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Substrate Synergism and the Steady-State Kinetic Reaction Mechanism for EPSP Synthase from *Escherichia coli*

Kenneth J. Gruys,* Mark C. Walker, and James A. Sikorski
New Products Division, Monsanto Agricultural Company, A Unit of Monsanto Company, 800 North Lindbergh Boulevard, St. Louis, Missouri 63167

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ABSTRACT: Previous studies of *Escherichia coli* 5-enolpyruvoylshikimate-3-phosphate synthase (EPSPS, EC 2.5.1.19) have suggested that the kinetic reaction mechanism for this enzyme in the forward direction is equilibrium ordered with shikimate 3-phosphate (S3P) binding first followed by phosphoenolpyruvate (PEP). Recent results from this laboratory, however, measuring direct binding of PEP and PEP analogues to free EPSPS suggest more random character to the enzyme. Steady-state kinetic and spectroscopic studies presented here indicate that *E. coli* EPSPS does indeed follow a random kinetic mechanism. Initial velocity studies with S3P and PEP show competitive substrate inhibition by PEP added to a normal intersecting pattern. Substrate inhibition is proposed to occur by competitive binding of PEP at the S3P site [$K_{i(PEP)}$ = 6–8 mM]. To test for a productive EPSPS·PEP binary complex, the reaction order of EPSPS was evaluated with shikimic acid and PEP as substrates. The mechanism for this reaction is equilibrium ordered with PEP binding first giving a K_{ia} value for PEP in agreement with the independently measured K_d of 0.39 mM (shikimate K_m = 25 mM). Results from this study also show that the 3-phosphate moiety of S3P offers 8.7 kcal/mol in binding energy versus a hydroxyl in this position. Over 60% of this binding energy is expressed in binding of substrate to enzyme rather than toward increasing k_{cat}. Glyphosate inhibition of shikimate turnover was poor with approximately 8×10^4 loss in binding capacity compared to the normal reaction, consistent with the independently measured K_d of 12 mM for the EPSPS·glyphosate binary complex. The EPSPS·glyphosate complex induces shikimate binding, however, by a factor of 7 greater than EPSPS·PEP. Carboxyallenyl phosphate and (*Z*)-3-fluoro-PEP were found to be strong inhibitors of the enzyme that have surprising affinity for the S3P binding domain in addition to the PEP site as measured both kinetically and by direct observation with ^{31}P NMR. The collective data indicate that the true kinetic mechanism for EPSPS in the forward direction is random with synergistic binding occurring between substrates and inhibitors. The synergism explains how the mechanism can be random with S3P and PEP, but yet equilibrium ordered with PEP binding first for shikimate turnover. Synergism also accounts for how glyphosate can be a strong inhibitor of the normal reaction, but poor versus shikimate turnover.

The enzyme 5-enolpyruvoylshikimate-3-phosphate synthase (EPSPS,[1] EC 2.5.1.19) catalyzes the unique transfer of a carboxyvinyl moiety from phosphoenolpyruvate (PEP) to shikimate 3-phosphate (S3P), forming EPSP (5-enolpyruvoylshikimate 3-phosphate) as shown in Scheme I [for reviews refer to Sikorski et al. (1991) and Anderson and Johnson (1990a)]. EPSPS plays an important role in the synthesis of essential aromatic amino acids and is the biological target for glyphosate, the active ingredient in Roundup herbicide (Amrhein et al., 1980; Franz, 1985). Data from this

* Author to whom correspondence should be addressed.

[1] EPSPS, 5-enolpyruvoylshikimate-3-phosphate synthase; S3P, shikimate 3-phosphate; PEP, phosphoenolpyruvate; EPSP, 5-enolpyruvoylshikimate 3-phosphate; EPS, 5-enolpyruvoylshikimate; 5-deoxy-S3P, 5-deoxyshikimate 3-phosphate; glyphosate, *N*-(phosphonomethyl)glycine; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*'-2-ethanesulfonic acid; P_i, inorganic phosphate; EPSP reaction, S3P and PEP turnover to produce EPSP; EPS reaction, shikimate and PEP turnover to produce EPS.

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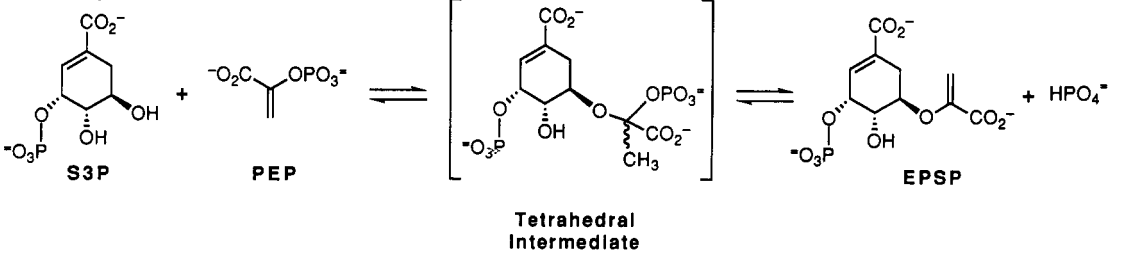
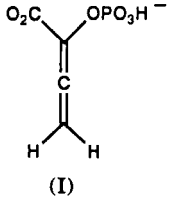
Scheme I: EPSPS Catalyzed Reaction



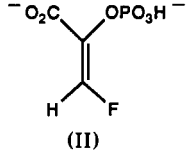
laboratory in collaboration with Prof. Kenneth A. Johnson at Pennsylvania State University have conclusively shown that the EPSPS reaction proceeds through a single, kinetically competent tetrahedral intermediate (Scheme I). This intermediate has been characterized by rapid quench kinetics (Anderson et al., 1988a), isolation (Anderson et al., 1988b), observation of the enzyme bound species by ^{13}C NMR (Anderson et al., 1990; Barlow et al., 1989), and its rapid reversion to the expected equilibrium concentration of substrates and products upon binding to enzyme (Anderson & Johnson, 1990b).

For *Escherichia coli* EPSPS, transient-state kinetic data presented by Anderson et al. (1988a) led to a proposed kinetic scheme for ordered binding of S3P followed by PEP. Relying on this model, further analysis of the pre-steady-state results suggested that during steady-state turnover of substrates, the steady-state kinetic reaction mechanism can be described as equilibrium ordered (Anderson & Johnson, 1990a). While this conclusion was consistent with the transient-state kinetic data, confirmation of a equilibrium ordered mechanism by steady-state kinetic analysis for the *E. coli* enzyme has not been pursued. Besides this work, steady-state kinetic analysis of EPSPS from sources other than *E. coli* have surprisingly resulted in varied proposed kinetic reaction mechanisms, none of which is equilibrium ordered. For example, the *Neurospora crassa* and *Sorghum bicolor* enzymes have been reported to follow steady-state ordered kinetics with S3P binding first (Boocock and Coggins, 1983; Boocock, 1985; Ream et al., 1988), whereas the *Klebsiella pneumoniae* enzyme is proposed to follow a random addition of substrates (Steinrücken & Amrhein, 1984a,b). Though possible, it seems unlikely that an enzyme with such high interspecies sequence homology (Gasser et al., 1988) could exhibit such wide diversity in steady-state kinetic reaction mechanism. From this it is apparent that a developed, comprehensive kinetic mechanism is lacking that can accommodate all of the literature observations.

Recently, the question of orderedness or randomness has resurfaced in the kinetic mechanism for the *E. coli* enzyme by our observation that PEP has good binding capacity for free enzyme with a K_d of 390 μM (Ream et al., 1992), only a factor of 20 greater than the K_m value to form ternary complex with EPSPS·S3P. In addition, recent kinetic work with a series of PEP analogue inhibitors including carboxyallenyl phosphate (I) and (*Z*)-3-fluoro-PEP (II) has shown



(I)



(II)

that there is good binding affinity for these inhibitors to free enzyme in addition to the EPSPS·S3P binary complex (Walker et al., 1991), an observation more consistent with random rather than ordered binding of substrates. In light of these findings for the *E. coli* enzyme, a thorough steady-state kinetic evaluation was felt essential toward developing a comprehensive kinetic model consistent with these new observations and those presented previously in the literature.

Data presented in this report utilizing steady-state kinetic techniques along with supporting ^{31}P NMR binding studies address the question of orderedness versus randomness in the *E. coli* EPSPS kinetic mechanism. These studies include (1) a thorough steady-state initial velocity study of the normal EPSP reaction, (2) an inhibition study of the EPSP reaction using 5-deoxy-S3P as a kinetic probe, (3) a steady-state kinetic analysis of the formation of 5-enolpyruvoylshikimate (EPS) using shikimic acid as an alternate substrate for S3P, (4) a complete kinetic description of inhibitors I and II on the EPSP reaction, (5) a ^{31}P NMR characterization of enzyme complexes containing I and II, and (6) a kinetic description of glyphosate's inhibition on the EPS reaction in comparison with the EPSP reaction. Results from these studies provide evidence that significantly modifies our understanding of the EPSPS kinetic reaction mechanism for the *E. coli* enzyme and suggest a more unified mechanism for EPSPS's with high sequence homology. In addition, the differences between shikimate and S3P in binding to enzyme and rates of turnover further demonstrate the importance of charged groups (salt bridges) and hydrogen bonds to the substrate specificity and catalytic efficiency of EPSPS.

Materials and Methods

Enzyme Purification. EPSPS was isolated from a cloned *E. coli* strain which overproduces the enzyme (Rogers et al., 1983). The enzyme was purified as described previously (Castellino et al., 1989). The enzyme concentration was determined using an extinction coefficient of 35 200 M^{-1} cm^{-1} at 280 nm or by the bicinchoninic acid (BCA) procedure of Pierce. A molecular weight of 46 000 was used for calculation of enzyme active site concentration and k_{cat} values.

Chemicals. S3P was synthesized enzymatically by treatment of shikimic acid (Sigma) with shikimate kinase (Millar et al., 1986) and purified using ion exchange HPLC techniques (Castellino et al., 1991). [U-^{14}C]S3P was synthesized and purified in a similar manner starting with uniformly labeled [^{14}C]shikimic acid (New England Nuclear). The specific activities of [U-^{14}C]S3P for PEP substrate inhibition studies and [U-^{14}C]shikimic acid for shikimate turnover studies were approximately 18–20 mCi/mmol. [2-^{14}C]PEP was purchased from New England Nuclear at approximately 30 mCi/mmol. Glyphosate was obtained from Monsanto internal stocks and was analytically pure (>99%). 5-Deoxy-S3P was synthesized as described previously (Pansegrau et al., 1991). Carboxyallenyl phosphate and (*Z*)-3-fluoro-PEP were generous gifts from Professor Marion O'Leary of the Center for Biological Chemistry at the University of Nebraska—Lincoln and Professor Ronald Somerville of the Department of Biochemistry at Purdue University. (*Z*)-3-Fluoro-PEP contained approx-

imately 10% of the *E* isomer and an additional 2% impurity that is turned over by EPSPS in the presence of S3P. This impurity, presumed to be PEP on the basis of identical chromatographic elution times for the turnover product and EPSP, was removed by incubation with S3P and catalytic amounts of EPSPS followed by acid quench and separation on Mono-Q (10.5 × 1.5 cm; Pharmacia). Fluoro-PEP elution off this column was then accomplished using a linear gradient from an initial condition of 50 mM triethylammonium bicarbonate, pH 8, to 250 mM in 10 min and holding at 250 mM for 30 min. All buffers and other reagents employed were of highest commercial purity. Barnstead Nanopure water was used for all solutions.

Kinetic Assays. All initial velocity experiments for the EPSP and EPS reactions were conducted at 30 °C in 200 mM HEPES buffer, pH 7.0, with 100 mM KCl. HEPES and KCl at these concentration levels were used to overwhelm any pH or ionic strength effects that could have been contributed by high substrate or glyphosate concentrations. As an extra precaution for pH control, all concentrated stock solutions of substrates or glyphosate were neutralized to pH 7 with 2 N KOH. Progress of the reaction was followed from the enzymatic conversion of [U-^{14}C]S3P or shikimate to [^{14}C]EPSP or [^{14}C]EPS, respectively. The reaction was initiated by the addition of enzyme and allowed to proceed from 5–15 min before quenching with a 1:1 (v/v) addition of 10% 1 M potassium acetate, pH 4.5, in ethanol. Turnover of substrates were always held to limits that ensured quenching in the steady-state portion of the reaction, yet maximized levels of product to give good precision. All reactions were done in duplicate. Quenched samples were centrifuged to remove precipitated protein (necessary for shikimate-based reactions), and the supernatant was transferred to vials to be used in an automated Waters HPLC gradient system. Reactions done in the presence of I or II as PEP analogue inhibitors or with 5-deoxy-S3P were done similarly to that described above with only minor changes in conditions (100 mM HEPES, 50 mM KCl, and [2-^{14}C]PEP or [U-^{14}C]S3P as radiolabeled probe). Concentrated stock solutions of I and II were prepared in 150 mM triethylammonium bicarbonate, pH 8.

A search for possible interfering side reactions was pursued when shikimate was used as substrate because of the high concentration of enzyme needed to monitor this reaction. Hydrolysis of PEP to pyruvate was of particular concern since previous work in this laboratory had suggested the existence of this slow EPSPS catalyzed reaction (Anderson et al., 1990). To follow this, a coupled assay system was utilized employing the reaction of pyruvate with NADH to form NAD^+ and lactate as catalyzed by lactic dehydrogenase (LDH). Loss of absorption at 340 nm is a convenient means to quantitate the rate of PEP hydrolysis spectrophotometrically (NADH ϵ = 6.22×10^3 cm^{-1} M^{-1}). The assay solution for this contained 200 mM HEPES, pH 7.0, 100 mM KCl, 0.25 mM NADH, and 20 units/mL LDH.

HPLC Analyses. Substrates and products from reaction mixtures were quantitated by HPLC using a continuous flow radioactivity detector (Radiomatic, model Flow-one/βeta). The cocktail used was Flo-Scint IV (Radiomatic) at a flow rate of 4.0 mL/min. Separation was based on anion exchange using a SynChropak AX-100 column (250 × 4.6 mm) with a flow rate of 1.0 mL/min. For reactions utilizing S3P as the substrate, separation was accomplished isocratically with 0.18–0.24 M KP_i (variability due to exact column properties), pH 6.5. Baseline resolution was accomplished in a total run time of 22 min. With reactions utilizing shikimate as substrate, gradient elution was necessary starting with 50 mM KP_i, pH 6.5, for 5 min and then a linear gradient from 5 to 11 min until 0.4 M KP_i was reached. Excellent baseline resolution resulted from this gradient elution, giving retention times for shikimate, EPS, and PEP at 4, 10, and 19 min, respectively.

Data Analyses. Kinetic data were fitted with the Fortran programs of Cleland (1979), some of which were modified to work on a MacIntosh computer (provided by Professor Paul Cook, Texas College of Osteopathic Medicine), or by using the commercial software GraFit (Leatherbarrow, 1990) with the derived velocity equations inserted as instructed by the program manual. Fitting data with either of these programs when using the same rate equation gave virtually identical results both in kinetic constants and their associated relative errors. The data were fitted directly using the equations below or in their log form.

$$v = VA/(K_a + A) \quad (1)$$

$$v = VAB/(K_{ia}K_b + K_bA + AB) \quad (2)$$

$$v = VAB/(K_{ia}K_b + K_aB + K_bA + AB) \quad (3)$$

$$v = VAB/(K_{ia}K_b + K_bA + AB + BK_{ia}K_b/K_i) \quad (4)$$

$$v = VAB/[K_{ia}K_b(1 + B/K_i) + K_aB(1 + B/K_i) + K_bA + AB] \quad (5)$$

$$v = VA/[K_a(1 + I/K_{is}) + A] \quad (6)$$

$$v = VA/[K_a + A(1 + I/K_{ii})] \quad (7)$$

$$v = VA/[K_a(1 + I/K_{is}) + A(1 + I/K_{ii})] \quad (8)$$

$$v = VAB/(K_{ia}K_b + K_bA + AB + IK_{ia}K_b/K_i + IBK_{ia}K_b/K_i\alpha K_b) \quad (9)$$

The individual terms define v, velocity; V, maximal velocity; K_{ia}, K_b, αK_b, etc., dissociation or Michaelis constants; K_i, K_{is}, or K_{ii}, inhibitory dissociation constants; A and B, substrate concentrations; and I, inhibitor concentration.

^{31}P NMR Studies. NMR spectra were accumulated on a Varian XL-300 FT spectrometer equipped with a Nalorac 4-nucleus Z-spec probe for 5-mm diameter NMR tubes. Samples were prepared in a buffered solution containing 50 mM HEPES, 50 mM KCl, 0.05 mM EDTA, and 5 mM β-mercaptoethanol, pH 7.25, containing 25% (v/v) D_2O. β-Mercaptoethanol served to stabilize the enzyme while EDTA was included to avoid signal broadening due to potential trace amounts of paramagnetic metals. D_2O was utilized for lock. ^{31}P NMR spectra were acquired at 7 °C with a one-pulse sequence using a 98° pulse width, with delay and acquisition times of 1.0 s each. Spectra were Waltz decoupled and subject to 1–5-Hz line broadening. Spectra were referenced to 0.0 ppm using a 1 M D_3PO_4 insert. A minimum of 3000 transients were required for experiments with enzyme.

Results

Initial Velocity Studies with S3P and PEP. The EPSPS catalyzed rates of product formation were determined at various fixed S3P concentrations over a PEP range of 0.010–20 mM. Figure 1 shows the results of this experiment and the excellent fit of the data using eq 5. This equation describes a normal intersecting pattern (sequential mechanism), but with competitive substrate inhibition by B (PEP). The data did not converge with an equilibrium ordered process plus dead-end combination of B with free enzyme (eq 4). The velocity patterns also cannot be supported by uncompetitive substrate inhibition where B forms a dead-end complex with the EQ product complex in a steady-state ordered mechanism (EQ

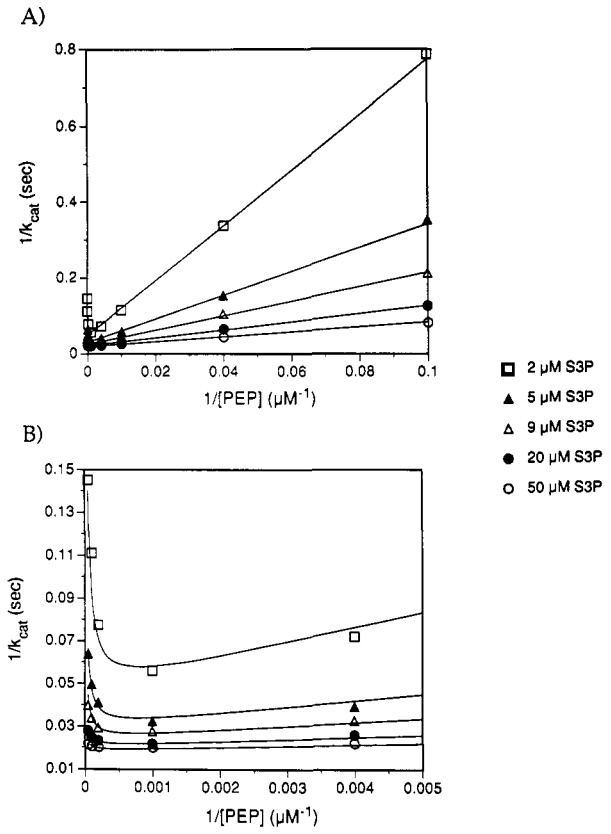


FIGURE 1: Competitive substrate inhibition of EPSPS catalyzed S3P turnover by PEP with (a) full data set and (b) expanded region at high PEP concentration.

Table I: Kinetic Constants for PEP Substrate Inhibition Study of EPSPS Catalyzed EPSP Formation from S3P and PEP[a]

kinetic constant	normal fit value	log fit value
V_m (k_{cat})	56.6 ± 0.5 s^{-1}	56.7 ± 0.8 s^{-1}
$K_{a(S3P)}$	3.6 ± 0.2 μM	3.2 ± 0.2 μM
$K_{b(PEP)}$	23 ± 2 μM	21 ± 1 μM
$K_{ia(S3P)}$	31 ± 4 μM	37 ± 3 μM
$K_{ib(PEP)}$	200 ± 20 μM	250 ± 20 μM
$K_{i(PEP)}$	8200 ± 600 μM	6100 ± 400 μM

[a] The fit was obtained with data from Figure 1 using eq 5, which describes both steady-state ordered and random mechanisms. K_{ib} values were calculated by replacing $K_{ia}K_b$ terms in the denominator with K_aK_{ib} in eq 5. This is valid only for a random mechanism where $K_{ia}K_b = K_aK_{ib}$.

in this case represents EPSPS·EPSP binary complex, formed after release of phosphate). Of significance in Table I is the fitted K_i value at 6–8 mM for the inhibitory complexes of PEP with EPSPS in the presence or absence of S3P. This is well over a factor of 10 greater than the K_d of 0.39 mM for the EPSPS·PEP binary complex as determined by microcalorimetry and equilibrium dialysis binding studies (Ream et al., 1992). Also of interest is the calculated PEP K_{ib} (i.e., dissociation contant for EPSPS·PEP binary complex for a random mechanism). At 0.20–0.25 mM, this value is within a factor of 2 of the previously measured K_d.

To aid in deciphering the mechanism, the inhibition of the EPSP reaction by 5-deoxy-S3P versus PEP at fixed 100 μM S3P was investigated (for variable S3P at 100 μM fixed PEP, 5-deoxy-S3P is strictly competitive with K_i = 12.6 ± 1.1 μM, fitted with eq 6, not shown). For an equilibrium ordered reaction, a competitive inhibitor of the first substrate should also show competitive inhibition versus the second substrate (Segel, 1975). A mixed inhibition pattern versus PEP, as shown in Figure 2, is further evidence that an equilibrium

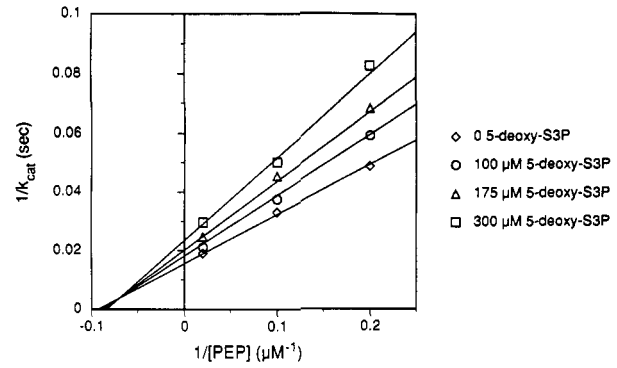


FIGURE 2: Inhibition of EPSPS catalyzed EPSP formation. Mixed inhibition against PEP with 5-deoxy-S3P (K_{is} = 450 ± 13 μM; K_{ii} = 580 ± 11 μM). S3P is fixed at 100 μM.

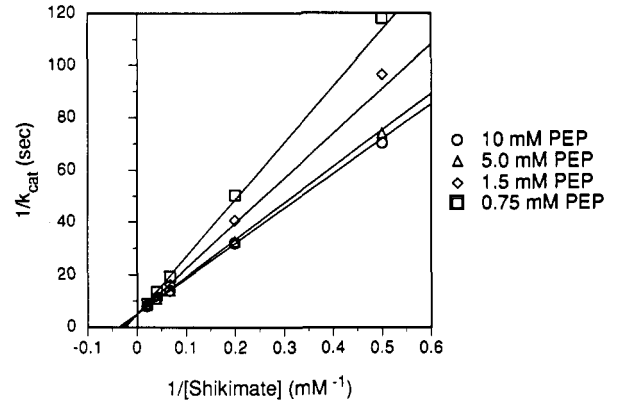


FIGURE 3: EPSPS equilibrium ordered addition of PEP and shikimate.

ordered process is not in place for the EPSP reaction. Mixed inhibition in this case is consistent with either a random or steady-state ordered mechanism, in line with the above results from the initial velocity studies. There is, however, an exception to the expected inhibition patterns that could allow for a mixed pattern in an equilibrium ordered reaction. If binding of 5-deoxy-S3P at the S3P site promotes binding of PEP, then an intercept effect like that seen in Figure 2 is possible (see below for glyphosate inhibition of the shikimate reaction as an example). This seems unlikely, however, given the above initial velocity results. In addition, recent studies to define the interdependence between the S3P and PEP sites for catalysis indicate that PEP binding is in fact reduced with 5-deoxy-S3P (M. C. Walker, J. E. Ream, R. B. Frazier, and J. A. Sikorski, unpublished results).

Initial Velocity Studies with Shikimate and PEP. The EPSPS catalyzed rate for the production of EPS from shikimate and PEP was monitored at fixed levels of PEP while varying shikimate. Verification that the product of this reaction is indeed EPS will be described elsewhere (M. J. Miller, K. S. Anderson, D. S. Braccolino, D. G. Cleary, C. Y. Han, K. C. Lin, J. E. Ream, R. D. Sammons, and J. A. Sikorski, unpublished results). No other detectable products are found on the basis of the ^{14}C elution profiles of the kinetic reaction mixtures. Only peaks identified as unreacted shikimate or EPS are detected (not shown). In addition, rates of PEP hydrolysis, a measurable side reaction catalyzed by EPSPS under the high enzyme concentrations utilized to monitor shikimate turnover, occur at a level no greater than 5% and more frequently at ≤1% of the rate for the shikimate reaction to form EPS under all experimental conditions (not shown). This side reaction is dependent on the presence of all three components needed for shikimate turnover, PEP, shikimate, and EPSPS, and thus suggests a minor break along the pathway to form EPS.

Table II: Kinetic Constants for EPSPS Catalyzed EPS Formation from Shikimate and PEP[a]

kinetic constant	normal fit value	log fit value
V_m (k_{cat})	0.187 ± 0.005 s^{-1}	0.198 ± 0.006 s^{-1}
$K_{ia(PEP)}$	0.38 ± 0.04 mM	0.56 ± 0.04 mM
$K_{b(shikimate)}$	24 ± 1 mM	25 ± 1 mM

[a] The fit was obtained with data from Figure 3 using eq 2, which describes an equilibrium ordered mechanism.

The rate data in reciprocal form for the shikimate reaction are shown in Figure 3 with fitting to eq 2. The plot shows intersection on the Y axis which is diagnostic of a equilibrium ordered reaction mechanism where PEP binds as the first substrate. Fit to a normal intersecting pattern (steady-state ordered or random mechanism, eq 3) was poor, resulting in negative K_m and K_{ia} values. The kinetically measured K_{ia} (0.38–0.56 mM, Table II) is in excellent agreement with the independently measured dissociation constant of 0.39 mM for the EPSPS·PEP binary complex (Ream et al., 1992). The k_{cat}/K_m for shikimate gives a value of 8.0 s^{-1} M^{-1} compared to 1.5 × 10^7 s^{-1} M^{-1} for S3P using values given in Tables II and I, respectively. This shows that the 3-phosphate moiety of S3P confers a factor of approximately 1.9 × 10^6 in specificity or 8.7 kcal/mol of binding energy as calculated by eq 10. This expression describes the incremental Gibbs free

$$\Delta\Delta G_b = -RT \ln [(k_{cat}/K_m)_{R-P_i}/(k_{cat}/K_m)_{R-OH}] \quad (10)$$

energy of transfer of the 3-phosphate group from enzyme to water, relative to the corresponding 3-hydroxyl group (Fersht, 1985).

EPSPS Inhibition of the EPSP Reaction by PEP Analogues. The inhibition of enzyme activity by I and II was examined by varying the concentration of PEP while holding the concentration of S3P at both saturating and nonsaturating levels with several fixed concentrations of inhibitor. Neither of these compounds was turned over by EPSPS as PEP substrate analogues nor exhibited any time-dependent inhibition (Walker et al., 1991). At 100 μM S3P, the inhibition data for both compounds versus PEP are best supported by a mechanism describing competitive inhibition which is shown graphically in Figure 4 with carboxyallenyl phosphate (I). This indicates that, with saturating S3P, these analogues strictly compete with PEP for binding at the PEP site to form a reversible, dead-end complex, an expected result for structural analogues of PEP. Calculated K_{is} values (fitted from eq 6) at 100 μM fixed S3P in Table III show that I has a binding affinity for EPSPS·S3P binary complex comparable to PEP (K_m = 20 μM) with II showing some enhanced binding over both PEP and I.

Results at fixed, nonsaturating S3P (5 μM) are surprising. Under these conditions, I and II gave mixed inhibition patterns with varying PEP (Table III, fitted with eq 8). If I and II bind exclusively to the PEP site, then their inhibition patterns

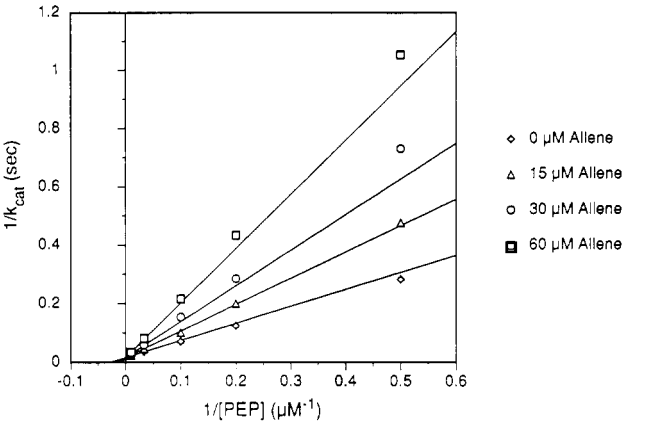


FIGURE 4: Inhibition of EPSPS catalyzed EPSP formation. Competitive inhibition against PEP with carboxyallenyl phosphate (I). S3P is fixed at 100 μM.

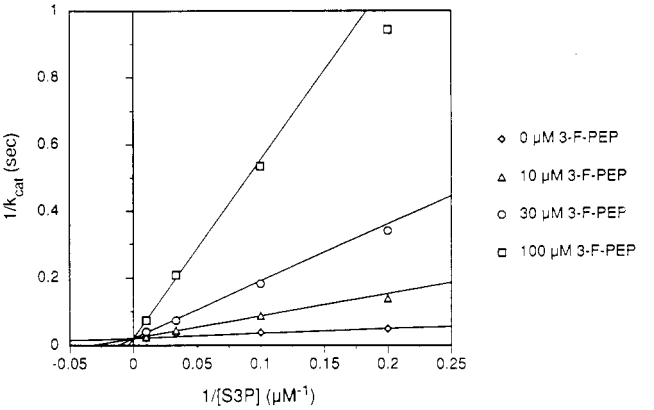


FIGURE 5: Inhibition of EPSPS catalyzed EPSP formation. Competitive inhibition against S3P with (*Z*)-3-fluoro-PEP (II). PEP is fixed at 100 μM.

should be competitive at all fixed concentrations of S3P. An intercept effect, as measured by K_{ii} in Table III, clearly shows this not to be the case. This implies that an inhibitory binding domain exists for I and II in addition to the PEP site.

The inhibition of EPSPS with S3P as the variable substrate was examined in a similar manner for I and II. With low and high concentrations of PEP, most of the data are best supported by a mechanism describing mixed inhibition (refer to Table III). This suggests binding affinity of I and II for free enzyme in addition to an EPSPS·S3P binary complex, a result more consistent with random rather than ordered addition of substrates. One surprising exception to this mixed inhibition pattern was the competitive behavior of II versus S3P at 100 μM fixed PEP as shown in Figure 5. This result was unexpected since it indicates that II has considerable affinity for enzyme in directed competition with S3P under conditions where PEP concentration is high enough to occupy much of

Table III: Inhibition Patterns Observed with (*Z*)-3-Fluoro-PEP (II) and Carboxyallenyl Phosphate(I) as Dead-End Inhibitors of EPSPS[a]

inhibitor	varied substrate	fixed substrate	pattern	K_{is} (μM)	K_{ii} (μM)	K_{is}/K_{ii}
(*Z*)-3-F-PEP	PEP	100 μM S3P	competitive	6.4 ± 0.7		
(*Z*)-3-F-PEP	PEP	5 μM S3P	mixed	6.5 ± 1.5	6.1 ± 0.8	1.1
(*Z*)-3-F-PEP	S3P	100 μM PEP	competitive	2.9 ± 0.2		
(*Z*)-3-F-PEP	S3P	5 μM PEP	mixed	7.5 ± 0.3	7.7 ± 0.2	0.97
allene	PEP	100 μM S3P	competitive	27 ± 1		
allene	PEP	5 μM S3P	mixed	7.3 ± 0.5	40 ± 6	0.18
allene	S3P	100 μM PEP	mixed	11 ± 1	110 ± 17	0.10
allene	S3P	20 μM PEP	mixed	7.5 ± 1.1	23 ± 2	0.33
allene	S3P	2 μM PEP	mixed	4.3 ± 0.6	9.1 ± 0.6	0.47

[a] Data were fitted to eqs 6 and 8 for competitive and mixed inhibition patterns, respectively.

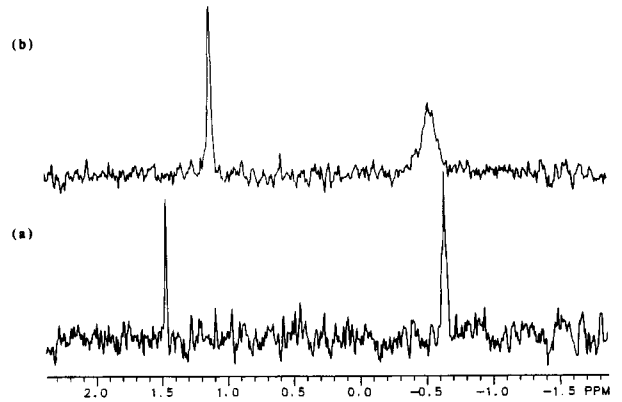


FIGURE 6: ^{31}P NMR spectra of (a) 1.0 mM carboxyallenyl phosphate (I) and (b) with 1.3 mM EPSPS.

the PEP site. The allene, under closer examination, interacts with the enzyme in a similar manner. Table III shows that at 100 μM fixed PEP, I is nearly competitive with S3P based on the high K_{ii} value relative to K_{is}. A competitive model for this data set in fact fits nearly as well as the mixed model. Like the mixed behavior versus PEP concentration described above, these results also imply that an inhibitory binding domain exists for I and II in addition to the PEP site.

The additional binding site for I and II is suggested by the results in Table III to be the S3P binding domain on the basis of the increasingly competitive nature of I and II inhibition versus S3P as fixed PEP concentration increases (i.e., the K_{is}/K_{ii} ratio decreases with increasing fixed PEP concentration). Complete competitive behavior at 100 μM fixed PEP ($\approx 5 \times K_m$) with II, while still partially mixed for I, can be rationalized on the basis of the different relative affinities of each inhibitor for both binding domains. It appears that II binds at the S3P domain tight enough to overwhelm any minor binding occurring at the PEP site that would be observed experimentally as an intercept effect. With 100 μM fixed S3P ($\approx 30 \times K_m$) and PEP as variable substrate, competitive behavior is seen for both inhibitors due to saturating conditions that make the S3P site unavailable for inhibitor binding. The K_i values in Table III would also suggest that, between I and II, II appears to have greater affinity for the S3P site. Such a direct interpretation may be too simplistic, however, since additional binding modes significantly increase complexity as will be addressed under Discussion. Direct verification that I and II have affinity for free enzyme and for the S3P binding domain is presented below with ^{31}P NMR observation of EPSPS·inhibitor species.

^{31}P NMR of EPSPS Complexes with PEP Analogues. ^{31}P NMR has been exploited previously as a convenient technique to directly probe the binding of ligands to EPSPS (Castellino et al., 1989; Leo et al., 1990). Buffered solutions of I exhibit a sharp resonance at −0.66 ppm which is assigned to the allene phosphate (Figure 6a). In the presence of EPSPS, a broad resonance at approximately −0.5 ppm was observed (Figure 6b). The observed line broadening and change in chemical shift is assigned to the formation of binary EPSPS·I complex. The sharp resonance observed at 1.49 ppm in Figure 6a is due to inorganic phosphate, which is a contaminant present in this sample. The phosphate peak also appears broadened and shifted upfield (Figure 6b), presumably due to binding with enzyme. Phosphate affinity for EPSPS is known to occur with enzyme at the concentrations used in this NMR experiment, either alone or in the presence of other ligands (Ream et al., 1992). A binary complex with II and EPSPS is also seen when this inhibitor is incubated with free enzyme. Upon addition

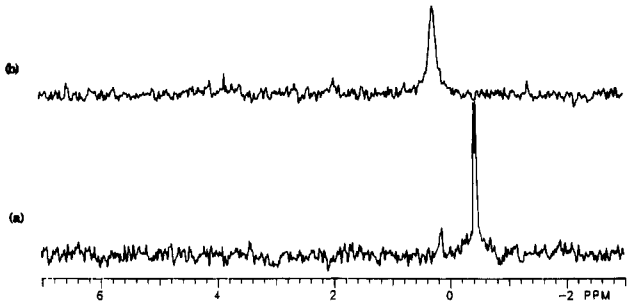


FIGURE 7: ^{31}P NMR spectra of (a) 1.0 mM 3-fluoro-PEP (II) and (b) with 1.5 mM EPSPS.

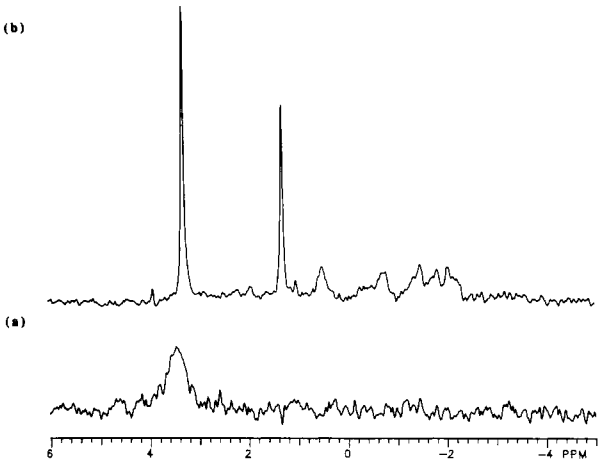


FIGURE 8: ^{31}P NMR spectra (a) preformed EPSPS·S3P binary complex in the absence of carboxyallenyl phosphate (I) and (b) in the presence of 2 equiv of I:EPSPS.

of EPSPS, the phosphate resonance for II broadens and shifts downfield from −0.38 to 0.3 ppm (Figure 7a,b). These binary complexes of I and II with EPSPS exist, supposedly by binding at the PEP site.

With the addition of I to a stoichiometric mixture of S3P and enzyme, the broad resonance assigned to the 3-phosphate in the EPSPS·S3P binary complex (δ = 3.5 ppm, Figure 8a) becomes sharper, signifying release of S3P to the solution (δ = 3.3 ppm, Figure 8b). New resonances corresponding to multiple EPSPS·I complexes also appear (δ = −0.5 to −2.2 ppm, Figure 8b). Considering the observed expulsion of bound S3P, these probably represent the different environments for allene in a EPSPS·(bis-I) complex when both the S3P and PEP sites are occupied. In addition, some degradation of I is known to occur under these conditions because of the time required for data collection. Consequently, some resonances such as phosphate at δ = 1.5 and those in the region for the allene are breakdown products.

The combined results from I and II ^{31}P NMR binding studies show that free EPSPS readily forms a binary complex with I and II. Therefore, the presence of S3P is not required for binding of these inhibitors to free enzyme. On the basis of enzyme and inhibitor concentrations used in the experiments shown in Figures 6 and 7, complete binding of I or II to EPSPS (i.e., there is no observed free inhibitor in solution) translates to maximum K_d values for these complexes in the mid-micromolar range, in line with the K_i values reported in Table III. The spectra in Figure 8 also demonstrate that PEP analogues have affinity for both the PEP and S3P binding sites on the enzyme. These results are consistent with the inhibition results discussed above.

Glyphosate Inhibition of the Shikimate Reaction. Glyphosate was tested as an inhibitor of the shikimate reaction

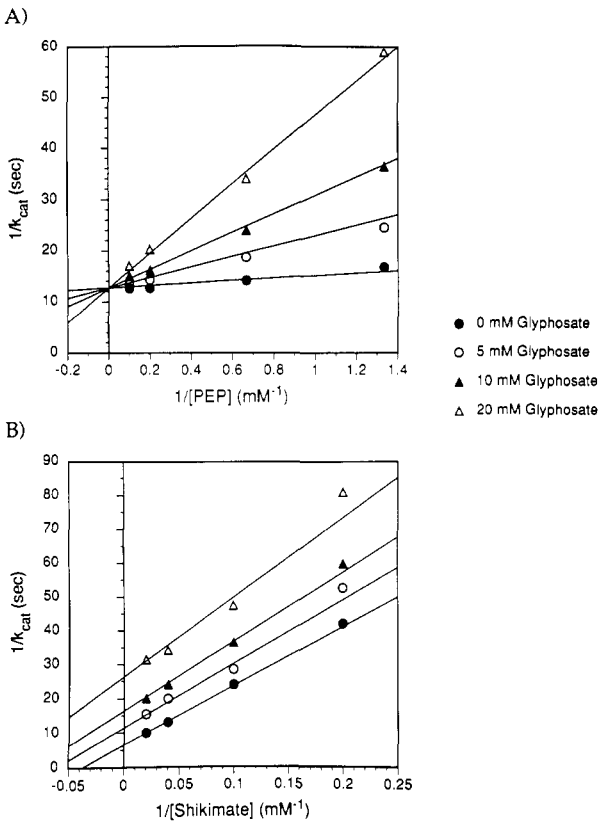


FIGURE 9: Glyphosate inhibition of EPSPS catalyzed EPS formation. (a) Competitive behavior versus PEP; shikimate is fixed at 25 mM. (b) Mixed behavior due to glyphosate-induced substrate binding of shikimate (refer to Scheme III); PEP is fixed at 1.5 mM.

by varying shikimate or PEP at fixed, nonsaturating levels of the cosubstrate (1.5 mM fixed PEP when varying shikimate and 25 mM fixed shikimate when varying PEP). As previously mentioned, an inhibitor that competes with the first substrate (PEP) would also be expected to show competitive inhibition against the second (shikimate) for an equilibrium ordered reaction. While glyphosate inhibition against PEP is consistent with this expectation (Figure 9a), against shikimate, glyphosate is quite clearly a mixed inhibitor (Figure 9b). In fact, fitting to an uncompetitive model (eq 7) produces a fit almost as good as for mixed inhibition (eq 8). This apparent discrepancy between an equilibrium ordered model for the EPS reaction and the glyphosate inhibition pattern versus shikimate is rationalized on the fact that competitive patterns versus the second substrate presumes that inhibitors competitive with PEP in this system do not promote the binding of shikimate. Contrary to this presumption, glyphosate bound to EPSPS in a binary complex does appear to promote binding of shikimate as suggested by fitting of the data to eq 9 (solid lines in Figure 9). This equation is based on an equilibrium ordered model that contains two dead-end complexes of an inhibitor I, EI and EIB (refer to Scheme III under Discussion), and can be defined as "inhibitor-induced substrate (shikimate) binding". This model predicts competitive behavior versus PEP and mixed inhibition versus shikimate with the intercept effect for the latter being dependent on the level of binding of shikimate to the EPSPS·glyphosate binary complex.

Table IV gives the fitted kinetic constants using eq 9 when both sets of glyphosate inhibition data were combined. While the fit to the data is good, because of five kinetic constants to resolve, along with three variable components in the reaction (i.e., shikimate, glyphosate, and PEP), there is low confidence in K_i and αK_b values. If, however, one utilizes the measured

Table IV: Kinetic Constants for Glyphosate Inhibition of EPSPS Catalyzed EPS Formation from Shikimate and PEP[a]

kinetic constant	floating K_i value	fixed K_i value
V_m (k_{cat})	0.153 ± 0.007 s^{-1}	0.151 ± 0.006 s^{-1}
$K_{b(shikimate)}$	23 ± 2 mM	22 ± 2 mM
$\alpha K_{b(shikimate)}$	2.2 ± 1.7 mM	3.2 ± 0.5 mM
$K_{ia(PEP)}$	0.39 ± 0.06 mM	0.37 ± 0.06 mM
$K_{i(glyphosate)}$	18 ± 13 mM	

[a] The fit was obtained with data from Figure 9a,b using eq 9. Refer to Scheme III for mechanism and definition of kinetic dissociation constants. The value for K_i when fixed was 12 mM as reported in Ream et al. (1992).

dissociation constant of 12 mM for the EPSPS·glyphosate binary complex (Ream et al., 1992) as a fixed value, a value for αK_b with good confidence results. With αK_b = 3.2 mM versus K_b = 22 mM (Table IV), it is evident that shikimate binds to the EPSPS·glyphosate binary complex about a factor of 7 greater than to EPSPS·PEP. These results also point out that while glyphosate does inhibit the EPS reaction, it does so at a significantly decreased level when compared to the EPSP reaction, which is in agreement with the dissociation constants of 12 mM (Ream et al., 1992) versus 0.15 μM (Ream et al., 1992; Anderson et al., 1988c) for glyphosate binding to free EPSPS and binary EPSPS·S3P, respectively. This represents an 80000-fold loss in binding capacity as determined by $K_{d(E\cdot glyphosate)}/K_{d(E\cdot S3P\cdot glyphosate)}$.

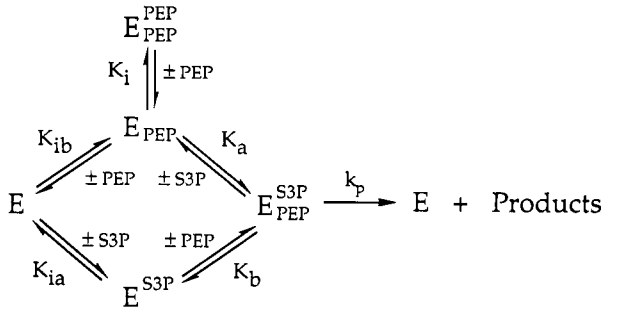
Discussion

EPSPS Kinetic Reaction Mechanism. The data and results presented in Figure 1 and Table I are consistent with two possible models: (1) the reaction is steady-state ordered with PEP acting as a competitive substrate inhibitor at high concentration by binding to free enzyme at either the S3P or PEP sites (i.e., strict orderedness in the reaction does not allow a productive EPSPS·PEP binary complex, and so it matters not whether PEP binding occurs at the PEP or S3P sites), or (2) the reaction is random with competitive substrate inhibition at high PEP concentration due to binding of PEP at the S3P site. Equilibrium ordered has already been dismissed by the initial velocity patterns along with results from 5-deoxy-S3P inhibition studies (refer to Results).

The K_i value for PEP in Table II at 6–8 mM is a factor of 15–20 greater than the measured K_d for PEP at 0.39 mM (Ream et al., 1992). For a steady-state ordered mechanism, the K_i value should match the measured K_d. While differences in techniques for measurement of a dissociation constant (i.e., kinetic vs direct binding) can account for a small disagreement in values, the quality of each fitted data set leads one to conclude that the differences are quite real and that these two values represent two different modes of binding for PEP. If this is the case, then a model must be brought forward to account for this behavior. A random mechanism is an attractive choice to explain these results. As defined by a random model, PEP binding to free EPSPS with a K_d of 0.39 mM represents the first step prior to S3P binding in the alternative pathway (Scheme II). The K_{ib} value in Table I for the EPSPS·PEP binary complex being within a factor of 2 of 0.39 mM is consistent with this interpretation. A random model also proposes that the competitive substrate inhibition exhibited by PEP (K_i = 6–8 mM, Table I) occurs through binding at the S3P site. Inhibition studies and ^{31}P NMR results showing PEP analogue affinity for the S3P site support this suggestion.

Another potential explanation can be made, however, regarding the 0.39 mM K_d for EPSPS·PEP binding formation. There is the possibility that there are two different PEP binding regions on the enzyme with the domain giving a K_d of 0.39

Scheme II: Random Kinetic Mechanism for Turnover of S3P and PEP As Catalyzed by EPSPS



mM being totally noncatalytic. If this is the case, then a steady-state ordered mechanism would still be possible. To further differentiate between random and steady-state ordered mechanisms, the exact nature of the PEP binary complex exhibiting a K_d of 0.39 mM, catalytic or not, needed to be resolved.

One approach to test the randomness in the mechanism is to investigate the orderedness of binding when utilizing alternate substrate analogues of S3P. One such substrate that was available for investigation with EPSPS was shikimic acid, which is identical to S3P, but lacks the 3-phosphate. If binding of S3P or an S3P analogue to free enzyme is not an absolute prerequisite for turnover (i.e., EPSPS·PEP is a productive intermediate along an alternate pathway), then one may be able to kinetically confirm EPSPS·PEP formation as a component binding process for catalysis in this alternative reaction. The fact that the data in Figure 3 point to an equilibrium ordered addition of substrates with PEP binding first, demonstrating a productive EPSPS·PEP complex as an obligatory step in the reaction, gives strong evidence to the overall random nature of this enzyme. The possibility that the K_d measured at 0.39 mM occurs at a noncatalytic domain can therefore be dismissed since the results presented in Tables II and IV demonstrate the absolute catalytic nature for this binding process. With the assignment of a random mechanism for the EPSP reaction, it can also be concluded that PEP substrate inhibition against S3P is indeed due to nonproductive competitive binding of PEP at the S3P site. A kinetic scheme for this is shown below with appropriately labeled dissociation constants that define the values presented in Table I.

Scheme II is simplified from a more complete equilibrium model in that only an EPSPS·(bis)PEP dead-end complex is considered. An EPSPS·PEP inhibitory complex where the single PEP is bound nonproductively at the S3P site is not included. This is justified from a consideration of dissociation constants. With a K_d of 6–8 mM for binding at the S3P site, an EPSPS·PEP inhibitory complex will not be present at a kinetically significant concentration. A productive EPSPS·PEP complex (K_d = 0.39 mM) will have previously formed. Therefore, an EPSPS·(bis)PEP complex is the only predominant dead-end complex present. This is supported by refitting of the data to eq 11, which is the rate equation for the

$$v = VAB/[K_{ia}K_b + K_aB + K_bA + AB + B^2K_a/K_i] \quad (11)$$

mechanism in Scheme II and a simplified version of eq 5. Here the term for the EPSPS·PEP inhibitory complex (one PEP molecule bound at the S3P site), $BK_{ia}K_b/K_i$, has been dropped. Results using this equation give identical values as those found in Table I.

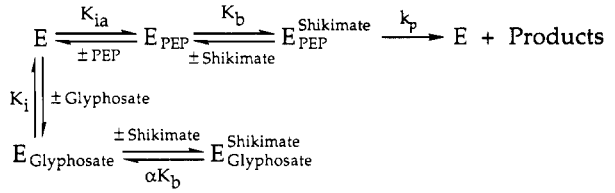
The kinetic constants of K_m for S3P (K_a = 3.2–3.6 μM) and PEP (K_b = 21–23 μM) shown in Table I are in good agreement with those found by Duncan et al. (1984) at 3.5 and 15 μM, respectively. The value for K_{ia} (i.e., the dissociation constant for the EPSPS·S3P binary complex) in Table I at 31–37 μM, however, is approximately 5 times that found by fluorescent binding measurements (Anderson et al., 1988c). This apparent discrepancy may partially be due to differences in measurement techniques but, to a much larger degree, is most likely a result of significant differences in reaction conditions. Initial velocity studies described here were done at 30 °C with an ionic strength $\mu \approx 300$ mM versus 20 °C and $\mu \approx 100$ mM for fluorescence binding studies.

Previous work on the *E. coli* enzyme led to the conclusion that EPSPS operates through a equilibrium ordered mechanism based on transient-state kinetic analysis and manual fitting of the data to obtain the best fit consistent with the pre-steady-state results (Anderson et al., 1988a; Anderson & Johnson, 1990a). Though this was an elegant study that gave the first strong evidence suggesting an EPSPS reaction mechanism occurring by an addition–elimination pathway through the formation of a tetrahedral intermediate, it is nevertheless clear that the steady-state rate data presented here are not consistent with a equilibrium ordered mechanism. The results from the previous transient-state kinetic analysis, however, can easily be integrated into a random model by considering that the reported microscopic rate constants are simply those for the bottom half of the pathway shown in Scheme II. Also, it is important to point out that a random kinetic mechanism is completely compatible with a chemical mechanism based on an addition–elimination reaction since, like equilibrium ordered, it is sequential in nature.

An equilibrium ordered mechanism was concluded by Anderson and Johnson (1990a), on the basis of a number of observations. First, binding of S3P to free enzyme occurs rapidly and with a dissociation constant close to the measured K_m. Data from trapping experiments also led to the conclusion that PEP does not bind to the enzyme in the absence of S3P in a mode kinetically competent to carry out catalysis. Only S3P appeared to fulfill the requirements as the first substrate in a equilibrium ordered reaction. These results are not inconsistent with the data presented here since, under the conditions of those experiments, an effective EPSPS·PEP binary complex did not exist at a significant concentration (<6%) based on a K_d of 0.39 mM. Therefore, it can be concluded that practical limitations of the experimental protocol prevented the observation of a competent EPSPS·PEP binary complex and therefore evidence for a random mechanism. The random character of the enzyme, in fact, is the probable explanation for only 70% trapping of [^{14}C]S3P in the reported pulse–chase experiments where 100% is the expected result for an equilibrium ordered reaction (Anderson et al., 1988a).

Glyphosate inhibition of the EPS reaction is interesting since a significant amount of inhibitory power is lost compared to the normal reaction. An explanation for this lies in the loss of synergistic binding that is specific for ordered addition of S3P followed by glyphosate. This is clearly demonstrated by Ream et al. (1992), where the K_d for EPSP·S3P·glyphosate ternary complex is 0.15 μM compared to 12 mM for EPSPS·glyphosate binary complex. In comparison, PEP gives dissociation constants of 22 and 390 μM for the ternary and binary complexes, respectively (Table I; Ream et al., 1992). Synergistic binding of S3P and glyphosate to EPSPS is therefore much stronger than of S3P and PEP by an approximate factor of 4500 as defined by $K_{d(E\cdot glyphosate)}K_{d(E\cdot S3P\cdot PEP)}/K_{d(E\cdot PEP)}K_{d(E\cdot S3P\cdot glyphosate)}$. It is important to point out that it is this strong synergism that accounts for the uncompetitive kinetic behavior versus S3P for

Scheme III: Equilibrium Ordered Kinetic Mechanism for Turnover of Shikimate and PEP As Catalyzed by EPSPS in the Presence of the Inhibitor Glyphosate



glyphosate inhibition of the EPSP reaction and not the orderedness in substrate addition (S3P followed by PEP) as has been previously proposed. Scheme III shows the equilibrium ordered kinetic mechanism for the EPS reaction and its inhibition with glyphosate. Equation 9 is the rate equation for this mechanism.

The glyphosate inhibition studies for the EPS reaction presented here and binding results presented by Ream et al. (1992) are the key to understanding why the literature contains a number of proposed steady-state kinetic mechanisms for EPSPS. Much of the weight for ordered mechanisms rested on the fact that glyphosate is an uncompetitive inhibitor of the enzyme versus S3P, but competitive versus PEP. The results presented here and the preceding paper show, however, that while glyphosate is competitive versus PEP, it shares little resemblance to the PEP ground-state structure in terms of affinity for free enzyme. Thus, as a tool for deciphering the steady-state kinetic reaction mechanism, glyphosate was a poor choice that gave misleading information. With this consideration, it seems probable that EPSPS from all sources may not be so kinetically diverse as the literature would suggest, since with exclusion of the glyphosate inhibition data, initial velocity studies are all consistent with a random mechanism.

While the data here are conclusive for the forward reaction, the reverse reaction has not been addressed. Transient steady-state kinetic analysis has suggested an equilibrium ordered process (Anderson & Johnson, 1990a). This has been independently supported by dual competitive inhibition patterns versus both EPSP and P_i using a potent inhibitor structurally based on the tetrahedral intermediate (Alberg & Bartlett, 1989). This pattern of inhibition is usually diagnostic of an equilibrium ordered mechanism, which, on the surface, can be taken as a steady-state check of the proposed mechanism. With potent inhibition, however, an inhibitor based on the tetrahedral intermediate will be bound to both the S3P and PEP sites. As such, the inhibitor combines only to free enzyme. Inhibitor binding to an EPSPS·substrate binary complex would occur only with a concurrent large increase in K_i since half of the inhibitor molecule is not being utilized for binding. With this in mind, it can be seen how competitive inhibition patterns for both substrates are consistent with both random and equilibrium ordered mechanisms. Therefore, an assignment for a equilibrium ordered process in the reverse reaction is still tentative and needs further verification.

EPSPS Inhibition by PEP Analogues. The data from PEP analogue inhibition studies, along with the supporting ^{31}P NMR binding results, conclusively show that binding of these compounds occurs readily to free enzyme in addition to the EPSPS·S3P binary complex at both the PEP and S3P sites. In combination with the initial velocity studies, it is evident that these analogues mimic, though to varying degrees, the binding behavior that is exhibited by PEP itself. This is in remarkable contrast to glyphosate, which exhibits relatively weak affinity for free enzyme and whose binding appears to be exclusive to the PEP site [The overlap between the glyphosate and PEP binding sites is based on (1) results from the EPS reaction (Figure 9 and Table IV), (2) competitive behavior vs PEP and uncompetitive behavior vs S3P in the EPSP reaction (Boocock & Coggins, 1983; Boocock, 1985), and (3) equilibrium dialysis and microcalorimetry measurements which demonstrate that a large excess of glyphosate will reduce PEP affinity for free enzyme by more than 95% (Ream et al., 1992)]. These results have significance from two viewpoints: (A) the pronounced difference in specificity stated above adds additional evidence to the original proposal of Steinrücken and Amrhein (1984b) that glyphosate is not a ground-state PEP mimic, but more likely a mimic of a transition-state structure of the protonated, C2-carbonium ion form of PEP; and (B) the binding capacities of I and II for free enzyme at both the S3P and PEP domains support a more random component to binding of substrates than has been previously suggested by Anderson et al. (1988a).

The dual binding modes for I and II to EPSPS as discussed above make it difficult to quantitate the kinetic results in terms of exact values for the inhibitor dissociation constants for binding at both the S3P or PEP sites. This is due to the multiple enzyme forms that can exist, including general complexes such as EPSPS·(bis)inhibitor, EPSPS·inhibitor·substrate, etc. For example, if PEP binding at the PEP site occurs under conditions where I or II are bound at the S3P site, then an intercept effect (K_{ii}) on variable PEP concentration would be amplified by the fact that an additional dead-end complex, EPSPS·inhibitor·PEP, is created. In this situation, PEP binding is induced by the dead-end EPSPS·inhibitor complex with the intercept effect being dependent on how well PEP binding is synergized (i.e., the greater the synergism, the greater the effect on the intercept). This type of behavior, synergistic binding of substrates with inhibitors, is demonstrated for EPSPS with glyphosate and in all likelihood occurs with I and II as well. Indeed, II appears to be a much weaker inhibitor of the EPS reaction than the EPSP reaction, presumably from significant loss of synergistic binding (K. J. Gruys and J. A. Sikorski, unpublished results). From this discussion, it is evident that a complete rate equation that defines this collective binding would be quite complex and not practical for data fitting. Nevertheless, considering the initial velocity studies, it is accurate to interpret the mixed inhibition behavior versus variable S3P as due both to the random character of the enzyme as well as the binding affinity of I and II for the S3P site.

The exact reason why I and II should have such binding capacity for the S3P site is unclear but may be based on the larger dipole moments of I and II versus PEP. The crystal structure for EPSPS predicts that the active site contains a high concentration of positively charged amino acid side chains which are available for the formation of stabilizing salt bridges upon substrate binding. Additionally, the multiple helices of each domain are oriented such that a positive dipole is focused in the vicinity of the substrate binding pocket (Stallings et al., 1991). This concentration of ionic charges and dipole interactions in the S3P site is apparently better realized by I and II, presumably because of their stronger dipole moment. While PEP binding at the S3P domain is measurable, it is clear that this occurs to a much smaller degree compared to I and II and at concentrations that are probably never realized in vivo. It should be noted that, similar to the results shown here, I has been shown to bind to 3-deoxy-D-*arabino*-heptulosonic acid-7-phosphate synthase, the first enzyme in the shikimate pathway, with two distinct K_i values, suggesting more than one binding site on this enzyme for I (Pilch & Somerville, 1976).

Contributions of the Shikimate 3-Phosphate Moiety to Specificity and Catalysis. As the data demonstrated, the absence of the 3-phosphate moiety on the shikimate ring results in a change in kinetic mechanism so that shikimate, in contrast to S3P, appears not to bind to EPSPS in the absence of PEP. This kinetic observation can be misleading, however, since presumably the overall mechanism is still random with synergistic binding of substrates. The most appropriate interpretation of the kinetic results for the EPS reaction is that substrate synergism is enhanced to the point where binding of shikimate to free enzyme cannot be observed kinetically. For example, if binding of shikimate to binary EPSPS·PEP versus free EPSPS was enhanced (synergized) by a factor of 25, an EPSPS·shikimate binary complex would have a K_d of 625 mM, which would not be measured, and would result in an initial velocity pattern identical to that observed experimentally (equilibrium ordered). With the EPSP reaction, substrate synergism accounts for an enhancement by a factor of 8.7–11.9 (Table I). Thus, the difference in substrate synergism for the EPS and EPSP reactions is one of degree, which in combination with much slower turnover of shikimate results experimentally in the different initial velocity patterns. A somewhat similar situation was seen by Knight and Cleland (1989) for glycerokinase. In that case, the order of substrate addition appears to reverse when going from a tightly bound substrate (glycerol) to a poorly bound slow one (aminoglycerol).

The value for K_{cat}/K_m includes both the activation and binding energies for a particular substrate and can be used for comparison of alternate substrates to help appraise how binding energy is utilized for particular functional groups, both in terms of substrate specificity and lowering of activation energy (Fersht, 1985). In the present case, when comparing the 3-phosphate group on the shikimate ring to a hydroxyl in this position (8.7 kcal/mol difference in binding energy), greater than 60% of binding energy is utilized in recognizing substrate at the expense of increasing catalytic efficiency. Nature has designed this specificity for S3P over shikimate since chorismate formation, the next reaction in the shikimate pathway catalyzed by chorismate synthase, absolutely requires the energetically favorable elimination of the 3-phosphate group. Avoiding shikimate turnover by EPSPS therefore eliminates a metabolically dead-end product.

It is clear that EPSPS takes full advantage of binding energy made available by the presence of the 3-phosphate group on the shikimate ring. The binding domain of the enzyme is known to be polycationic and is thus designed to recognize polyanions such as phosphate to form salt bridges and hydrogen bonds (Stallings et al., 1991). It is known that the contribution of hydrogen bonds to enzyme–substrate binding energy results from entropy rise through release of hydrogen-bonded water which can add a significant amount of binding energy to the enzyme·substrate complex. The 3-phosphate on S3P appears to accomplish this favorable process to some degree as evidenced by a positive entropy change of 5.4 cal/mol·K as measured by microcalorimetry (Ream et al., 1992) for binding of S3P to enzyme. The poor binding of shikimate to EPSPS then is probably due to both leaving an enzyme salt bridge exposed and unneutralized by substrate and by not taking advantage of positive hydrogen-bonding entropy effects through release of water. This has obvious implications to the design of inhibitors for EPSPS in that tight binding will absolutely require a functional group that very closely resembles the spatial and ionic characteristics of the 3-phosphate moiety.

Another consideration to weak binding of shikimate is the appropriate solution structure of substrate that the enzyme recognizes. Recent results from this laboratory have elucidated the preferred conformation of S3P in the pH range of 5–9, which brackets the second ionization of the 3-phosphate group (Castellino et al., 1991). The 3-phosphate on S3P helps lock the conformation of S3P to a half-chair with phosphate in an axial position. This has recently been confirmed as the bound active site structure of S3P on the enzyme (Leo et al., 1992). Shikimate, by lacking the 3-phosphate group, is not so constrained and has a wider population of conformers in solution. A possible conclusion from this analysis is that, with shikimate, enzyme either has less available substrate because of a population of nonbinding conformers, or binding of all conformers is allowed but binding energy must then be utilized for rearrangement to a preferred structure for catalysis. Either case would result in weaker binding of shikimate.

Conclusions. Collectively, the results presented here strongly suggest that the steady-state reaction mechanism for EPSPS from *E. coli* in the forward direction is appropriately classed as random but subject to synergistic binding of substrates and inhibitors. For S3P and PEP as substrates, the EPSP reaction is random, with substrate synergism accounting for a lowering in K_d by an approximate factor of 10 when binding as the second substrate (Table I). Shikimate binding to free enzyme, on the other hand, is not kinetically measurable and depends on stronger synergism created by EPSPS·PEP formation to function as a substrate for EPSPS. Because of this, the EPS reaction is equilibrium ordered. This synergistic behavior explains why glyphosate acts as a strong inhibitor of the EPSP reaction, appearing as an uncompetitive inhibitor of S3P, but expresses poor inhibition of the EPS reaction. Synergism also accounts for how shikimate binds to the EPSPS·glyphosate binary complex stronger than to EPSPS·PEP. In addition to EPSPS, two examples of similar behavior have been described for glycerokinase and yeast hexokinase (Knight & Cleland, 1989; Viola et al., 1982). It seems quite plausible that many supposedly ordered mechanisms for other enzymes are of this type and are inherently more random in character.

The combined studies of steady-state and transient-state kinetic analysis for EPSPS demonstrate the importance of relying on both to understand enzymatic reaction mechanisms. Steady-state results provide the leads to active site reactions that can then be tested by transient-state kinetic techniques. Having an accurate picture for the steady-state kinetic mechanism, therefore, provides an absolutely essential framework from which to design non-steady-state experiments with enzyme as a stoichiometric reactant. As such, it can be seen that an incomplete picture for the steady-state reaction mechanism will likely lead to an incomplete picture for the reactions occurring at the active site. Both approaches, therefore, act as checks and balances for inconsistencies in deciphering the overall mechanism and provide a more complete view of the kinetic pathway. In this study, results from ^{31}P NMR and steady-state kinetic analysis utilizing alternate substrates and inhibitors to probe the S3P and PEP binding sites provided an important complement to the previous transient-state kinetic analysis.

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