Analysis of Fluoromethyl Group Chirality Establishes a Common Stereochemical Course for the Enolpyruvyl Transfers Catalyzed by EPSP Synthase and UDP-GlcNAc Enolpyruvyl Transferase†

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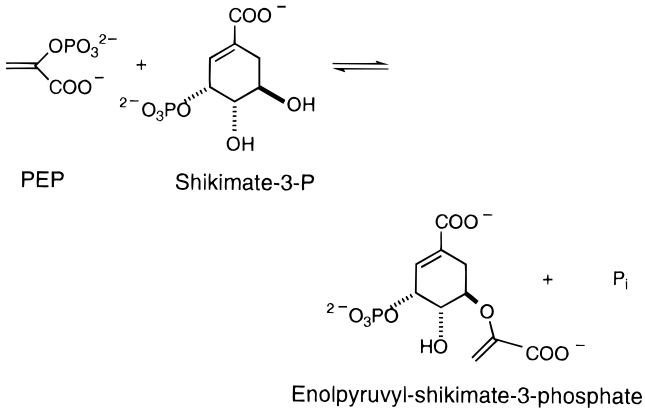
ABSTRACT: The stereochemistry of transient methyl group formation at C-3 of phosphoenolpyruvate (PEP) in the reaction catalyzed by 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase has been examined using the pseudosubstrates, (*E*)- and (*Z*)-3-fluorophosphoenolpyruvate (FPEP). Kinetically stable, chiral [^{1}H,^{2}H]fluoromethyl analogs of the reaction tetrahedral intermediate were isolated and subjected to decomposition and stereochemical analysis. EPSP synthase was found to catalyze the 2-*re* face addition of solvent-derived hydrogen to C-3 of FPEP (corresponding to the 2-*si* face of PEP). Comparison of these data with our prior analogous work on the MurA reaction [Kim, D. H., Lees, W. J., & Walsh, C. T. (1995) *J. Am. Chem. Soc. 117,* 6380−6381] suggests that the two enolpyruvyl transferases share a common stereochemical course, further strengthening the mechanistic, structural, and evolutionary relationship between the two enzymes.

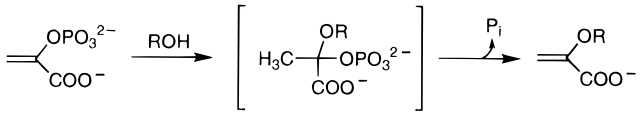
The shikimic acid pathway is the common biosynthetic route for plant and microbial biosynthesis of aromatic amino acids and a diverse array of primary and secondary aromatic metabolites (Haslam, 1993). The absence of the pathway from mammals has focused attention on the shikimic acid pathway as a target for herbicide or antimicrobial action, particularly since the discovery that the enzyme catalyzing the sixth step in the pathway, 5-enolpyruvylshikimate 3-phosphate (EPSP)[1] synthase, is the molecular target of glyphosate (Steinrücken & Amrhein, 1980, 1984; Comai et al., 1983), the active ingredient of the herbicide Roundup.

EPSP synthase catalyzes the transfer of an enolpyruvyl moiety from phosphoenolpyruvate (PEP) to the 5-OH of shikimate 3-phosphate (S3P), an unusual reaction of PEP that involves its reactivity as a C-2 electrophile with subsequent cleavage of the C−O bond of PEP (Scheme 1). The bacterial peptidoglycan biosynthetic enzyme UDP-GlcNAc enolpyruvyl transferase (MurA), targeted by the antibiotic fosfomycin (Kahan et al., 1974), is the only other enzyme known to catalyze an analogous enol ether formation from PEP. The reaction pathways of both EPSP synthase (Anderson & Johnson, 1990) and MurA (Marquardt et al., 1993; Brown et al., 1994; Kim et al., 1995a) have been established to proceed by an addition−elimination mechanism (Bondinell et al., 1971) through a tetrahedral intermediate with an sp^3 methyl group reversibly formed and decomposed at the enzyme active site (Scheme 2).

Scheme 1: Reaction Catalyzed by EPSP Synthase



Scheme 2: Addition−Elimination Mechanism for Enzymatic Enolpyruvyl Transfer[a]



[a] Legend: ROH = S3P for EPSP synthase; ROH = UDP-GlcNAc for MurA.

Stereochemical analysis of the EPSP product generated from stereospecifically isotope-substituted PEP has shown that the stereochemistry about the double bond of PEP is retained in the enol ether product, indicating that addition and elimination steps proceed with opposite stereochemistry (Grimshaw et al., 1984; Lee et al., 1984; Asano et al., 1985). That is, if addition to the double bond is *syn*, then elimination is *anti*, or the converse (*anti*/*syn*). Recently, investigation of the MurA reaction using (*E*)-phosphoenolbutyrate (Lees & Walsh, 1995) has also established that the addition and

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[1] Abbreviations: EPSP, 5-enolpyruvylshikimate 3-phosphate; S3P, shikimate 3-phosphate; PEP, phosphoenolpyruvate; FPEP, 3-fluorophosphoenolpyruvate; ATP, adenosine triphosphate; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-(2-ethanesulfonic acid); Tris, tris(hydroxymethyl)aminomethane; TEAB, triethylammonium bicarbonate; NADH, nicotinamide adenine dinucleotide (reduced form); HPLC, high-performance liquid chromatography; ^{1}H−^{1}H DQF-COSY, ^{1}H−^{1}H double quantum filtered correlation nuclear magnetic resonance spectroscopy.

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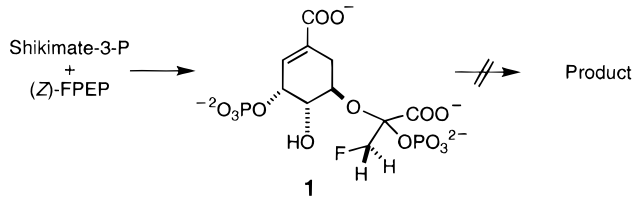
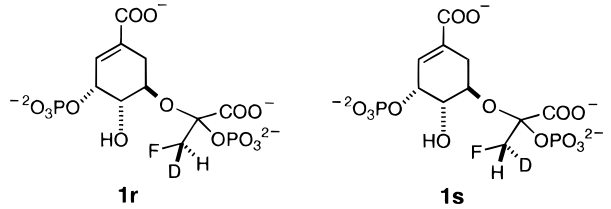
Scheme 3: Inactivation of EPSP Synthase by (*Z*)-FPEP



Scheme 4: Chiral Fluoromethyl Tetrahedral Adducts Formed at the Active Site of EPSP Synthase from (*E*)- and (*Z*)-FPEP in D_2O



elimination steps proceed *syn*/*anti* or *anti*/*syn*.

The fluorinated PEP analogs, (*E*)- and (*Z*)-3-fluorophosphoenolpyruvate (FPEP) (Stubbe & Kenyon, 1972), have been particularly useful in studies of the enolpyruvyl transfers catalyzed by EPSP synthase and MurA. Walker et al. (1992) reported that (*Z*)-FPEP, but not the (*E*)-isomer, was a pseudosubstrate for EPSP synthase, leading to the accumulation of a stable fluoromethyl analog of the tetrahedral intermediate, **1** (see Scheme 3), at the active site of the enzyme. We have observed similar pseudosubstrate behavior with both (*E*)- and (*Z*)-FPEP in the MurA reaction and have carried out a detailed kinetic analysis of the reaction pathway of MurA using FPEP (Kim et al., 1994, 1995a).

Although studies that characterize the stereochemistry of the enol ether product provide information on the overall stereochemical course of enzymatic enolpyruvyl transfer, the elucidation of the absolute stereochemistry of the enzyme-catalyzed reaction requires analysis of the transiently-formed tetrahedral intermediate. We have recently utilized both the kinetic stability and the chirality of the fluoromethyl group of the tetrahedral intermediate analog formed from (*E*)- and (*Z*)-FPEP at the active site of MurA when the reaction is performed in D_2O (resulting in formation of a [^{1}H, ^{2}H]-fluoromethyl group) to evaluate the stereochemistry of addition at C-3 of FPEP in the MurA reaction (Kim et al., 1995b).

In the present paper we extend this approach to the study of the stereochemical course of the EPSP synthase reaction, using both (*E*)- and (*Z*)-FPEP as pseudosubstrates of EPSP synthase in D_2O to generate chiral fluoromethyl analogs of **1**, **1r**, and **1s** (Scheme 4), followed by stereochemical analysis of the compounds. The results are compared to those obtained previously for MurA, and the implications on the mechanism and evolutionary relationship of the two enolpyruvyl transferases are discussed.

EXPERIMENTAL SECTION

Subcloning, Sequencing, Overexpression, and Purification of EPSP Synthase. The *Escherichia coli aroA* gene (Duncan et al., 1984) was amplified from a single colony of *E. coli* strain XA90 using the polymerase chain reaction (PCR), Vent polymerase (New England Biolabs), and the oligonucleotide primers DK1B (5′-GGTCTAGACCCATGGAATCCCTGACGTTACAAC-3′) and DK2 (5′-GCGGGATCCTCAGGCTGCCTGGCTAATCC-3′), subcloned into the *Nco*I-*Bam*HI site of the pET-15b expression vector (Novagen), and sequenced. The nucleotide sequences of the PCR products derived from *E. coli* strain XA90 and two additional *E. coli* K-12 strains, BW13711 (kindly provided by Prof. B. L. Wanner, Purdue University) and ZK126 (W3110 derivative provided by Prof. R. Kolter, Harvard Medical School), were identical (ruling out the possibility of mutations due to PCR) and revealed two single nucleotide differences at nucleotide positions 68 and 989 when compared to the previously published *E. coli* sequence (Duncan et al., 1984). A G-for-C difference at position 68 results in a Thr-to-Ser change at amino acid position 23, whereas the G-for-C difference at position 989 results in an Arg-to-Thr at residue 330. Alignment comparison to fourteen other *aroA* sequences (Marquardt, 1993) shows that all other sequences other than the *E. coli* sequence have Ser at residue 23, and while there is no consensus at residue 330, the majority of bacterial sequences have Thr at position 330. The two differences in sequence may reflect an error in the original sequence or genetic variation in strain. Following standard procedures (Novagen), overexpression of the pET-15b-aroA plasmid (pAROA.7) in *E. coli* strain BL21(DE3) gave a yield of EPSP synthase of greater than 20% total cell protein in crude extracts as assessed by SDS−PAGE. EPSP synthase was purified to homogeneity by a sequence of ammonium sulfate precipitation, G-25 Sephadex, Fast Flow Q-Sepharose and butyl Sepharose chromatography (Pharmacia), with a yield of 80 mg of pure EPSP synthase per liter of culture.

Synthesis of Shikimate 3-Phosphate (S3P). The *E. coli aroL* gene encoding shikimate kinase II (Millar et al., 1986) was amplified from a single colony of *E. coli* strain XA90 using PCR, Vent polymerase, and the oligonucleotide primers DK3 (5′-GGTCTAGACATATGACACAACCTCTTTTTCTGATC-3′) and DK4 (5′-GCGGGATCCTCAACAATTGATCGTCTGTGCC-3′). The resulting PCR product was subcloned into the *Nde*I-*Bam*HI site of pET22b vector (Novagen), and the pET22b-aroL plasmid (pAROL.1) was expressed in *E. coli* strain BL21(DE3) following standard procedures (Novagen). This resulted in ∼200-fold overexpression [as assessed by measurement of specific activity in crude extracts containing the overexpressed protein and comparison of this activity to previously published values (Millar et al., 1986)]. S3P was synthesized using shikimate kinase II from crude extracts, shikimic acid (Sigma), and ATP (Sigma) in the following manner: HEPES (50 mM, pH 7.0), KCl (50 mM), $MgCl_2$ (5 mM), ATP (10 mM), and shikimic acid (10 mM, Sigma) were combined in 200 mL, and the pH was adjusted to 7.0 by the addition of KOH. Six units of shikimate kinase II was added to the reaction, which was incubated for 18 h at 25 °C. S3P was purified by anion-exchange chromatography (Bio-Rad AG1-X8) using a triethylammonium bicarbonate (TEAB), pH 8.0, gradient (0.0−1.0 M). S3P was observed to elute at approximately 0.6 M TEAB.

Isolation and Characterization of Fluoromethyl Tetrahedral Adducts. (*E*)-FPEP [88% (*E*), 12% (*Z*)] was the generous gift of Prof. Timor Baasov (Department of Chemistry, Technion−Israel Institute of Technology). (*Z*)-FPEP [90% (*Z*), 5% (*E*), 5% PEP] was the generous gift of Prof. Ronald Somerville (Department of Biochemistry, Purdue University). **1** was prepared by incubation of S3P (1 mM), (*Z*)-FPEP (0.5 mM), and EPSP synthase (0.13 mM) in

Tris·HCl buffer (50 mM, pH 7.8) in a total volume of 15 mL for 20 min, followed by quenching of the reaction in 0.2 N KOH and filtration through a 10 kDa molecular weight cutoff filter (Centriplus-10, Amicon). **1** was purified from the filtrate by anion-exchange HPLC (Mono Q 5/5, Pharmacia) using a triethylammonium bicarbonate (TEAB) (pH 8.0) gradient (flow rate, 2 mL/min; initial, 0.0 M; 1−6 min, 0.0−0.7 M TEAB; 6−16 min, 0.7 M TEAB; **1** eluted at approximately 12 min), by detecting the absorbance at 230 nm. The fraction containing **1** (∼0.5 μmol) was exchanged into D_2O by repeated vacuum evaporation.

The FHDC− tetrahedral adducts, **1r** and **1s**, were prepared from (*E*)- and (*Z*)-FPEP, respectively, by incubation, in D_2O, of S3P (1 mM), (*E*)- or (*Z*)-FPEP (0.5 mM), and EPSP synthase (0.2 mM) (exchanged into deuterated buffer by dialysis) in Tris·DCl/D_2O buffer (20 mM, pD 7.8) in a total volume of 5 mL. Isolation and purification were performed as described above for **1**. Decomposition of **1r** and **1s** to enantiomers of 3-[^{2}H]-3-fluoropyruvate was performed by incubation of **1r** and **1s** (∼0.3 μmol) with 120 units of alkaline phosphatase (calf intestine, Boehringer-Mannheim) in Tris·HCl (50 mM, pH 8.0) and EDTA (0.1 mM) in a volume of 100 μL for 20 min at 25 °C. 3-Fluoropyruvate formation was followed by incubation of an aliquot of the reaction with lactate dehydrogenase (pig heart, Boehringer-Mannheim) and NADH. The alkaline phosphatase reaction was filtered through a 30 kDa molecular weight cutoff filter (Microcon-30, Amicon), and the filtrate was added to a reaction mixture consisting of pyruvate carboxylase (5 units, Sigma), malate dehydrogenase (60 units, Boehringer Mannheim), acetyl-CoA (0.1 mM), NADH (2 mM), ATP (5 mM), $KHCO_3$ (20 mM), $MgCl_2$ (10 mM), and Tris·HCl (pH 8.0, 50 mM), in a total volume of 1 mL for 3 h at 25 °C. 3-Fluoropyruvate consumption was followed by incubation of an aliquot of the reaction with lactate dehydrogenase and NADH. The reaction mixture was filtered through a 30 kDa filter, and 0.1 mL D_2O was added for ^{19}F-NMR measurements.

NMR Data Collection and Processing. 1D ^{1}H-NMR spectra were collected on a Varian VXR500 NMR spectrometer operating at 500 MHz and 25 °C. Sample was not spun. ^{19}F-NMR spectra were collected on a Bruker AM400 operating at 376 MHz and 25 °C and were referenced to an external standard of 1% trifluoroacetic acid (δ −76.53 ppm). The ^{1}H−^{1}H Double quantum filtered (DQF) COSY experiment was run on a Varian VXR 500 NMR spectrometer operating at 500 MHz and 25 °C. 2048 complex data points were collected in the directly detected dimension. 512 complex experiments were collected in the indirect dimension using the States/TPPI method for quadrature detection (Marion et al., 1989). The data was transferred to a Silicon Graphics workstation and processed using Felix 2.30 (Biosym). Time domain data in each dimension were apodized with a skewed sine bell function, and the data zero filled to give a 2048 × 1024 point real matrix at the end of processing. The t_1 noise ridges were reduced using the algorithm of Manoleras and Norton (1992).

RESULTS AND DISCUSSION

Our strategy for the determination of stereochemistry of addition at C-3 of FPEP in the EPSP synthase reaction consisted of two steps: (1) isolation of **1r** and **1s** from reactions of EPSP synthase with (*E*)- and (*Z*)-FPEP, and (2) stereochemical analysis using the method described previously (Kim et al., 1995b), involving decomposition of **1r** and **1s** to (2*R*,3*R*)-3-fluoromalates in three enzymatic steps. The analysis of fluoromethyl group chirality (Goldstein et al., 1978; Rétey et al., 1980; Walsh, 1982; Hoving et al., 1985) represents an alternative to the classic methods of chiral methyl group analysis (Cornforth et al., 1969; Lüthy et al., 1969) that is particularly suited to the present problem because of the stoichiometric accumulation of the fluoromethyl analog, **1**, at the active site of EPSP synthase, in contrast to the transient formation and catalytic decomposition of the methyl group of the native tetrahedral intermediate.

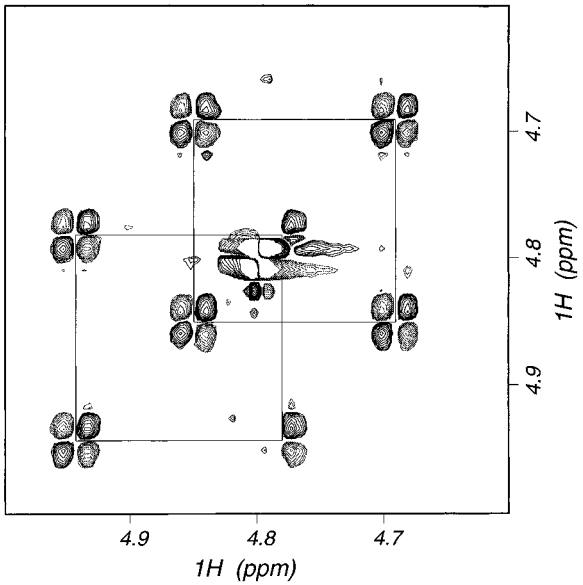


FIGURE 1: ^{1}H−^{1}H DQF-COSY spectrum of **1**. Note the repeated cross-peak pattern (highlighted by the boxes along the diagonal) representing the passive H−F coupling. Spectrometer conditions and data processing are as described in the Experimental Section.

Figure 1 shows the fluoromethyl group hydrogen region of the ^{1}H−^{1}H DQF-COSY spectrum of **1** formed at the active site of EPSP synthase from S3P and (*Z*)-FPEP in H_2O. Because this spectral region also includes overlapping resonances from H-3 of the shikimate ring (δ 5.0 ppm) and the off-scale resonance due to HOD (δ 4.8 ppm), the COSY spectrum provides a clearer representation of the resonances arising from the fluoromethyl group when compared to the 1D ^{1}H spectrum of Figure 2 (top spectrum). The resonances due to the diastereotopic hydrogens of the fluoromethyl group of **1** are separated by 0.15 ppm, and the spectrum in Figure 1 clearly shows the coupling of the diastereotopic hydrogens to each other (as indicated by the cross-peaks; J_{H-H} = 9.2 Hz) and the passive coupling of each hydrogen to fluorine (J_{F-H} = 46 Hz).

Figure 2 shows the 1D ^{1}H-NMR spectrum of **1** (top spectrum) compared to the corresponding spectra of the fluoro tetrahedral adducts, **1r** and **1s**, formed from the reaction of EPSP synthase and S3P with (*E*)- and (*Z*)-FPEP in D_2O (Figure 2, middle and bottom spectra, respectively). Comparison of the spectra of Figure 2 demonstrates that reaction in D_2O results in the formation of a monodeuterated fluoromethyl group, as evidenced by the disappearance of the signal corresponding to one of the two diastereotopic hydrogens due to deuterium substitution (as well as a loss

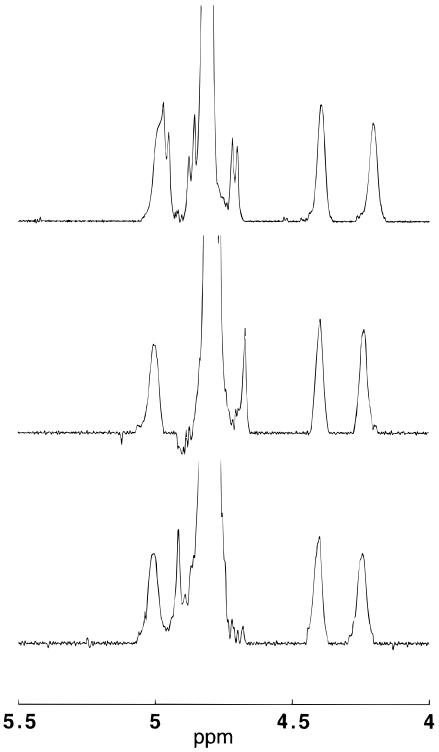


FIGURE 2: Stereospecific addition to both (*E*)- and (*Z*)-FPEP by EPSP synthase. 1H-NMR spectra of **1** (top, **1r** (middle), and **1s** (bottom). The off-scale resonance at 4.8 ppm is due to HOD peak. The resonances at 5.0, 4.4, and 4.2 ppm were assigned to H-3, H-5, and H-4 of the shikimate ring, respectively.

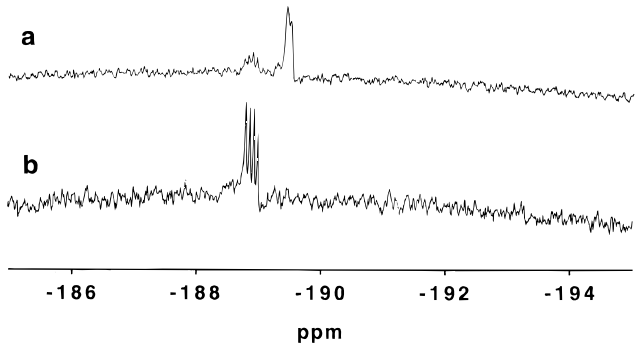


FIGURE 3: ^{19}F-NMR spectra of (2*R*,3*R*)-3-fluoromalates resulting from stereochemical analysis of **1r** (spectrum a) and **1s** (spectrum b).

of the H−H coupling in the resonance of the remaining hydrogen). The complementarity of the spectra of Figure 2, middle and bottom, establishes that addition at C-3 of (*E*)- or (*Z*)-FPEP proceeds stereospecifically. Furthermore, these spectra establish that the (*E*)-isomer of FPEP is also a pseudosubstrate for EPSP synthase, as has been found to be the case with MurA (Kim et al., 1994). Although Walker et al. (1992) previously reported formation of **1** from (*Z*)-FPEP, with no evidence for binding of the (*E*)-FPEP, the availability of a purified (*E*)-FPEP preparation allowed us to investigate the behavior of the (*E*)-isomer in the present study.

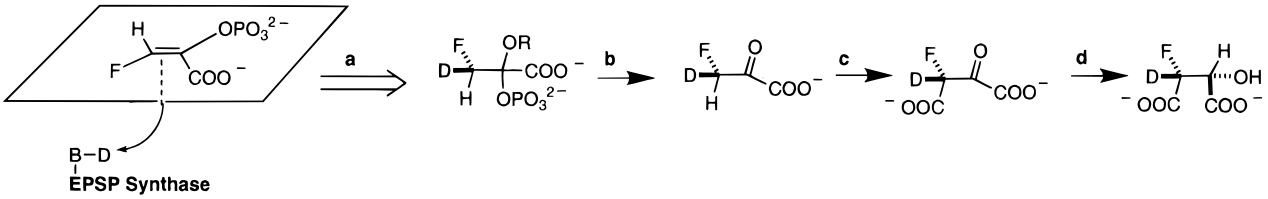
Assignment of the chirality of the fluoromethyl groups of **1r** and **1s** was performed by enzymatic decomposition and stereospecific conversion of these adducts using alkaline phosphatase/pyruvate carboxylase/malate dehydrogenase as described previously (Kim et al., 1995b). Figure 3 shows the ^{19}F-NMR spectra of the (2*R*,3*R*)-3-fluoromalates from **1r** and **1s**, respectively. Spectra corresponding to (2*R*,3*R*)-3-[^{2}H]-3-fluoromalate (spectrum a) and (2*R*,3*R*)-3-[^{1}H]-3-fluoromalate are distinguished on the basis of chemical shift (deuterium causes an upfield shift of 0.5 ppm) and the absence or presence of coupling to H-3 ($J_{F-H(3)} = 49$ Hz; $J_{F-D(3)} = 7$ Hz, $I = 1$; note that the other splitting is due to $J_{F-H(2)} = 25$ Hz) (Keck et al., 1980; Hoving et al., 1985; Kim et al., 1995b). The presence of 12% (*Z*)-isomer in the (*E*)-FPEP preparation and possible slow exchange from 3-fluoropyruvate (Goldstein et al., 1978) during the course of stereochemical analysis contribute to the small amount of (2*R*,3*R*)-3-[^{1}H]-3-fluoromalate present in spectrum a. As depicted in Scheme 5 for (*E*)-FPEP, the formation of 3-[^{2}H]-3-fluoromalate from **1r** [formed from (*E*)-FPEP] and the complementary formation of 3-[^{1}H]-3-fluoromalate from **1s** [formed from (*Z*)-FPEP] allowed for the assignment of the chirality of the fluoromethyl group of **1r** as *R* and **1s** as *S* and furthermore demonstrated that addition of D^+ to C-3 of FPEP proceeds from the 2-*re* face (Scheme 4). Extension of this result to the normal physiological reaction with PEP suggests that addition to C-3 proceeds from the 2-*si* face (note that the presence of the fluorine substituent results in an inversion of the *re*/*si* designation).

We have previously found that MurA also catalyzes the 2-*si* face addition at C-3 of PEP (Kim et al., 1995b). The assignment of fluoromethyl group chirality in the tetrahedral adducts formed from EPSP synthase and MurA permits the assignment of ^{1}H chemical shifts of the prochiral hydrogens of the FH_2C- group in each adduct. A comparison of the chemical shifts for the resonances arising from the diastereotopic hydrogens of the fluoromethyl group for each tetrahedral adduct is presented in Table 1. In each case, the pro-*R* hydrogen is shifted downfield of the pro-*S* hydrogen, strongly suggesting that the absolute configuration at C-2 of each adduct is the same—that is, the stereochemistry of addition at C-2 of PEP proceeds from the same face for the two enzymes. However, the absolute stereochemistry at C-2 of PEP has not been established for either enolpyruvyl transferase and remains to be determined in order to complete the stereochemical description of the enzymatic reactions.

Because of the same constraint on the relative stereochemistry of the addition and elimination steps (*syn*/*anti* or *anti*/*syn*) (Grimshaw et al., 1984; Lee et al., 1984; Lees & Walsh, 1995) and the determination of 2-*si* face addition at C-3 of PEP for both EPSP synthase and MurA, the implication of the same stereochemistry of addition at C-2 of PEP for both enzymes is that the enzymes share a common stereochemical course, following the same one of two possible routes in Scheme 6. Thus, the analysis of fluoromethyl chirality, while undertaken to elucidate the stereochemistry of addition at C-3 of PEP unambiguously, has also provided indirect evidence with respect to the stereochemistry at C-2 of PEP.

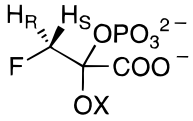
Homology modeling, based on the crystal structure of EPSP synthase (Stallings et al., 1991) and the ~20% sequence identity between EPSP synthase and MurA (Wanke et al., 1992; Marquardt et al., 1992), suggests that the two enzymes have a similarity in domain structure and overall fold (Marquardt, 1993). The commonality in stereochemical course further strengthens the mechanistic, structural and evolutionary relationship between the two enolpyruvyl transferases. Although enolpyruvyl transfer represents an unusual class of PEP reaction, 2-*si* face addition at C-3 of PEP is in

Scheme 5: Method for the Determination of Stereochemistry of Addition at C-3 of FPEP [(*Z*)-Isomer Shown][a]



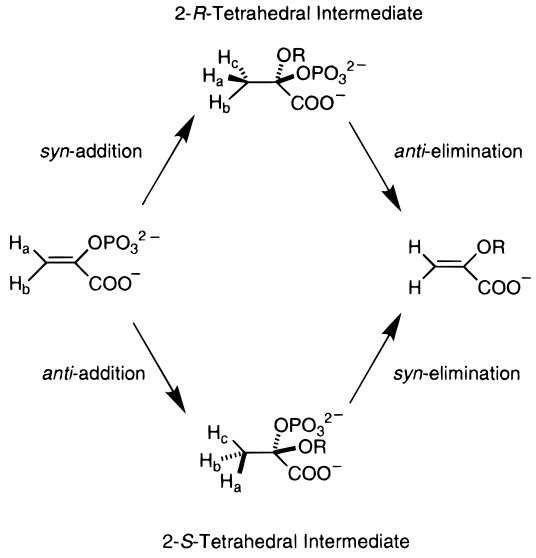
[a] Legend: (a) Formation of **1r** and **1s** by EPSP synthase from (*E*)- and (*Z*)-FPEP and S3P. (b) Decomposition to enantiomers of 3-fluoropyruvate using alkaline phosphatase. (c, d) Stereospecific conversion to either (2*R*,3*R*)-3-[^{2}H]-3-fluoromalate [as shown, from (*Z*)-FPEP] or (2*R*,3*R*)-3-[^{1}H]-3-fluoromalate [from (*E*)-FPEP], using pyruvate carboxylase and malate dehydrogenase.

Table 1: Comparison of ^{1}H-NMR Chemical Shifts for the Prochiral Hydrogens of the Fluoromethyl Group of the Respective Tetrahedral Adducts from EPSP Synthase (This Work) and MurA (Kim et al., 1995b)



	^{1}H-δ (ppm)	
X	pro-*R*	pro-*S*
shikimate 3-P	4.89	4.74
UDP-GlcNAc	4.90	4.70

Scheme 6: Two Possible Routes for the Stereochemical Course of EPSP Synthase and MurA



agreement with nearly all PEP-utilizing enzymes,[2] posing interesting evolutionary questions regarding a common PEP-binding motif (Rose, 1972). Resolution of these questions awaits further structural characterization of PEP-utilizing enzymes.

ACKNOWLEDGMENT

We thank Prof. Timor Baasov (Technion—Israel Institute of Technology) and Prof. Ronald Somerville (Department of Biochemistry, Purdue University) for their gifts of (*E*)- and (*Z*)-FPEP, respectively.

[2] PEP-utilizing enzymes for which the 2-*si* face of addition at C-3 of PEP has been determined: pyruvate kinase (Rose, 1970), enzymes catalyzing PEP carboxylation (Rose et al., 1969), 3-deoxy-D-*arabino*-2-heptulosonate 7-phosphate (DAHP) synthase (Floss et al., 1972), 3-deoxy-D-*manno*-2-octulosonate 8-phosphate (KDO8P) synthase (Kohen et al., 1993; Dotson et al., 1993). The single exception to the preference for the 2-*si* face is enzyme I of the bacterial PEP-dependent sugar transport system, which appears to catalyze 2-*re* face protonation at C-3 of PEP (Hoving et al., 1983).

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