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Overexpression, Purification, and Use of Phosphoenol Pyruvate Synthetase in the Synthesis of PEP Analogues

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The *Escherichia coli* enzyme phosphoenol pyruvate synthetase has been overexpressed and purified in a single chromatographic step. The enzyme catalyzes the synthesis of phosphoenol pyruvate (PEP), from adenosine triphosphate and pyruvate, and has enabled the synthesis of uniformly labeled [1,2,3-$^{13}C_3$]PEP, which is a key molecule in structural and mechanistic studies of enolpyruvyl transferases. Fluoropyruvate was also used as substrate for the enzyme and gave only (*Z*)-phoephoenol-3-fluoropyruvate, albeit at a slower rate.

INTRODUCTION

Phosphoenol pyruvate (PEP) is used throughout biological systems as a building block for larger carbon units. It is an intermediate of the glycolysis pathway, used in two steps of the shikimate pathway and an integral unit in the biosynthesis of the bacterial cell wall. Our research has focused on PEP utilizing enzymes over the past decade (*1–3*), and NMR experiments using labeled substrates have contributed insight into their mechanisms. The enzyme, 5-enolpyruvyl-shikimate-3-phosphate (EPSP) synthase, catalyzes the condensation of shikimate-3-phosphate and PEP in the shikimate pathway and has been subject of intense research after the discovery that it is the primary site of action of the broad-spectrum postemergence herbicide glyphosate (GLP). The mechanism of EPSP synthase is widely accepted as a nucleophilic attack of the 5-OH of shikimate-3-phosphate (S3P) at C-2 of PEP, giving rise to an unusually stable enzyme intermediate (*4*). We were the first to show the existence of this enzyme-bound tetrahedral intermediate by ^{13}C NMR spectroscopy under equilibrium (*1*) and pre-steady-state conditions (*3*, *5*). [1,2,3-$^{13}C_3$]PEP would further facilitate our understanding of EPSP synthase through its use in the determination of the stereochemistry of the tetrahedral intermediate, and measurement of the dihedral angle between the carboxylate and the C=C double bond of enzyme-bound PEP. [1,2,3-$^{13}C_3$]PEP is not commercially available and if made by custom synthesis would probably cost significantly more than [2-^{13}C]PEP, which retails for \$17,000 g^{-1} (Cambridge Isotopes Laboratories Inc. 1997–1998).

PEP synthesis from *E. coli* was reported in 1965 (*6*). A highly purified PEP synthetase from *E. coli* was first described and characterized shortly thereafter

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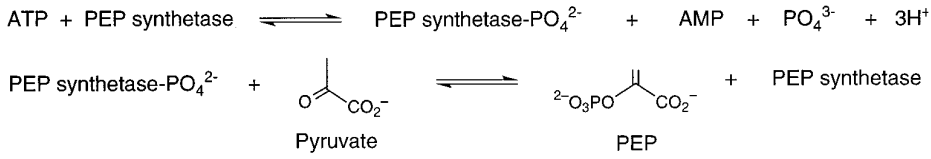

SCHEME 1. The reaction catalyzed by PEP synthetase.

(*7–10*). Scheme **1** shows the chemical reaction catalyzed by PEP synthetase which reversibly converts pyruvate to PEP. Through radiolabeling studies, it was deduced that the enzyme proceeds via a two-step mechanism (*9*). More recently, the *ppsA* gene was cloned into a pBR322-derived plasmid and its amino acid sequence compared to orthophosphate dikinases and pyruvate phosphate dikinase (PPDK) (*11*), which catalyze similar reactions. Elegant research on PPDK (*12*, *13*) has deduced a swiveling-domain mechanism whereby His-455 transfers the phosphate group between substrates which are bound to the enzyme in different domains. PEP synthetase has five regions that show a high degree of sequence similarity with PPDK (the overall sequence identity is 21% (*11*)), and it also contains a phosphorylated histidine (His-421) located within the second highly similar region. The mechanism for PEP synthetase has been suggested (*9*) as two steps (Scheme **1**), and given its sequence similarity to PPDK it is tempting to speculate that the enzyme mechanisms are similar.

While the mechanism of PEP synthetase is not the subject of this paper, the cloning and overexpression reported here will open the door to further mechanistic studies, in addition to enabling the facile synthesis of PEP analogs.

MATERIALS AND METHODS

Bacterial strains and plasmids. pBCP58 (an ampicillin (amp) resistant pBR322-derived plasmid containing the *E. coli ppsA* gene) was a generous gift from P. J. Postma, University of Amsterdam (*11*). pCR-Script Cam SK(+) cloning kit was supplied by Stratagene (La Jolla, CA). The overexpression system was pET 24b purchased from Novagen (Madison, WI). Standard microbial techniques were used throughout, bacteria were grown aerobically at 37°C in LB liquid media (300 rpm) or on LB agar plates. Antibiotics were added to media at the following concentrations: amp, 100 μg mL^{-1}; kanamycin (kan), 50 μg mL^{-1}; and chloroamphenicol (cam), 30 μg mL^{-1}.

Chemicals and enzymes. All chemicals were of reagent quality. [1,2,3-$^{13}C_3$]pyruvate was obtained from Cambridge Isotope Labs (Andover, MA). pCR primers were purchased from Gibco BRL (Gaithersburg, MD). *Pfu* DNA polymerase was purchased from Stratagene. Restriction endonucleases were purchased from New England Biolabs (Beverly, MA). All enzyme manipulations were carried out at 4°C except for PEP synthesis using PEPs which was carried out at 20°C.

DNA manipulation. The pBCP58 was isolated using a Wizard plus minipreps DNA purification system (Promega, WI) from overnight cultures in LB and used as a template for *ppsA* amplification. A Perkin Elmer Cetus DNA thermocycler was used for the PCR with 30 cycles of 95°C (45 s), 59°C (45 s), and 72°C (7 min). The reaction was run using cloned *Pfu* polymerase and followed Stratagene directions (*Pfu polymerase* instruction manual) with primer 1 (25 mer, 5′-CATATGTCCAACAATGGCTCGTCAG-3′, a *Nde*I cleavage site coupled onto the 5′-end of *ppsA*) and primer 2 (26 mer, 5′-AAGCTTATTTCTTCAGTTCAGCCAGG-3′, a *Hind*III cleavage site coupled onto the 3′-end of *ppsA*). The cloned DNA was purified by selective precipitation and ligated into the blunt-ended *Srf* I site in the pCR-Script Cam SK(+) cloning vector and transformed into *E. coli* XL-1-blue MRF′ Kan supercompetent cells to give plasmid pDJ096. pET 24b and pDJ096 were prepared for ligation by cutting with *Hind*III and *Nde*I and purified from low-melting point agarose (1%). T4 DNA ligase was used for an ingel ligation at 20°C overnight (plasmid pDJ102) which was subsequently transformed into $CaCl_2$-competent BL21 λDE3 plysE cells.

Protein expression and purification. E. coli BL21 λDE3 plysE pDJ102 is an expression system under control of the T7 RNA polymerase gene on λDE3 under the control of the *lac*UV5 promoter, which is inducible with isopropyl-1-thio-β-D-glactopyranoside (IPTG). Cells were grown to a cell density of $OD_{600} \approx 0.7$, when expression of *ppsA* was induced by addition of IPTG to a final concentration of 0.2 mM. After 4 h induction cells were harvested by centrifugation (11,000*g*) and frozen at −78°C. Typical growths gave 10 g cells (wet wt) which were lysed using the lysozyme/DNase I method (*14*) with a lysis buffer of Tris–HCl (5 mM, pH 7.4). Ammonium sulfate was added to a concentration of 242 g L^{-1} (40%) (*7*) and the resulting precipitate removed by centrifugation (11,000*g*), a further ammonium sulfate aliquot (62 g L^{-1}, 50%) was added to the supernatant, and protein precipitate collected by centrifugation. The precipitate (5 g wet wt) was dissolved in Tris–HCl (50 mM, pH 6.8), DTT (1 mM), NaN_3 (1.5 mM) and run down a gel filtration column (Pharmacia Biotech analytical Superose 12 HR 26/30) at 0.3 mL min^{-1} in five batches. The fractions eluting at 75–90 mL were combined and concentrated to give protein (300 mg) as determined by the method of Bradford (*15*), with bovine serum albumin (BSA) as standard and was stored at −78°C.

Enzymatic synthesis of PEP and [*1,2,3-*$^{13}C_3$]*PEP*. Adenosine triphosphate (ATP, 280 mg, 510 μM), pyruvate (58 mg, 527 μM), and $MgCl_2$ (500 μL of 1 M, 500 μM) were dissolved in Tris–HCl (5 mL, 50 mM) and adjusted to pH 8.4. PEP synthetase (400 μL, 7 mg mL^{-1}) was added and the pH kept above 7.5 by addition of NaOH (1 M). The reaction mixture was passed through a 30-kDa molecular weight cutoff membrane and loaded onto a FPLC (Pharmacia) MonoQ 16/10 column. Elution with a nonlinear gradient of ammonium bicarbonate (0–0.3 M, pH 9.0) gave PEP (81 mg, 47%, at 0.2 M). Spectroscopic data were identical to commercial PEP. [1,2,3-$^{13}C_3$]PEP was prepared via an identical experiment except that [1,2,3-$^{13}C_3$]pyruvate was used as substrate. δ_C (D_2O) 103.6 (d, $^1J_{CC}$ 80.1 Hz, C3), 152.4 (ddd, $^2J_{CP}$ 7.8 Hz, C2), 174.8 (d, $^1J_{CC}$ 76.7 Hz, C3).

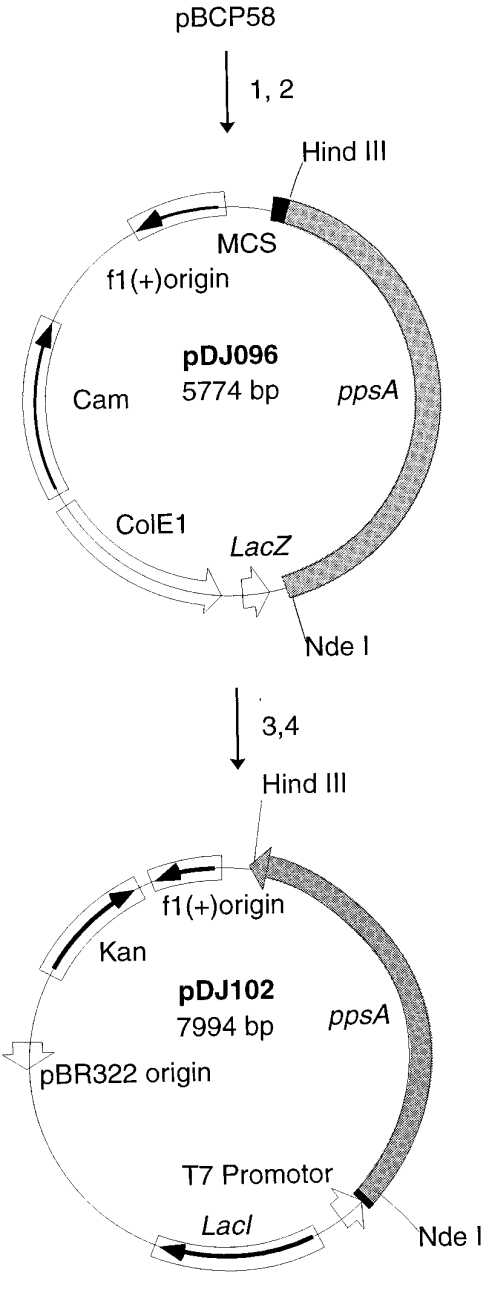

FIG. 1. Synthesis of overexpression plasmid pDJ102. (1) PCR amplify *ppsA* gene with *Pfu* DNA polymerase using primer 1 and primer 2. (2) Blunt end ligate PCR product into pCR-Script CAM SK(+) cloning vector *Srf*I site. The *ppsA* is shown in one orientation (pDJ096); blunt end ligation may result in the opposite orientation. (3) Cut out *ppsA* with *Nde*I and *Hind*III. (4) Ligate *ppsA* into precut pET 24b and transform into BL21. MCS, multiple cloning site. Arrows indicate direction of transcription.