=2-hydroxyethylphosphonic acid, HPP=2-hydroxypropylphosphonic acid, FM=fosfomycin.

ity to NP-7¹¹). As NP-7 could convert AEP and HEP to FM presumably *via* phosphonoacetaldehyde (PnAA)¹⁰, its mutational point should be step 1 or step 2 reaction. Thus, we concluded that NP-7 was defective in step 2 reaction, decarboxylation of phosphonopyruvic acid.

In order to reveal the pathway from HEP to FM, the conversion of two putative chemically synthesized intermediates *cis*-propenylphosphonic acid (PPOH) and 2-hydroxypropylphosphonic acid (HPP)¹² to FM was tested using mutant NP-7 as a converter. Although epoxidation of *cis*-propenylphosphonic acid (PPOH) seemed to be the most likely route to FM, all attempts to convert PPOH to FM using mutant NP-7 were unsuccessful. On the other hand, NP-7 produced FM by the addition of HPP. In agreement with our results, Hammerschmidt *et al.* observed the failure of the incorporation of ¹⁸O₂ into FM by *Streptomyces fradiae*^{13,14}. It was thus concluded that the epoxide ring of FM was formed by dehydrogenation of HPP not by the addition of molecular oxygen to the double bond of PPOH. This type of epoxidation has never been reported in the biosynthesis of any other natural products.

In the course of screening for FM non-

producing mutants, we found that mutant A16 defective in the biosynthesis of methylcobalamine could not produce FM¹⁵. Since mutant A16 could convert HPP to FM, it was suggested that incorporation of the methyl group into the FM molecule should occur during a stage between HEP and HPP, and that methylcobalamine was required for methylation of a putative C₂ biosynthetic intermediate, possibly phosphonoacetaldehyde (PnAA) or its analogue. To elucidate the origin of the methyl group of FM, incorporation of [methyl-¹⁴C]methylcobalamin into FM was examined. The radioactive FM was detected in the broth filtrate of mutant A16 cultivated in FM production medium supplemented with [methyl-¹⁴C]methylcobalamin¹⁴. Thus the methyl group of FM appears to be derived from methylcobalamin.

We have recently reported that methylcobalamin served as the direct methyl donor for methylation of the phosphorus atom of the phosphonic acid function in a bialaphos biosynthetic intermediate using a cell free system¹⁶. Since the carbonyl carbon of PnAA is positively charged as is the phosphorus atom in the phosphinic acid function, it is likely that PnAA is methylated by a nucleophilic attack of the

Fig. 2. The fosfomycin biosynthetic gene cluster of *S. wedmorensis*. Open reading frames are indicated by allows. Numbers under the map correspond to the fosfomycin biosynthetic steps.

methyl anion derived from methylcobalamin to generate HPP.

Fig. 1 summarizes FM biosynthetic pathway consisting four steps; C-P bond formation catalyzed by PEP phosphomutase (step 1), decarboxylation of PnPy (step 2), methylation of PnAA (step 3), epoxide formation (dehydrogenation of HPP, step 4).

2. Fosfomycin biosynthetic genes of *S. wedmorensis*.

As described above, mutant NP-7 was proven to be defective in step 2 reaction of FM biosynthesis¹¹. Thus we first cloned a 4.8 kb DNA fragment that restored FM productivity in this mutant¹⁷. A complementation assay using a bialaphos non-producing mutant (E26) deficient in PEP phosphomutase reaction revealed that the 4.8 kb fragment also contained step 1 gene¹⁷.

In order to reveal the organization of FM biosynthetic genes, we next cloned approximately 30 kb DNA fragment which contained the previously cloned 4.8 kb area. Since this fragment could restore FM productivity to NP-17 defective in step 3 conversion, it was concluded that step 3 gene localize on this region. Subcloning experiments defined 6.0 kb fragment for step 3 gene.

Since we could not isolate a step 4 mutant of

S. wedmorensis, *S. lividans* was used as the host for step 4 gene cloning. The above described 30 kb fragment containing step 1 to 3 genes was digested with *Bam*HI, ligated to pIJ702 and use to transform *S. lividans*. Then, the conversion of HPP to FM by the transformants was tested. As a result, step 4 gene (epoxidase gene) was identified on the chromosomal region next to step 3 gene.

Fig. 2 shows the FM biosynthetic gene cluster of *S. wedmorensis*. Ten ORFs were identified by the nucleotide sequencing of the 11.0 kb *Pst*II-*Bam*HI fragment¹⁸. Subcloning experiments showed that ORF-1 to -4 should code for step 1 to 4 enzymes, respectively. Both of Step 1 and step 2 enzymes, PEP phosphomutase and PnPy decarboxylase, seem to be essential for the biosynthesis of all C-P compounds. The PEP phosphomutase gene of *S. wedmorensis* showed significant homology to these of bialaphos producing *S. hygroscopicus*¹⁹ (Fig. 3, (a)) and *Tetrahymena pyriformis*²⁰ an protozoan producing 2-aminoethyl phosphonic acid suggesting conservation of this enzyme in all C-P compound-producing organisms.

We recently succeeded in cloning the step 2 gene of *S. hygroscopicus* (bialaphos producer) encoding the enzyme catalyzing decarboxylation of PnPy (PnPy decarboxylase)²¹. Approximately 17% identity was observed between

Fig. 3. Similarities of the deduced amino acid sequences of C-P bond forming enzymes. The conserved amino acids are marked by asterisks. The numbers indicate the positions of the last amino acid in each line. (a) Homology between the amino acid sequences of PEP phosphomutases, FM1: PEP phosphomutase of *S. wedmorensis*, BA1: PEP phosphomutase of *S. hygroscopicus*. (b) Homology between the amino acid sequences of PnPy decarboxylases, FM2: PnPy decarboxylase of *S. wedmorensis*, BA2: PnPy decarboxylase of *S. hygroscopicus*.

Although we could not isolate blocked mutants defective in epoxide formation, we found that the epoxidase gene could express in

Other than ORFs-1 to -4, six ORFs (-A to -F) were found in the sequenced fragment. A data-

base search revealed that the deduced products of ORF-C and ORF-F were similar to yeast alcohol dehydrogenase (28% similarity) and a putative amino acid racemase (24.8% similarity)²⁵⁾, respectively. ORFs-A, -B, -D and -E showed no significant similarity to proteins in the database. We recently noticed that ORF-A and ORF-B might play a role in determining fosfomycin resistance. Further investigation into the functions of ORF-A and ORF-B is now under way.

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