STUDIES ON THE BIOSYNTHESIS OF BIALAPHOS (SF-1293) 9. BIOCHEMICAL MECHANISM OF C-P BOND FORMATION IN BIALAPHOS: DISCOVERY OF PHOSPHOENOLPYRUVATE PHOSPHOMUTASE WHICH CATALYZES THE FORMATION OF PHOSPHONOPYRUVATE FROM PHOSPHOENOLPYRUVATE†

Sir:

In recent years, several antibiotics and physiologically active substances containing a unique C-P bond have been isolated, mainly as microbial metabolites. These include bialaphos (BA)[1], fosfomycin[2], FR-33289[3], plumbemycin[4], fosfazinomycin[5], and K-26[6] (Fig. 1). Biosynthetic studies of the first three compounds[7~9] using labeled precursors indicated that their C-P bonds were formed by intramolecular rearrangement of phosphoenolpyruvate (PEP) to phosphonopyruvate (Pnpy) *via* the same mechanism[10] as proposed for the formation of 2-aminoethylphosphonic acid (Fig. 2).

Since the discovery of the first natural C-P compound[11], the biosynthetic mechanism of the C-P bond formation has attracted considerable interest in the past two decades; all attempts to purify the enzyme catalyzing the formation of Pnpy from PEP, however, have been unsuccessful and confirmatory evidence in support of the widely accepted intramolecular rearrangement mechanism has not been available.

During biosynthetic studies of BA[12], a tripeptide herbicide possessing the unique C-P-C bond which is produced by *Streptomyces hygroscopicus* SF-1293[1], we proved *inter alia* by obtaining appropriate blocked mutants[13~15] and by cloning the relevant biosynthetic genes[16] that the biosynthetic pathway of BA consisted of at least 13 steps, including the C-P bond formation step which presumably proceeded *via* isomerization of PEP to Pnpy (Fig. 2, Step 1). Since the investigation of this intriguing step seemed to be very important for improving the production yield of BA, we have attempted to purify from *S. hygroscopicus* the enzyme PEP phosphomutase catalyzing the rearrangement of PEP to a C-P compound (presumably Pnpy).

In this communication, we describe the discovery in *S. hygroscopicus* of the enzyme PEP phosphomutase which catalyzes the conversion of PEP to Pnpy. Since there existed no appropriate methods for detecting the formation of the C-P bond, we first employed an ADP/pyruvate kinase-NADH/lactate dehydrogenase coupled spectrophotometric system[17] (pH 7.5, 30°C with Pnpy as the substrate) as a convenient assay method for this enzyme. This system depends on the quantitative assay of PEP which would be formed from Pnpy by the reverse reaction.

During purification of PEP phosphomutase, we surprisingly found that the enzyme showed very strong ability to catalyze the reverse reaction and that the equilibrium between PEP and Pnpy was in favor of the formation of the phosphate ester (in the ratio of more than 100 : 1)††. This result was quite exceptional to us, since it has been generally accepted that the C-P bond is always more stable than the C-O-P bond[19]. Thus, at the initial stage of our enzyme

Fig. 2. Proposed biosynthetic pathway of AEP from PEP.

PEP: Phosphoenolpyruvate, Pnpy: phosphonopyruvate, AEP: 2-aminoethylphosphonic acid.

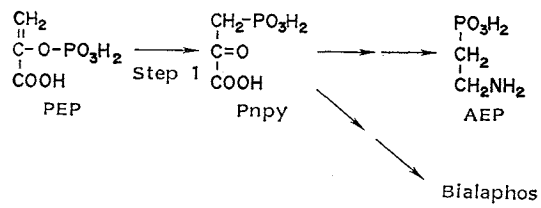
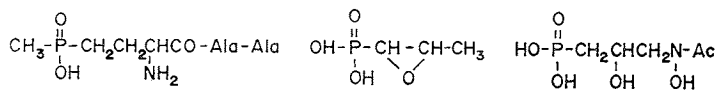


Fig. 1. The structures of bialaphos and related C-P compounds.



† For part 8[12].

†† The same result has been recently reported by DUNAWAY-MARIANO *et al.*[18].

purification, all attempts to catalyze the forward reaction were unsuccessful†. As a consequence, one question which occurred to us was whether the enzyme we were trying to purify was the true enzyme involved in the biosynthesis of BA.

In order to answer this question, we isolated two blocked mutants defective in Step 1 (Fig. 2). One of them, NP47 was obtained by the conventional NTG treatment[13] and the other, E26 was prepared by the gene replacement technique developed by ANZAI *et al.*[20] (unpublished data). This technique permitted the introduction of an *in vitro* derived specific frame-shift mutation in the Step 1 gene.

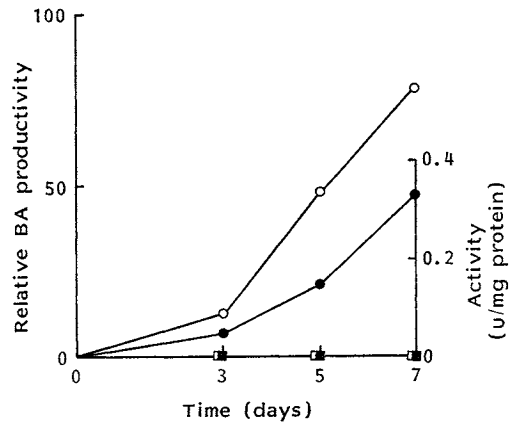
Both BA non-producing mutants, NP47 and E26, which could transform Pnpy to BA, lacked the ability not only to form the C-P bond but also to catalyze the formation of PEP from Pnpy, suggesting a strictly positive correlation between these two reactions. In addition, the PEP phosphomutase activity of the producing strains was proportional to their BA productivity as shown in Fig. 3. Thus we concluded that PEP phosphomutase catalyzes the first C-P bond formation in the BA biosynthetic pathway.

In addition to the method describe above, we employed an additional method to follow the formation of Pnpy from PEP using NADH/malate dehydrogenase (MDH). Since the equilibrium of the PEP phosphomutase reaction lies extremely towards PEP, the reaction product supposed to be Pnpy must be removed from the reaction system to catalyze the forward reaction. Thus, ^{14}C-labeled PEP was used as the substrate and the ^{14}C-labeled 3-phosphonolactic acid formed from Pnpy by the action of NADH/MDH was detected by autoradiography of TLC plates. The product from the NADH/MDH reaction with Pnpy as the substrate has been identified as 3-phosphonolactate by ^{1}H NMR.

The purification of the enzyme from *S. hygroscopicus* is summarized in Table 1. Due to the extreme instability of PEP phosphomutase, we have not yet succeeded in obtaining the enzyme in a pure form. The partially purified enzyme had a specific activity of 15.5 U/mg protein for the reverse reaction and catalyzed the formation of 3-phosphonopyruvate from PEP in the presence of NADH/MDH (Fig. 4, detected as phosphonolactic acid). These results clearly showed that the C-P bond formation and the reverse reaction are catalyzed by the same enzyme. C-P bond formation from PEP by cell free extracts of *Tetrahymena pyriformis* was also reported by TAKADA and HORIGUCHI[21] recently.

In order to confirm absolutely the positive

Fig. 3. Time course of bialaphos (BA) production and changes in PEP phosphomutase activity of a high producing strain HP5-29 and a blocked mutant E26.



High producing strain HP5-29 (○, ●), *in vitro* derived mutant E26 (□, ■), BA productivity (open symbols), specific activity (closed symbols) of PEP phosphomutase of cell free extract measured by ADP/pyruvate kinase-NADH/lactate dehydrogenase.

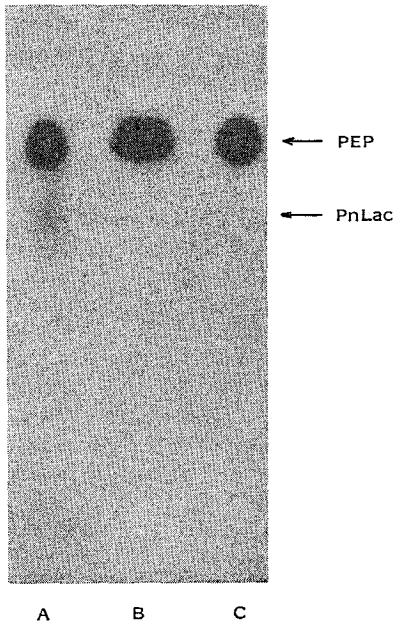
Table 1. Purification of PEP phosphomutase.

Step	Total protein (mg)	Total U	Specific activity (U/mg protein)
1. Crude extract	2,068	650	0.31
2. DEAE-cellulose	124	300	2.42
3. Hydroxylapatite	32	142	4.47
4. FPLC (Mono Q)	1.8	28	15.5

1 U=1 μmol substrate/minute.

† This is probably the reason why all attempts to isolate PEP phosphomutase by several groups in the past were not successful.

Fig. 4. Cellulose TLC autoradiograph of the product from reaction of ^{14}C-labeled PEP with partially purified PEP phosphomutase and NADH/malate dehydrogenase (MDH).



A complete incubation mixture contained 50 mM MES (pH 6.5), 0.3 mM NADH, 10 mM [1-^{14}C]PEP (5 μCi/mmol), 10 U of MDH and partially purified PEP phosphomutase (active fraction of FPLC, 2 U for the reversed reaction). (A) Complete (PnLac represents phosphonolactic acid), (B) PEP phosphomutase, (C) NADH/MDH. After incubation at 30°C for 2 hours, the samples were applied to a cellulose thin-layer plate and developed with BuOH - CH_3COOH - H_2O (2 : 1 : 1).

relationship between the reverse reaction and C-P bond formation in general, we checked the enzymatic ability to catalyze the reverse reaction with cell free extracts of *Streptomyces wedmorensis*, *Streptomyces rubellomurinus*, *Streptomyces plumbeus*, and actinomycetes K-26 (NRRL 12379) which produce fosfomycin, FR-33289, plumbemycin and K-26, respectively, by employing the ADP/pyruvate kinase-NADH/lactate dehydrogenase coupled spectrophotometric system. Among these, only the FR-33289 producing organism showed the expected activity.

The failure to dectect the reverse reaction, in particular, with the fosfomycin-producing organism, which had been improved as a very high fosfomycin-producing organism (*S. wedmorensis* 209-97, unpublished data), and which therefore should possess a strong activity to catalyze the C-P bond formation, may imply that the enzyme ability to catalyze the reverse reaction and C-P bond formation are not always in parallel relationship. In this regard, it may be necessary to establish a system which catalyzes the forward reaction (possibly by decarboxylation or transamination) of the PEP phosphomutase isolated from *Tetrahymena* very recently by collaboration between the Harvard group and ourselves[22].

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