STUDIES ON THE BIOSYNTHESIS OF BIALAPHOS (SF-1293) 12.

C-P BOND FORMATION MECHANISM OF BIALAPHOS: DISCOVERY OF A *P*-METHYLATION ENZYME†

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An enzymatic activity catalyzing *P*-methylation of *N*-acetyldemethylphosphinothricin, a biosynthetic intermediate of the herbicide bialaphos, was detected in a cell extract of *Streptomyces hygroscopicus* SF-1293, a bialaphos producing organism. The gene coding for this *P*-methylation enzyme in the bialaphos biosynthetic gene cluster was also expressed in *Streptomyces lividans*. The methyl donor of the reaction was determined to be methylcobalamin. The *P*-methylation enzyme utilized both *N*-acetyldemethylbialaphos and *N*-acetyldemethylphosphinothricin as substrates.

Bialaphos (BA) is a tripeptide produced by *Streptomyces hygroscopicus* SF-1293, and is now in use as a herbicide[2]. This compound possesses a direct carbon-phosphorus-carbon bond system that is quite unique among natural products. We have previously characterized most of the biosynthetic pathway of BA consisting of at least 13 steps including three C-P bond-formation steps. One is the intramolecular rearrangement of phosphoenolpyruvic acid (PEP) in step 1 catalyzed by PEP phosphomutase[3]. Another is the intramolecular rearrangement of carboxyphosphonoenolpyruvate (CPEP) in step 5 catalyzed by CPEP phosphonomutase[4], and the last is *P*-methylation of *N*-acetyldemethylphosphinothricin (*N*-Ac DMPT) in step 12[5]. We have recently isolated PEP phosphomutase[3] and CPEP phosphonomutase[1,4,6] and determined their corresponding nucleotide sequences[7]. The biosynthetic mechanism of the C-P-C bond formation which presumably proceeds by *P*-methylation of a phosphinic acid derivative(s), however, has remained unclear.

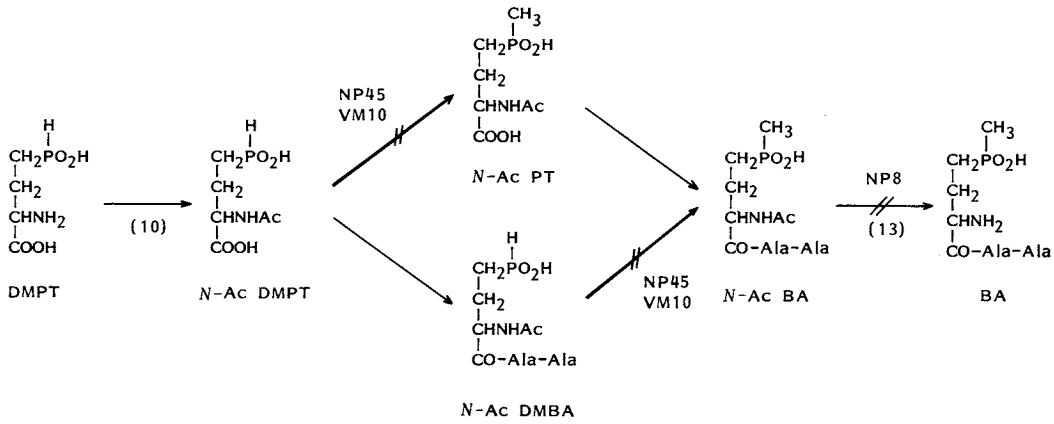
In previous reports[5,8,9], we determined the pathway from demethylphosphinothricin (DMPT) to BA as shown in Fig. 1. Mutant NP45 lacks the ability to catalyze methylation of phosphinic acid derivatives such as *N*-Ac DMPT or *N*-acetyldemethylbialaphos (*N*-Ac DMBA). The detailed reaction sequence, however, remained to be clarified. We also reported that DMPT and demethylbialaphos (DMBA) were accumulated in the fermentation broth of the parent strain cultivated in the absence of cobalt ion[5]. Thus we assumed that cobalt ion was essential for this *P*-methylation. Since this seemed to be one of the key steps in the BA biosynthesis, we have attempted to purify from *S. hygroscopicus* the enzyme catalyzing the formation of *P*-methyl derivative(s) from phosphinic acid(s).

Here we report the detection of the *P*-methylation activity in cell extracts of the BA producing organism and its expression in *Streptomyces lividans*.

† For part 11[1].

Fig. 1. Biosynthetic pathway from demethylphosphinothricin to bialaphos.

DMPT = Demethylphosphinothricin, *N*-Ac DMPT = *N*-acetyldemethylphosphinothricin, *N*-Ac PT = *N*-acetylphosphinothricin, *N*-Ac DMBA = *N*-acetyldemethylbialaphos, *N*-Ac BA = *N*-acetylbialaphos, BA = bialaphos. // Represent blocked points of mutants, NP8, NP45 and VM10. Figure in parentheses means the step in biosynthesis of BA.



Materials and Methods

Bacterial Strains

BA non-producing mutants, NP8 and NP45[8] were obtained from the Meiji Seika Kaisha Culture Collection. NP8 lacks the enzyme system to remove the *N*-acetyl group from *N*-Ac BA (step 13). NP45 is defective in the ability to catalyze methylation of phosphinic acid derivatives as described above. VM10 was prepared by a gene replacement technique developed by ANZAI *et al.*[10] and showed the same co-synthetic pattern as NP45. It was derived from a BA producer by introduction of a frame shift mutation into the step 12 gene region which is capable of restoring BA productivity to NP45. Fig. 1 shows the mutational point of VM10. *S. lividans* 66 was obtained from the John Innes Culture Collection.

DNA Manipulation and Transformation

Plasmid preparations from *S. lividans* and transformation and regeneration of *S. lividans* protoplasts were carried out as described by THOMPSON *et al.*[11].

Preparation of [*Acetyl*-1-^{14}C]*N*-Ac DMPT

[*Acetyl*-1-^{14}C]*N*-Ac DMPT was prepared by acetylating DMPT with [1-^{14}C]acetic anhydride as follows. A buffer containing 16 μl of 34% sodium hydroxide and 68 μl of 3.3 M sodium carbonate was added to an aqueous solution of DMPT (33.4 mg/0.4 ml). After a few minutes, [1-^{14}C]acetic anhydride (37 MBq) was added and the reaction mixture was allowed to stand for 30 minutes. The reaction mixture was passed through a Dowex 50 column (H^+, 1.0 × 7.0 cm) and the column was washed with 8 ml of water. The effluent was adsorbed to a DEAE-Sephadex column (Cl^-, 1.0 × 3.5 cm) which was developed with each 6 ml of water, 0.05 M NaCl and 0.5 M NaCl. The 0.5 M NaCl eluate was concentrated *in vacuo* and dissolved in 1 ml of water to give [*acetyl*-1-^{14}C]*N*-Ac DMPT (100,000 dpm/μl).

Preparation of [*Methyl*-^{14}C]methylcobalamin[12,13]

A solution of 45.6 mg of sodium borohydride in 10 ml of ethanol was added to an aqueous solution of hydroxocobalamin (162.5 mg/10 ml) and the mixture was stirred for 30 minutes under nitrogen gas. Then [^{14}C]methyl iodide (9.25 MBq, cooled in a dry ice-acetone bath) was added and the reaction mixture was stirred for 2 hours. After removal of the ethanol by evaporation, the reaction mixture was adsorbed to an Diaion HP-20 column (1.0 × 10 cm). After washing the column with 50 ml of water and 50 ml of 3%

ethanol, [*methyl*-^{14}C]methylcobalamin was eluted with 100 ml of 30% ethanol. This fraction was concentrated *in vacuo* and dissolved in water to give a solution of [*methyl*-^{14}C]methylcobalamin (150,000 dpm/μl).

Assay for *P*-Methylation Activity

BA non-producing mutants NP8, NP45 and VM10, and *S. lividans* were cultured at 27°C for 5 days in 500-ml Erlenmeyer flasks containing 60 ml of the BA production medium[8] consisting of glucose 70 (g/liter), wheat germ 39, Sungrain 25, KH_2PO_4 1, $CoCl_2 \cdot 6H_2O$ 0.001 and silicon KM-72 antifoam 0.01 (pH 6.8). The mycelium of each strain was then harvested by centrifugation and washed twice with 50 mM phosphate buffer (pH 6.5) and then suspended in 30 ml of the same buffer.

1. Assay for *P*-methylation activity in washed mycelium

Washed mycelia of mutants (100 μl) were incubated with 20 μl of [*acetyl*-1-^{14}C]*N*-Ac DMPT (100,000 dpm/μl) at 30°C for 18 hours and then a portion of the reaction mixture (30 μl) was applied to a cellulose thin layer plate (20 × 20 cm, Merck). The plate was developed with C_2H_5OH - NH_4OH - H_2O (8 : 1 : 2) in the first dimension and BuOH - AcOH - H_2O (8 : 3 : 2) in the second dimension, and spots were made visible by autoradiography. In the first dimension, Rf values were 0.67 (*N*-Ac DMPT) and 0.64 *N*-acetylphosphinothricin ((*N*-Ac PT)) and in the second dimension, they were 0.52 and 0.65. *N*-Ac DMPT and *N*-Ac PT were detected by the $FeCl_3$-sulfosalicylic acid color reaction.

2. Assay for *P*-methylation activity in cell free extracts

Washed mycelia of mutants were sonicated for 10 minutes at 4°C with a 600 W ultrasonic disintegrator (2N-100; Toyo Rika Co.). Unbroken cells and cell debris were removed by centrifugation (15,000 × *g*, 25 minutes). Reaction mixtures containing 50 μl of cell extract and 2 μl of [*acetyl*-1-^{14}C]*N*-Ac DMPT were incubated at 30°C for 3 hours and then analyzed in the same way as for the assay 1. For quantitative determination, the spot of [*acetyl*-1-^{14}C]*N*-Ac PT was counted by a liquid scintillation counter.

For preparation of low-molecular-weight fraction, cell extract of NP8 was passed through a Sephadex G-25 PD-10 column (Pharmacia) equilibrated with 50 mM phosphate buffer (pH 6.5).

Results

Detection of *P*-Methylation Activity in the Washed Mycelium of NP8 Strain

The activity of the *P*-methylation enzyme was demonstrated by the following experiments. Use of NP8, which lacked the enzyme system to remove the *N*-acetyl group from *N*-Ac BA or *N*-Ac PT[8], as the enzyme source for the reaction enable to employ [*acetyl*-1-^{14}C]*N*-Ac DMPT as the substrate. The conversion of *N*-Ac DMPT to *N*-Ac PT by washed mycelium of NP8 was followed by biological assay against *Bacillus subtilis*, TLC analysis, and ^{31}P NMR measurements. *P*-Methylation activity was, however, too weak to be detected by these methods. Therefore, a radioactive substrate, [*acetyl*-1-^{14}C]*N*-Ac DMPT was utilized. As shown in Fig. 2 and Table 1, washed mycelium of NP8 converted 0.53% of [*acetyl*-1-^{14}C]*N*-Ac DMPT to [*acetyl*-1-^{14}C]*N*-Ac PT upon incubation in phosphate buffer at 30°C for 18 hours.

Having detected the *P*-methylation activity in washed mycelium, this reaction was carried out using a cell-free system. [*Acetyl*-1-^{14}C]*N*-Ac DMPT was added to the cell extract of NP8, and the formation of [*acetyl*-1-^{14}C]*N*-Ac PT was measured as described in Materials and Methods. As shown in Table 1, the *P*-methylation activity was also detected in the cell free system. Cell extract of NP8 showed, however, only 22.6% of the activity of the washed mycelium. Thus, expression of this enzyme activity in *S. lividans* was attempted by using high expression vectors.

Expression of the *P*-Methylation Enzyme in *S. lividans*

Fig. 3 shows BA biosynthetic gene cluster cloned by MURAKAMI *et al.*[14] They also identified the DNA fragment which restored BA productivity to NP45 (Fig. 3, step 12 gene). Based on these results, plasmid

Fig. 2. Cellulose TLC autoradiograph of the reaction product formed from [*acetyl*-1-^{14}C]*N*-Ac DMPT by the washed mycelia of blocked mutants, NP8 and VM10.

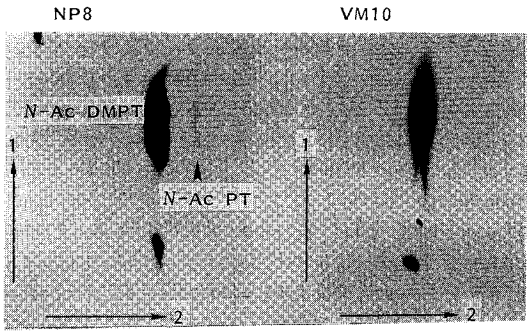
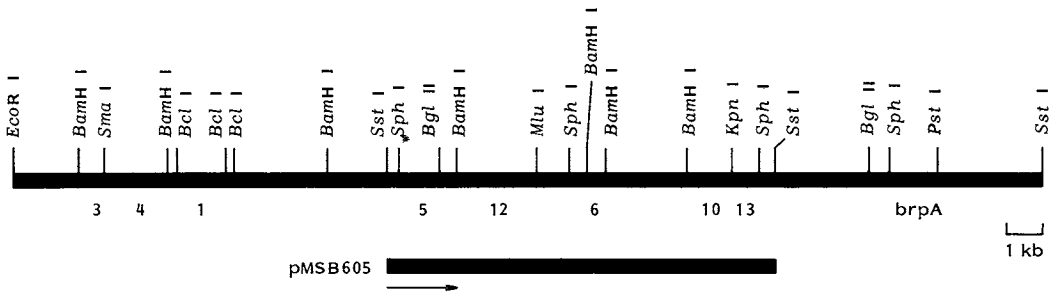


Table 1. *P*-Methylation activity of blocked mutants of *Streptomyces hygroscopicus* and *Streptomyces lividans* harboring pAK114 and pMSB605.

Mutant	Incorporation of radioactivity into *N*-Ac DMPT (%)	
	Washed mycelium	Cell extract
NP8	0.53	0.12
NP8 (heat treated)	0.05	0.05
NP45	0.06	0.05
VM10	0.06	0.04
S. lividans (pAK114)	—	0.04
S. lividans (pMSB605)	—	0.26

Fig. 3. The bialaphos biosynthetic gene cluster of *Streptomyces hygroscopicus*.

Numbers below the map show the bialaphos biosynthetic steps. The arrow adjacent pMSB605 indicates the direction of vector promoter with respect to the cloned fragment.



pMSB605 was constructed by insertion of a 9.8 kb *Sst*I DNA fragment including step 12 gene into the *Sst*I site of pAK114 followed by introduction into *S. lividans*. pKA114 is an expression vector constructed by MURAKAMI *et al.*[15] with a *tip* A promoter that was induced by thiostrepton in *S. lividans*. *S. lividans* (pMSB605) was cultured in the BA production medium and the cell extract was prepared in the same manner as described for *S. hygroscopicus*. A reaction mixture containing 30 μl of cell extract, 2 μl of [*acetyl*-1-^{14}C]*N*-Ac DMPT and 18 μl of low-molecular-weight fraction of NP8 cell extract (as a methyl-group donor) was incubated at 30°C for 18 hours and then the formation of *N*-Ac PT was detected by autoradiography. As shown in Table 1, *P*-methylation activity of *S. lividans* (pMSB605) is twice as strong as that of NP8.

Determination of the Methyl-group Donor in *P*-Methylation Reaction

In order to determine the methyl-group donor of this *P*-methylation, various plausible methyl-group donors, *S*-adenosyl-L-methionine, L-methionine, betaine, 5-methyl-5,6,7,8-tetrahydrofolic acid and methylcobalamin were separately added to the reaction system of *S. lividans* (pMSB605) in place of the low-molecular-weight fraction of NP8. Relatively strong *P*-methylation activity was detected when methylcobalamin was added to the reaction mixture (Table 2). Thus, the methyl donor for the *P*-methylation reaction was suggested to be methylcobalamin. In order to confirm these results, [*methyl*-^{14}C]methylcobalamin was prepared and incubated with cell extract of *S. lividans* (pMSB605) and *N*-Ac

Table 2. Effects of methyl donor on *P*-methylation activity of cell extract of *Streptomyces lividans* (pMSB605)*.

Methyl donor	Relative activity (%)
None	50**
Low-molecular-weight fraction of NP8	100
Methylcobalamin	272
SAM	40
Methionine	35
Betaine	34
5-Methyl-H_4-folate	40

* Reaction mixtures containing 50 μl of cell extract, 2 μl of [*acetyl*-1-^{14}C]*N*-Ac DMPT, various methyl donors (2 mM, final concentration) were incubated at 30°C for 3 hours and the *P*-methylation activities were measured as described in Materials and Methods.

** This activity is presumably due to endogenous methylcobalamin of *S. lividans* (pMSB605).

SAM = *S*-adenosyl-L-methionine, 5-methyl-H_4-folate = 5-methyl-5,6,7,8-tetrahydrofolic acid.

Fig. 4. Cellulose TLC autoradiograph of the reaction products formed from *N*-Ac DMPT and [*methyl*-^{14}C]methylcobalamin by the cell extract of *Streptomyces lividans* (pMSB605) (A) and by the same extract with heat treated (B).

A reaction mixture contained 50 μl of cell extract, 2 μl of 10 mM *N*-Ac DMPT and 1 μl of [*methyl*-^{14}C]methylcobalamin. After incubation at 37°C for 3 hours, the reaction mixture was applied to a cellulose thin-layer plate and developed as described in Materials and Methods.

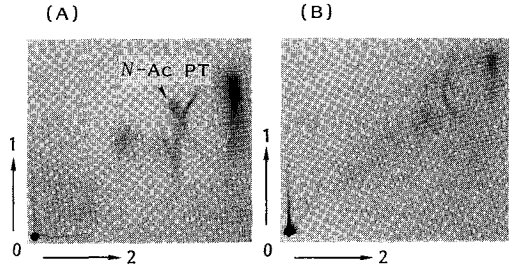


Table 3. Substrate specificity of *P*-methylation enzyme.

Substrate	Relative activity (%)*
DMPT	0
N-Ac DMPT	100
DMBA	0
N-Ac DMBA	105

* Details of the procedures are given in the text.

DMPT. As shown in Fig. 4, radioactive *N*-Ac PT was detected by autoradiography. We thus confirmed that the methyl donor of the *P*-methylation was methylcobalamin.

Substrate Specificity of *P*-Methylation Enzyme

Plausible substrates for the *P*-methylation reaction, such as DMPT, *N*-Ac DMPT, DMBA and *N*-Ac DMBA (10 mM, final concentration) were separately added to the reaction mixtures containing 50 μl of cell-free extracts of *S. lividans* (pMSB605) and 1 μl of [*methyl*-1-^{14}C]methylcobalamin. Table 3 shows that acetylated compounds, *N*-Ac DMPT and *N*-Ac DMBA, were methylated by cell extract of *S. lividans* (pMSB605). On the other hand, no enzymatic reaction was detected with DMPT or DMBA. These results indicate that the *N*-acetyl group is an essential structural requirement for the substrates of *P*-methylation.

Discussion

In this study, we have demonstrated for the first time that the *P*-methylation enzyme formed the most characteristic structural feature of BA, *i.e.*, C-P-C bond, from methylcobalamin and phosphinic acid derivatives. In contrast to well known and ubiquitous enzymes catalyzing *N*-, *O*-, *C*- and *S*-methylations, *P*-methylation enzyme has never been reported so far. We previously isolated two C-P bond forming enzymes, PEP phosphomutase[3] and CPEP phosphonomutase[4] involved in the BA biosynthetic pathway. Both enzymes catalyze intramolecular rearrangements of phosphate esters to form a C-P bond and their properties are quite different from those of the *P*-methylation enzyme. Although its important role in the biosynthesis of BA warrants further detailed studies, low methylation activities of cell free systems prepared from NP8 and the parent strain hamper its purification for the moment.

Introduction of the *P*-methylation gene into *S. lividans* using pAK114, the expression vector with a thiostrepton induced promoter, resulted in only a 2-fold improvement of the *P*-methylation activity as compared to that of *S. hygroscopicus*. However, success of the expression of *P*-methylation activity has

enabled us to identify the methyl-group donor and acceptors of this reaction.

S-Adenosyl-L-methionine is well known as a methyl donor for most methyl transfer reactions. The *P*-methylation enzyme, however, did not utilize *S*-adenosyl-L-[*methyl*-^{14}C]methionine to form *N*-Ac PT or *N*-Ac BA (data not shown). On the other hand, [*methyl*-^{14}C]methylcobalamin was a good methyl donor for formation of *N*-Ac PT and *N*-Ac BA in the cell free system of *S. lividans* (pMSB605). Thus, the methyl donor of the *P*-methylation was determined to be methylcobalamin. The requirement of cobalt ion for the production of *P*-methylated compounds such as *N*-Ac PT, *N*-Ac BA and BA[4] is rationalized by the essential role of the metal for the biosynthesis of methylcobalamin. OKAMURA *et al.* reported that [*methyl*-^{3}H]methylcobalamin showed high incorporation into fortimicin A indicating that the methyl group of methylcobalamin was directly incorporated into fortimicins[16]. TESTA *et al.* also reported the involvement of cobalt dependent methylation for gentamicin fermentation[17]. However, detailed mechanisms of these cobalamin mediated methylations remain unclear. In this paper, we prove that the methyl group of methylcobalamin is directly incorporated into the *P*-methyl group of *N*-Ac PT or *N*-Ac BA. Since the phosphorus atom in the phosphinic acid function is linked directly to negatively charged oxygens, it is most unlikely that *P*-methylation takes place *via* electrophilic attack of methyl cation to the phosphorus as in the case of usual methylation reactions. Therefore, involvement of methylcobalamin, which can generate methyl anions, in *P*-methylation seems to be quit reasonable.

The *P*-methylation enzyme recognized only *N*-acetylated derivatives such as *N*-Ac DMPT and *N*-Ac DMBA. Thus, the protection of the amino group of the substrate amino acids is concluded to be essential for *P*-methylation to take place. Consequently, the sequence from *N*-Ac DMPT to *N*-Ac BA was determined as shown in Fig. 1. Presently it can not be concluded which one of the two routes, *i.e.*, *P*-methylation before alanylalanylation (upper route), and alanylalanylation before *P*-methylation (lower route) is working in the biosynthesis of BA.

Acknowledgments

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