Hydrogen maleate (**3**) and hydrogen phthalate (**4**) anions are the paradigms of a symmetric hydrogen bond.[1] Yet previous studies[4] were carried out in crystal or in nonpolar solvents. This is the first study of hydrogen-bond structure in aqueous solution. The internal hydrogen bond of **3** and **4** is still present even in aqueous solution, as judged from the large difference between first and second pK_as.[7] We suggest that the asymmetry that we find is caused by the disordered aqueous environment,[8] and this possibility is currently under investigation.

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Biosynthetic Mechanism of C–P Bond Formation. Isolation of Carboxyphosphonoenolpyruvate and Its Conversion to Phosphinopyruvate

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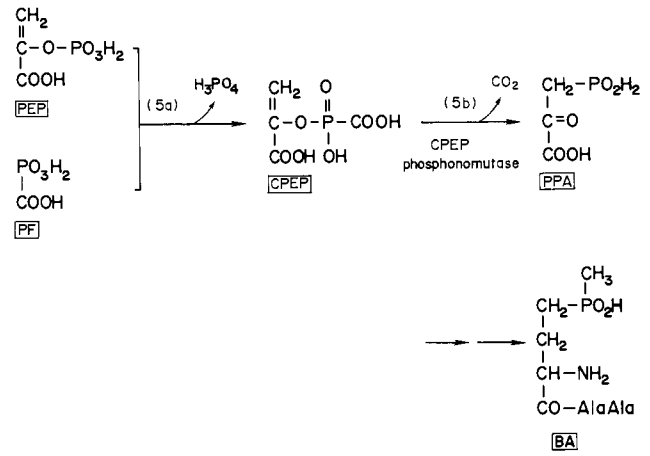
Since the first discovery of natural phosphonate, 2-aminoethylphosphonic acid,[1,2] the very unique C–P bond formation mechanism has attracted considerable interests in the past three decades; all the attempts, however, were so far unsuccessful to verify the enzymatic formation of the plausible intermediate phosphonopyruvate from phosphoenolpyruvate (PEP).

Recently Seidel,[3] Bowman,[4] Takada,[5] and our group[6] have reported the purification of PEP phosphomutase from *Tetrahymena* or from *Streptomyces hygroscopicus*. Although this enzyme is verified to be responsible for the C–P bond formation, only the reverse reaction, i.e., transformation of phosphonopyruvate to PEP, has been shown to occur due to the enzymatic reaction equilibrium which is far in favor of the formation of PEP.[3,4,6] We report herein another example of the C–P bond formation, i.e., the transformation of a newly isolated compound, carboxyphosphonoenolpyruvate (CPEP) to phosphinopyruvate, catalyzed by a new enzyme carboxyphosphonoenolpyruvate (CPEP) phosphonomutase.[7]

During the biosynthetic studies of a tripeptide herbicide bialaphos (BA),[8] which is produced by *Streptomyces hygroscopicus* SF-1293, we have shown that the first natural phosphinate, phosphinopyruvate (PPA),[9] was formed by condensation between

Scheme I. The Proposed Mechanism of C–P Bond Formation (Step 5) in Bialaphos Biosynthesis[a]



[a] AlaAla = alanylalanine.

PEP and phosphonoformate (PF)[10] as illustrated in Scheme I. Involvement of the latter substrate in this reaction named step 5 was clarified by its accumulation in a deficient mutant NP213 obtained by NTG treatment.[10] Consideration of this reaction mechanism strongly suggested the involvement of a hitherto unknown biosynthetic intermediate with a carboxylated phosphonate ester (CPEP in Scheme I). This plausible mechanism, however, remained unanswered due to the failure to obtain any blocked mutants, which will accumulate the suggested intermediate, by conventional methods.

Very recently we have succeeded in preparation of a desirable new deficient mutant NP71[7] by the use of a new gene replacement technique, in vitro derived mutation.[11] Although this organism could not catalyze the step 5 reaction, it was complementary to NP213. Cosynthesis experiments between these two mutants suggested the accumulation of an unknown intermediate of BA biosynthesis in NP213 and the presence of a new enzyme catalyzing C–P bond formation in the cells of NP71. This intermediate was exhausted upon starvation.

The new enzyme named CPEP phosphonomutase was purified by conventional methods about 200-fold in four steps to give a homologous protein by SDS-PAGE to be a monomer of 32000.[7] With the purified enzyme in hand, it became possible to isolate the substrate of CPEP phosphonomutase from the fermentation broth of the mutant NP213. CPEP was purified from the broth filtrate[12] (1.2 L) by treatments with active carbon, Dowex 1 (Cl^-), Diaion HP-20, DEAE Sephadex A-25 (Cl^-), and finally by Sephadex G-10 column chromatography to give a pure sample (1 mg). CPEP was obtained as white amorphous powder [mp 220 °C>; δ_P −1.65 ppm, IR (KBr) 1585, 1411 cm^{-1}. Anal. ($C_4H_2O_7PNa_3 \cdot 2H_2O$) H, Na; C calcd, 16.12; found 16.73, P calcd, 10.39; found, 9.90]. Its ^1H NMR spectrum (D_2O) showed only two broad singlets at 5.20 and 5.54 ppm and was very similar to that of PEP. The structural similarity between CPEP and PEP was also confirmed by the ^{13}C NMR signals of CPEP at 171.1 (C-1, COOH), 149.7 (C-2, O—C=), and 104.2 ppm (C-3, CH_2=). However, an additional resonance was observed at 177.4 ppm (C-4, P-COOH, doublet, J_{C-P} = 240.6 Hz). Thus, CPEP is determined to be a P-carboxylated derivative of PEP (Scheme I). The proposed structure was supported by the long range

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(12) Fractions under purification were incubated at 27 °C overnight with starved mycelium of NP213 and CPEP phosphonomutase, and then the amount of BA produced was determined by antimicrobial activity against *Bacillus subtilis*.

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couplings observed between the phosphorus and C-3 ($^3J_{C-P}$ = 3.7 Hz) and C-2 ($^2J_{C-P}$ = 9.5 Hz).

Incubation of CPEP with CPEP phosphonomutase[13] resulted in the formation of PPA which was identified by comparison with an authentic sample. The reaction was also followed by ^{31}P NMR which showed appearance and increase of only one signal assignable to the phosphorus in phosphinopyruvate (δ_P 19.1) with the accompanied decrease of the signal due to CPEP.[14] Thus CPEP phosphonomutase is considered to catalyze an intramolecular rearrangement of CPEP to form the C-P bond of phosphinopyruvate (step 5b in Scheme I). Unlike the reaction catalyzed by PEP mutase, the concomitant decarboxylation probably drives the accumulation of the C-P compound. On the basis of these experimental results, the step 5 proved to consist of two reactions 5a (transesterification) and 5b (rearrangement and decarboxylation), and the blocked sites of NP71 and NP213 turned out to be (5a) and (5b) in Scheme I, respectively.

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(13) The reaction mixture (10 mL) containing 50 mM MES buffer (pH 6.5), 2 mM $MnCl_2$, 10 mM CPEP, and 10 units of CPEP phosphonomutase was incubated at 30 °C for 3 h. The product was purified by DEAE Sephadex A-25 and Sephadex G-10 column chromatographies.

(14) The reaction was carried out as described in ref 13, and the reaction mixture was analyzed after ultrafiltration by ^{31}P NMR after 1, 2, and 3 h of incubation.

Biosynthetic Site-Specific Incorporation of a Non-Natural Amino Acid into a Polypeptide

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Despite the enormous significance of site-specific mutagenesis in protein research, this technique suffers from the major limitation that substitutions are confined to the 20 primary amino acids normally present in proteins. As a result, the introduction of chemically unique residues into proteins is not possible with this approach. In spite of the development of strategies for circumventing this limitation,[1] a truly general method of site-specific protein modification would almost certainly require intervention during translation, in order to avoid the innate chemical selectivity problems associated with posttranslational modification. In this paper, a potential means of incorporating non-natural amino acids at specified sites during protein biosynthesis is described.

Biosynthetic incorporation of a non-natural amino acid has been achieved during normal translation with an appropriate "misacylated tRNA",[2] but that procedure results in the insertion of the amino acid at multiple sites—in competition with the cognate amino acid—because both the acylated tRNA and the wild-type tRNA compete for the same codon. The same would be true for all other codons except the three termination codons (UAG, UGA, and UAA), for which there are no corresponding tRNAs. Because these codons normally terminate translation, point mutations that insert any of them at an inappropriate site in a vital gene lead to nonfunctional products and cellular death.[3] In these instances, "read-through"—random insertion of an amino acid—cannot effectively compete with termination, and truncated protein is the major translated product. However, in some mutants, suppressor tRNAs have arisen that specifically recognize the misplaced termination codon (i.e., a *nonsense suppression site*) and thereby increase the amount of functional protein that is produced. This theme has evolved even further in an *Escherichia coli* strain that actually produces a UGA suppressor tRNA that incorporates a non-natural amino acid, selenocysteine, into formate dehydrogenase during translation.[4] Development of an analogous synthetic version of this theme could be accomplished by construction of a "chemically misacylated" nonsense suppressor tRNA employing methods developed by the Hecht group.[5] The addition of this acylated tRNA to a translation system containing a gene with a nonsense suppression site would result in the incorporation of the non-natural amino acid at the corresponding site in the protein. This strategy has recently been reported by the Schultz group, who employed a chemically misacylated yeast nonsense suppressor tRNA, prepared by anticodon loop replacement, to incorporate several phenylalanine analogues into β-lactamase.[6]

We have independently assessed a similar strategy to incorporate the non-natural amino acid iodotyrosine into a polypeptide. A relatively simple target, a 16-residue polypeptide, was initially chosen in order to quantitatively and unambiguously assess suppression efficiency, read-through, and site-specificity for this process. Accordingly, we prepared an acylated tRNA ^{125}I-Tyr-tRNA$^{Gly}_{CUA}$-dCA[7] without base hypermodifications[8] to avoid the need for laborious isolation from a cellular source as previously performed[5,6] and with it have demonstrated high suppression efficiency with no detectable read-through, as well as unequivocal site-specificity of incorporation of the non-natural amino acid [^{125}I]-tyrosine. The construction of ^{125}I-Tyr-tRNA$^{Gly}_{CUA}$-dCA was accomplished by (1) chemical synthesis of a 2′(3′)-O-acylated dinucleotide,[9] (2) run-off transcription of tRNA$^{Gly}_{CUA}$-C$_{OH}$,[10] and (3) enzymatic coupling of these fragments. Acylation of the protected dinucleotide with tyrosine[11] was achieved in high yield,

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