(Z)-9-Fluoro-EPSP Is Not a Substrate for EPSP Synthase: Implications for the Enzyme Mechanism

Christopher T. Seto and Paul A. Bartlett*

Department of Chemistry, University of California, Berkeley, California 94720-1460

Received June 1, 1994

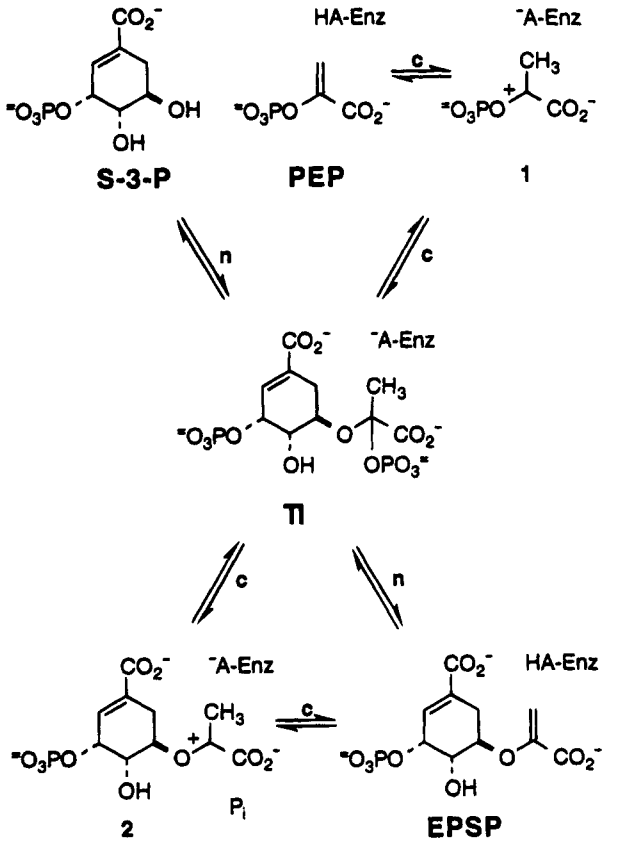
Introduction

The shikimic acid pathway is used by plants and bacteria to synthesize aromatic amino acids and other aromatic metabolites from carbohydrate precursors. One enzyme in the pathway, 5-enolpyruvylshikimate 3-phosphate (EPSP) synthase (EC 2.5.1.19) catalyzes transfer of the carboxyvinyl group of phosphoenol pyruvate (PEP) to the C-5 hydroxyl group of shikimate 3-phosphate (S-3-P).[1-4] This enzyme occupies an important position in the sequence, since it is the target of the herbicide glyphosate, and considerable efforts have been made to elucidate the details of its mechanism.

The tetrahedral adduct TI, first postulated by Sprinson,[1] has been isolated and characterized by Anderson et al. and shown to be a kinetically competent intermediate along the reaction pathway.[4] All of the kinetic constants for binding of substrates to enzyme, conversion to TI and to the products, and for release of the products from the enzyme have been determined.[5] Two limiting mechanisms can be proposed for formation and decomposition of the tetrahedral adduct. In a *cationic sequence* (steps c in Scheme 1), formation of TI involves initial protonation of the double bond of PEP to give cationic intermediate **1**, followed by subsequent addition of the C-5 hydroxyl; the tetrahedral intermediate TI then loses phosphate to give oxonium ion **2**, followed by deprotonation of the methyl group to form EPSP. Alternatively, in the *charge-neutral sequence* (steps n in Scheme 1), addition to the double bond of PEP and elimination of phosphate from TI occur with simultaneous formation or cleavage of the bonds to hydrogen and oxygen, respectively. In this mechanism, little charge builds up on the α-carbon or -oxygen of the pyruvyl unit. These two paths represent mechanistic extremes, and the true reaction sequence is likely to lie between them.

Using isotopically-substituted derivatives of PEP, Knowles[6] and Floss[7] have shown that the overall reaction occurs with retention of configuration of the enol double bond. This observation requires that the addition and elimination steps occur with the opposite stereochemistry; i.e., if the addition of the C-5 hydroxyl group of S-3-P to the double bond of PEP occurs with *anti* stereochemistry, then loss of phosphate from TI involves *syn* elimination. Abeles,[8] and more recently Knowles,[9] have shown that the enzyme catalyzes slow exchange of the vinyl protons of PEP with solvent in the presence of 5-deoxy analogs of S-3-P. This result implies either that the cationic species **1** can be formed in the enzyme active site or that PEP may be transferred first to an enzyme bound group, and then to S-3-P. Interestingly, enzyme-catalyzed exchange of solvent protons into EPSP has not been reported in the absence of P_i.

Scheme 1



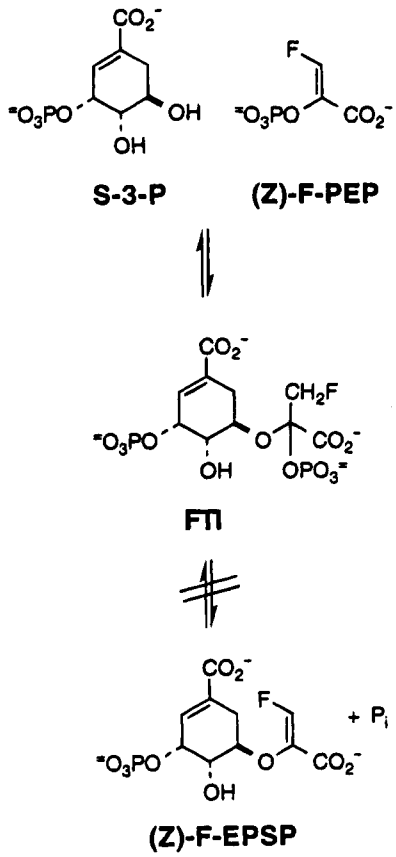
Recently, Walker et al. demonstrated that (Z)-F-PEP, but not the (E)-isomer, is a pseudo-substrate for EPSP synthase from *E coli*.[10] The enzyme catalyzes formation of fluoro-tetrahedral intermediate (FTI) from (Z)-F-PEP and S-3-P, albeit in a reaction that is much slower than transformation of the natural substrate (Scheme 2). $t_{1/2}$ for formation of FTI from S-3-P and F-PEP is less than 1 min, while $t_{1/2}$ for the natural substrate is below 1 μs. In the back reaction, S-3-P is formed from a stoichiometric mixture of FTI and EPSP synthase with a $t_{1/2}$ of 34 min,[10] which represents a reduction of 2.9×10^5-fold in comparison to the rate constant for formation of S-3-P and PEP from TI itself.[5] The large reduction in reaction rates with the fluorinated species is consistent with destabilization of the positively charged intermediate **1** by the electron-withdrawing fluorine substituent.

FTI is a potent inhibitor of EPSP synthase from *E. coli*, with a K_i value of approximately 0.6 nM.[10] It does not appear to be processed further by the enzyme, since there

(1) Levin, J. G.; Sprinson, D. B. *J. Biol. Chem.* **1964**, *239*, 1142–1150.
(2) Bondinell, W. E.; Vnek, J.; Knowles, P. F.; Sprechner, M.; Sprinson, D. B. *J. Biol. Chem.* **1971**, *246*, 6191–6196.
(3) Alberg, D. G.; Lauhon, C. T.; Nyfeler, R.; Fässler, A.; Bartlett, P. A. *J. Am. Chem. Soc.* **1992**, *114*, 3535–3546.
(4) Anderson, K. S.; Johnson, K. A. *Chem. Rev.* **1990**, *90*, 1131–1149 and references cited therein.
(5) Anderson, K. S.; Sikorski, J. A.; Johnson, K. A. *Biochemistry* **1988**, *27*, 7395–7406.
(6) Grimshaw, C. E.; Sogo, S. G.; Copley, S. D.; Knowles, J. R. *J. Am. Chem. Soc.* **1984**, *106*, 2699–2700.
(7) Lee, J. J.; Asano, Y.; Shieh, T.-L.; Spreafico, F.; Lee, K.; Floss, H. G. *J. Am. Chem. Soc.* **1984**, *106*, 3367–3368.
(8) Anton, D. L.; Hedstrom, L.; Fish, S. M.; Abeles, R. H. *Biochemistry* **1983**, *22*, 5903–5908.
(9) Pansegrau, P. D.; Anderson, K. S.; Widlanski, T.; Ream, J. E.; Sammons, R. D.; Sikorski, J. A.; Knowles, J. R. *Tetrahedron Lett.* **1991**, *32*, 2589–2592.
(10) Walker, M. C.; Jones, C. R.; Somerville, R. L.; Sikorski, J. A. *J. Am. Chem. Soc.* **1992**, *114*, 7601–7603.

0022-3263/94/1959-7130$04.50/0

Scheme 2



was no evidence of formation of F-EPSP under the conditions reported. However, this negative observation may reflect either an altered position of equilibrium (FTI stabilized relative to F-EPSP, in comparison to the nonfluorinated substrates) or a significantly greater destabilization of the second transition state (greater cationic character via intermediate **2**). Thus, the issue remained open as to whether the enzyme can catalyze interconversion of FTI and F-EPSP. In this paper, we describe the use of (*Z*)-F-EPSP[3] to probe the reverse reaction: formation of FTI by addition of P_i to F-EPSP. We show that (*Z*)-F-EPSP is not a substrate or pseudosubstrate for EPSP synthase from *Petunia hybrida* (the analog is not converted to either F-PEP or FTI) and discuss the implications that these results have for the reaction mechanism.[11]

Results

Kinetic Assay for Conversion of (*Z*)-F-EPSP to FTI. The specificity of EPSP synthase for the (*Z*)-isomer of F-PEP and the retention of double bond geometry in the normal transformation dictate that the (*Z*)-isomer of F-EPSP should be explored for potential interconversion with FTI.[12] Incubation of (*Z*)-F-EPSP with EPSP synthase in the presence of P_i should lead to inhibition of the enzyme, if the rate of formation of FTI is significant in comparison to the reversion of FTI to F-PEP and S-3-P ($t_{1/2}$ ca. 34 min). A buffered solution of EPSP synthase containing 50 μM (*Z*)-F-EPSP and 50 mM P_i was kept at 25 °C for 24 h and then diluted 77-fold into a standard assay mixture. Under these conditions, the enzyme is effectively saturated with (*Z*)-F-EPSP prior to dilution (reversible $K_i = 17$ μM[3]), but not inhibited by this analog during the assay (final concentration of (*Z*)-F-EPSP = 0.65 μM).

Parallel experiments involving incubation of the enzyme in the presence and absence of (*Z*)-F-EPSP gave no statistically significant difference between the observed kinetic parameters, while demonstrating that the enzyme was essentially stable: for enzyme incubated in the presence of the fluoro analog, $V_{max} = 0.321 \pm 0.012$ μM min^{-1} and $K_m = 4.4 \pm 0.6$ μM; for enzyme incubated alone, $V_{max} = 0.316 \pm 0.006$ μM min^{-1} and $K_m = 3.9 \pm 0.3$ μM. It is clear that there is no buildup of enzyme·FTI complex under these conditions, i.e., that formation of FTI from (*Z*)-F-EPSP and P_i is slower than 0.02 min^{-1}, the rate at which FTI reverts to F-PEP and S-3-P.

^{19}F-NMR Assay for Conversion of (*Z*)-F-EPSP to FTI or (*Z*)-F-PEP. The requirement that detectable amounts of enzyme·FTI complex accumulate limits the sensitivity of the kinetic experiments described above. In order to circumvent these problems, we used ^{19}F-NMR spectroscopy to look for any conversion of (*Z*)-F-EPSP to FTI, and thence to F-PEP, in the presence of EPSP synthase. The fluoro analog (24 mM), P_i (50 mM), and enzyme (4.1 μM) were combined with buffer in an NMR tube, and the reaction was monitored for disappearance of (*Z*)-F-EPSP ($\delta = -143.2$ ppm, doublet, $J_{FH} = 76.4$ Hz), appearance of (*Z*)-F-PEP ($\delta = -141.2$ ppm, doublet, $J_{FH} = 77.2$ Hz), or appearance of FTI ($\delta = -230.9$ ppm, triplet, $J_{FH} = 45.8$ Hz).[10] We did not observe appearance of either F-EPSP or FTI in the ^{19}F-NMR spectrum of this reaction mixture over 21 days. We estimate that additional fluorine resonances corresponding to 1% of the magnitude of (*Z*)-F-EPSP or ca. 500 μM concentration could have been detected in this experiment; thus, enzymatic conversion of (*Z*)-F-EPSP to either of these materials occurs at a rate of less than (500 μM product/4 μM enzyme/21 d) = 7×10^{-5} s^{-1}.[13]

Discussion

According to the ^{19}F-NMR experiments, F-EPSP is not converted to detectable amounts of FTI or F-PEP over 21 days in the presence of a large amount of enzyme. We believe this is a kinetic, as opposed to thermodynamic, phenomenon, because fluorine substitution should affect the free energy of formation of EPSP and PEP similarly, and because conversion of E·FTI to S-3-P and F-PEP is readily observed.[10] We can compare the upper limit for the reverse rate constant for E·F-EPSP to E·FTI (7×10^{-5} s^{-1}) to the rate constant of 240 s^{-1} determined by Anderson et al. for the corresponding step with the

(11) Extensive structural and kinetic similarity between the *E. coli* and *P. hybrida* EPSP synthases indicate that they are mechanistically related. See *inter alia*: Padgette, S. R.; Re, B. B.; Gasser, C. S.; Eichholtz, D. A.; Frazier, R. B.; Hironaka, C. M.; Levine, E. B.; Shah, D. M.; Fraley, R. T.; Kishore, G. M. *J. Biol. Chem.* **1991**, *266*, 22364–22369.

(12) A synthesis of the (*E*)-isomer of F-EPSP has yet to be described.

(13) Catalytic activity under these conditions was not measured directly; however, the enzyme is in any event likely to be stabilized through complexation with inhibitor (F-EPSP), as suggested by the results of the kinetic experiments. We did observe slow appearance of a ^{19}F-NMR peak ($\delta = -142.5$ ppm, doublet, $J_{FH} = 76.8$ Hz) representing approximately 10% of the material after 21 d; the new peak did not correspond to (*Z*)-F-PEP or FTI. Nor was this material the (*E*)-F-EPSP isomer, according to the carbon–fluorine coupling pattern observed in the ^{13}C NMR spectrum (Bosch, M. P.; Camps, F.; Gabrias, G.; Guerrero, A. *Magn. Reson. Chem.* **1987**, *25*, 347–351). It is conceivable that the peak arises from slow hydrolysis of the 3-phosphate group, although this possibility could not be probed directly because of the excess phosphate already present in the incubation mixture.

natural substrate.[14] Thus, fluorine substitution has destabilized this transition state (relative to the enolpyruvyl ground state) by at least 9 kcal mol^{-1} at 25 °C. In contrast, the first step of the transformation, conversion of S-3-P and PEP to TI, is slowed by at most a factor of 10^5 from fluorine substitution, corresponding to an increase in $\Delta G^{\ddagger}$ of <6.8 kcal mol^{-1}.[10]

What does the differential effect of fluorine on the two parts of the reaction reveal about the mechanism of the enzyme-catalyzed process? The high affinities observed for FTI (K_i = 0.6 nM) and F-EPSP (K_i = 17 μM vs $K_{m(EPSP)}$ = 4 μM) indicate that this substituent does not have a significant steric effect on the binding of these analogs. Since the primary influence of the electron-withdrawing fluorine substituent is destabilization of cationic species, our results imply that oxonium ion **2** contributes to the transition state of the second step to a greater extent than oxonium ion **1** does to the first (Scheme 1). The ether oxygen of **2** can stabilize the adjacent carbocation better than the phosphate oxygen stabilizes **1** (compare the pK_a values of the corresponding ROH species: ca. 15 and 12, respectively). Thus, it is intuitively reasonable that **2** would be involved to a greater extent than **1** in the normal enzymatic mechanism. Nevertheless, the fact that exchange of the methylene hydrogens on PEP is catalyzed by the enzyme in the presence of S-3-P surrogates that are unable to undergo addition suggests that generation of **1** in the active site is not impossible energetically. However, the very slowness of this exchange process, which is reduced ≈900-fold from the rate at which TI is formed in the presence of S-3-P itself,[8] is further evidence that **1** is not a discrete species along the reaction pathway.[15] This interpretation has significant implications regarding the postulated mode of action of the herbicide glyphosate, long thought to mimic the cationic species **1**.[16]

Conclusion

We have shown that F-EPSP is not processed by EPSP synthase, and inferred from this result that the transition state for interconversion of the tetrahedral intermediate and EPSP has more cationic character than that for formation of the tetrahedral intermediate from PEP and S-3-P. This work provides a further example of the use of a fluorinated substrate analog to probe the mechanism of an enzymatic transformation.[17]

Experimental Section

General. ^{19}F-NMR spectra were obtained at 376.5 MHz. Samples were prepared in H_2O with a capillary containing $CDCl_3$ for the lock signal. Spectra were referenced to $CFCl_3$ at 0 ppm. (*Z*)-F-EPSP was prepared according to the procedure of Alberg et al.[3] EPSP was prepared by the method of Chouinard and Bartlett.[18] EPSP synthase from *Petunia hybrida* cloned and expressed from *E. coli* was obtained as a generous gift from Dr. Ganesh Kishore of the Monsanto Co. The enzyme stock solution contained 1.96 mg/mL protein in a buffer of 50% glycerol, 50 mM K-HEPES (pH 7.5), 70 mM KCl, and 2.2 mM dithiothreitol.

Enzyme Assays of EPSP Synthase Incubated with (*Z*)-F-EPSP. A 4-μL portion of the EPSP synthase stock solution was diluted to 2.0 mL with a buffer containing 50 mM K-HEPES (pH 7.5), 50 mM K-phosphate, 4 mM $MgCl_2$, 20 mM KCl, 5 mM dithiothreitol, and 5 mg/mL of bovine serum albumin. This solution was split into two portions of 900 μL each. To one portion was added 29.0 μL of a 1.6 mM solution of (*Z*)-F-EPSP; to the other was added 29.0 μL of H_2O. The enzyme solution containing (*Z*)-F-EPSP had a final concentration of this analog of 50 μM. The two 929-μL enzyme solutions were incubated for 24 h in a circulating water bath at 25 °C. Enzyme assays were carried out using 13 μL of these enzyme solutions per assay following previously described procedures.[3] The (*Z*)-F-EPSP concentration in the assays that contained this material was 0.65 μM. The following kinetic parameters were determined: for enzyme incubated in the presence of the fluoro analog, V_{max} = 0.321 ± 0.012 μM min^{-1} and K_m = 4.4 ± 0.6 μM; for enzyme incubated alone, V_{max} = 0.316 ± 0.006 μM min^{-1} and K_m = 3.9 ± 0.3 μM.

Reaction of EPSP Synthase with (*Z*)-F-EPSP Monitored by ^{19}F-NMR Spectroscopy. (*Z*)-F-EPSP (5.1 mg, 11.9 μmol) was dissolved in 0.5 mL of buffer containing 100 mM K-HEPES (pH 7.5), 50 mM K-phosphate (pH 7.5), 50 mM KCl, and 50 μL of the EPSP synthase stock solution, and the solution was transferred to an NMR tube. The final concentration of (*Z*)-F-EPSP was 23.7 mM. The reaction mixture was incubated for 21 d in a circulating water bath at 25 °C. The ^{19}F-NMR spectrum of the sample was recorded after 2, 5, 9, 14, and 21 d.

Acknowledgment. This work was supported by the National Institutes of Health (Grant GM-28965). We thank Dr. Ganesh Kishore, Dr. James Sikorski, and Dr. Ken Gruys (Monsanto Co.) for providing us with EPSP synthase and a sample of (*Z*)-fluoro-PEP.

(14) Anderson, K. A.; Sikorski, J. A.; Johnson, K. A. *Biochemistry* **1988**, *27*, 7395–7406.

(15) The possibility that the surrogate exchange reaction reflects formation of a tetrahedral PEP–enzyme adduct, whether involved in the normal reaction mechanism or not, would only strengthen this argument.

(16) For an alternative viewpoint on the transition state analogy of glyphosate, see: Marzabadi, M. R.; Font, J. L.; Gruys, K. J.; Pansegrau, P. D.; Sikorski, J. A. *Bioorg. Med. Chem. Lett.* **1992**, *2*, 1435–1440.

(17) Poulter, C. D.; Argyle, J. C.; Marsh, E. A. *J. Biol. Chem.* **1978**, *253*, 7227–7233.

(18) Chouinard, P. M.; Bartlett, P. A. *J. Org. Chem.* **1986**, *51*, 75–78.