Reevaluating Glyphosate as a Transition-State Inhibitor of EPSP Synthase: Identification of an EPSP Synthase•EPSP•Glyphosate Ternary Complex

R. Douglas Sammons,[‡] Kenneth J. Gruys,*[,‡] Karen S. Anderson,[‡,§] Kenneth A. Johnson,[||] and James A. Sikorski*[,⊥]

New Products Division, The Agricultural Group, and Monsanto Corporate Research, Units of Monsanto Company, 700 Chesterfield Parkway North, St. Louis, Missouri 63198, and Department of Molecular and Cell Biology, The Pennsylvania State University, University Park, Pennsylvania 16802

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ABSTRACT: Numerous studies have confirmed that glyphosate forms a tight ternary complex with EPSP synthase and shikimate 3-phosphate. It has been proposed [Anton, D., Hedstrom, L., Fish, S., & Abeles, R. (1983) *Biochemistry 22,* 5903−5908; Steinrücken, H. C., & Amrhein, N. (1984) *Eur. J. Biochem. 143,* 351−357] that in this complex glyphosate functions as a transition-state analog of the putative phosphoenolpyruvoyl oxonium ion. For this to be true, glyphosate must occupy the space in the enzyme active site that is normally associated with PEP and, through turnover, the carboxyvinyl group of the product EPSP. According to this model, one would predict that, in the reverse EPSP synthase reaction with EPSP and phosphate as substrates, there should be little if any interaction of glyphosate with enzyme or enzyme•substrate complexes. In contrast to this expectation, rapid gel filtration experiments provided direct evidence that glyphosate could be trapped on the enzyme in the presence of EPSP to form a ternary complex of EPSPS•EPSP•glyphosate. The experimentally determined stoichiometry for this complex, 0.62 equiv of glyphosate/mole of EPSPS, is similar to that found for the EPSPS•S3P•glyphosate ternary complex (0.66). This direct binding result was corroborated and quantitated by fluorescence titration experiments which demonstrated that glyphosate forms a reasonably tight (K_d = 56 ± 1 μM) ternary complex with enzyme and EPSP. This finding was further verified, and its impact on substrate turnover analyzed, by steady-state kinetics. Glyphosate was found to be an uncompetitive inhibitor versus EPSP with $K_{ii(app)}$ = 54 ± 2 μM. Taking these results together, it is apparent that the carboxyvinyl group in EPSP does not prevent glyphosate binding, and in fact it strongly facilitates the binding of this inhibitor to the enzyme. It has been previously demonstrated that glyphosate has little (K_d = 12 mM) interaction with free enzyme [Ream, J. E., Yuen, H. K., Frazier, R. B., & Sikorski, J. A. (1992) *Biochemistry 31,* 5528−5534]. Interestingly, glyphosate was a mixed inhibitor ($K_{is(app)}$ = 18.2 ± 0.4 μM, $K_{ii(app)}$ = 23.9 ± 0.3 μM) versus phosphate. This surprising result suggests that the formation of an EPSPS•EPSP•glyphosate ternary complex does not preclude phosphate binding and that a quaternary complex of enzyme containing both substrates plus inhibitor can occur. The proposed model for glyphosate functioning as a transition state analog inhibitor, which has defined the paradigm for glyphosate's molecular mode of action for more than a decade, is very difficult to reconcile with these results. Glyphosate does not meet the key criteria required of a transition-state analog or, alternatively, an intermediate mimic inhibitor. Glyphosate, therefore, can no longer be classified as such for this system.

The enzyme 5-enolpyruvoylshikimate 3-phosphate synthase (EPSPS,[1] E.C. 2.5.1.19) has been the subject of numerous biochemical studies since it functions as the biological target for the commercially successful herbicide glyphosate **2** [*N*-(phosphonomethyl)glycine] (Franz, 1985; Amrhein et al., 1980). EPSPS catalyzes an unusual transfer reaction of the carboxyvinyl portion of phosphoenolpyruvate (PEP) regiospecifically to the 5-OH of shikimate 3-phosphate (S3P) forming EPSP and inorganic phosphate (P_i) [for reviews, see Sikorski et al. (1991, and Anderson and Johnson (1990a)]. The *Escherichia coli* enzyme exhibits a random kinetic mechanism in the forward and reverse reactions (Gruys et al., 1992, 1993) through a single, kinetically competent (Anderson et al., 1988a,b), tightly bound (Anderson & Johnson, 1990b), tetrahedral intermediate, **1** (Scheme 1).

For all EPSP synthases studied to date, glyphosate has been characterized as a reversible competitive inhibitor versus PEP and an uncompetitive inhibitor versus S3P (Kishore & Shah, 1988). In the past, these inhibition patterns have been utilized in part to provide insight into the kinetic and chemical mechanisms. As such, glyphosate's effect on EPSPS kinetics appeared to support the ordered binding of S3P followed by PEP (Anderson & Johnson, 1990a). As

* Authors to whom correspondence should be addressed. Tel: (314) 537-7345 (for K.J.G.) or (314) 537-6375 (for J.A.S.).

‡ New Products Division, The Agricultural Group, Monsanto Company.

§ Present address: Department of Pharmacology, Yale University Medical School, 333 Cedar Street, Box 3333, New Haven, CT 06150.

|| The Pennsylvania State University.

⊥ Monsanto Corporate Research, Monsanto Company.

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[1] Abbreviations used: EPSPS, 5-enolpyruvoylshikimate 3-phosphate synthase; S3P, shikimate 3-phosphate; PEP, phosphoenolpyruvate; EPSP, 5-enolpyruvoylshikimate 3-phosphate; P_i, inorganic phosphate; glyphosate, *N*-(phosphonomethyl)glycine; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PK, pyruvate kinase; LDH, lactic dehydrogenase; ADP, adenosine 5′-diphosphate; UDP-GlcNAc, UDP-*N*-acetylglucosamine; UDP-GlcNAc enolpyruvyl transferase, UDP-*N*-acetylglucosamine enolpyruvoyl transferase.

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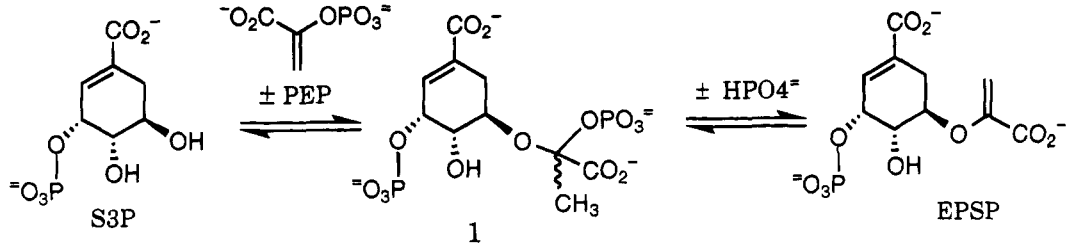
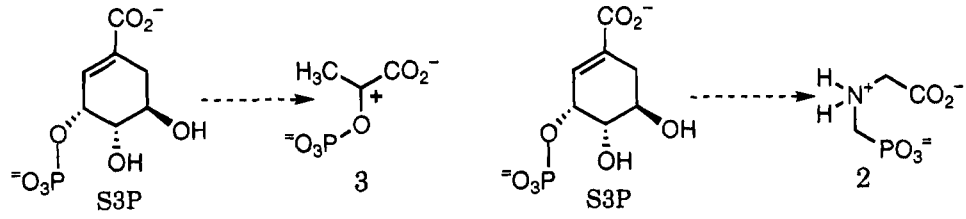
Scheme 1: EPSPS-Catalyzed Reaction



Scheme 2: PEP Oxonium Ion **3**, Formed Transiently during Catalysis, and Transition-State Analogy Proposed for Glyphosate **2**



mentioned above, however, we have recently shown unequivocally that the steady-state kinetic mechanism proceeds through random addition of substrates in both the forward and reverse directions (Gruys et al., 1992, 1993). A random kinetic mechanism appears to be inconsistent with the glyphosate inhibition patterns, but can be explained by the unique binding characteristics of the inhibitor to EPSPS. Glyphosate binds to the EPSPS·S3P binary complex over free enzyme by almost 4 orders of magnitude. In contrast, the binding of PEP to the EPSPS·S3P binary complex is enhanced by only a factor of 10−20 versus free enzyme (Ream et al., 1992; Gruys et al., 1992). This leads one to conclude that while glyphosate is competitive versus PEP, it shares little resemblance to the PEP ground-state structure. As such, the inhibition patterns for glyphosate versus PEP and S3P are misleading in the context of understanding the kinetic mechanism of the enzyme.

Three additional lines of evidence support the conclusion that glyphosate is not a ground-state mimic of PEP: (1) UDP-GlcNAc enolpyruvyltransferase, the only other enzyme besides EPSPS that is known to catalyze a carboxyvinyl transfer from PEP to a secondary alcohol, is not inhibited by glyphosate (Steinrücken & Amrhein, 1984). (2) Glyphosate does not significantly inhibit any other PEP-utilizing enzyme (Steinrücken & Amrhein, 1984). (3) Dead-end inhibitors of EPSPS that are clear structural mimics of PEP demonstrate mixed, not uncompetitive, kinetics versus S3P (Walker et al., 1991; Gruys et al., 1992). If glyphosate is not a ground-state mimic of PEP, and yet is a competitive inhibitor versus this substrate, the question arises as to what then mechanistically defines glyphosate's unique interaction with EPSPS.

Numerous studies using solution (Castellino et al., 1989) and solid-state (Christensen & Schaefer, 1993) NMR, fluorescence (Anderson et al. 1988c), gel filtration (Castellino et al., 1989), differential scanning (Merabet et al., 1993), and titration calorimetry (Ream et al., 1992) corroborate the formation of a tight ternary complex between S3P, EPSPS, and glyphosate and implicate this complex as the likely herbicidal species present *in planta*. Glyphosate exhibits a highly specific interaction in this complex, since minor structural variations induce major changes in enzyme affinity (Steinrücken & Amrhein, 1984; Knowles et al., 1993). The combined competitive kinetic patterns observed versus PEP and the high structural specificity at the glyphosate binding site have been used to support the proposal that glyphosate functions as a transition-state analog inhibitor of the putative PEP oxonium ion **3** formed transiently during catalysis (Scheme 2) (Steinrücken & Amrhein, 1984; Anton et al., 1983). More recently, as an alternative to the transition-state analogy, the tetrahedral geometry at the quaternary center in **1** and its tight interaction with enzyme led to the proposal that the bound EPSPS·S3P·glyphosate ternary complex might mimic the enzyme-bound conformation stabilizing **1** (Anderson & Johnson, 1990a). Here we report results from an investigation of the glyphosate·EPSPS binding interaction in the presence of EPSP along with glyphosate inhibition studies of the *E. coli* EPSPS reverse reaction which cast serious doubt on these two proposals.

MATERIALS AND METHODS

Enzyme Purification. EPSPS was isolated from a cloned *E. coli* strain which overproduces the enzyme (Rogers et al., 1983) and 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) kit procedure from Pierce Chemical Co. 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 treating shikimic acid (Sigma) with shikimate kinase (Millar et al., 1986) and was purified using ion-exchange HPLC techniques (Castellino et al., 1991). EPSP and [2′-^{14}C]EPSP were enzymatically synthesized from S3P and PEP (Sigma) or [2-^{14}C]PEP (New England Nuclear), respectively, using catalytic amounts of EPSPS, and purified as previously described (Anderson et al., 1988b). The EPSP used in these studies was analytically pure (≥98%) and contained ≤1% S3P by HPLC analyses. Glyphosate and [1-^{14}C]glyphosate were obtained from Monsanto internal stocks and were analytically pure (>99%). HEPES, NADH, ADP, lactic dehydrogenase (LDH), and pyruvate kinase (PK) were purchased from Sigma Chemical Company. All other reagents were of highest commercial purity. Barnstead Nanopure water was used for all solutions.

Rapid Gel Filtration Experiments. The procedures for all rapid gel filtration experiments (Penefsky, 1979) were

followed as outlined by Castellino et al. (1989). Four combinations of enzyme (approximately 60 μM for each) with S3P, EPSP, and glyphosate were evaluated for equivalents of radiolabeled ligand bound to EPSPS: (1) attempted isolation of an EPSPS·[1-^{14}C]glyphosate binary complex using EPSPS and 10× [1-^{14}C]glyphosate, (2) isolation of the dead-end ternary complex of EPSPS·S3P·[1-^{14}C]glyphosate using EPSPS and 10× each of S3P and [1-^{14}C]glyphosate, (3) isolation of a ternary complex of EPSPS·EPSP·[1-^{14}C]-glyphosate using EPSPS and 10× each of EPSP and [1-^{14}C]-glyphosate, and (4) isolation of a ternary complex of EPSPS·[2′-^{14}C]EPSP·glyphosate using EPSPS and 10× each of [2′-^{14}C]EPSP and glyphosate.

Fluorescence Titration Experiments. EPSPS fluorescence measurements were performed following the methods described by Anderson et al. (1988c) using an SLM 4900 fluorometer (Urbana, IL) thermostatically controlled at 20 °C in a 2.5-mL cell. The excitation wavelength was 280 nm, and the emission wavelength was 360 nm (8-nm bandpass). Buffer was 50 mM Hepes and 50 mM KCl, pH 7.0. The decrease in fluorescence was monitored as ligand was added in small increments (0.5 μL) to the buffered solution of EPSPS (2.0 mL). Fluorescence values were determined at each addition from an average of four readings and with correction for dilution. These values were plotted versus the total ligand concentration, and a fitted curve was calculated by nonlinear regression using eq 1, which relates observed fluorescence F to ligand concentration [L]:

$$F = F_i - \Delta F[(K_d + [E_t] + [L]) - ((K_d + [E_t] + [L])^2 - 4[E_t][L])^{1/2}]/2[E_t] \quad (1)$$

E_t represents the total enzyme concentration and was fixed for all experiments at 0.5 μM. The dissociation constant (K_d), initial fluorescence (F_i), and total change in fluorescence (ΔF) were determined by a least-squares fit of each data set. The calculated K_d for glyphosate binding to the EPSPS·EPSP binary complex was determined by first saturating the enzyme with [EPSP] = 100 μM so as to ensure complete formation of binary complex. This assumes a minimal model for glyphosate addition that occurs in two steps with EPSP binding first followed by glyphosate as indicated in eqs 2 and 3.

$$E + EPSP \overset{K_{d,EPSP}}{\Leftrightarrow} E{\cdot}EPSP \quad (2)$$

$$E{\cdot}EPSP + glyphosate \overset{K_{d,glyphosate}}{\Leftrightarrow} E{\cdot}EPSP{\cdot}glyphosate \quad (3)$$

An intermediate step (i.e., an enzyme isomerization occurring between the formation of the EPSPS·EPSP binary complex and the addition of glyphosate), if such an intermediate exists in a kinetically distinct step, would not be detected under the employed equilibrium conditions. The calculated K_d in such a situation would then be an apparent K_d (i.e., the product of the true K_d for glyphosate binding and any preequilibrating step).

Kinetic Assays. All kinetic studies of the reverse reaction were monitored using a continuous spectrophotometric coupled assay that follows the loss of NADH absorbance at 340 nm ($\epsilon = 6.22 \times 10^3$ cm^{-1} M^{-1}) due to oxidation to NAD$^+$ (Gruys et al., 1993; Alberg et al., 1992). This conversion was accomplished utilizing PK and LDH as the coupling enzymes to capture EPSPS-produced PEP. Reactions were run at 25 °C with 100 mM potassium HEPES, pH 7.5, 4 mM $MgCl_2$, 2.5 mM ADP, and additional components listed below that varied depending on the particular experiments. The UV/vis instrument used was a Hewlett Packard Model 8452A diode array spectrophotometer controlled by an MS-DOS computer using the HP UV/vis kinetics software. Initial steady-state rates were calculated by the software in the first 0−60 s of reaction depending on exact conditions. All reactions were initiated with EPSPS. The final concentration of enzyme varied from 1.5 to 7.5 nM.

For glyphosate inhibition studies with variable EPSP (KP_i was fixed at 50 mM), reactions were performed using a 10-cm path length cylindrical cell in a total volume of 4 mL using 25 μM NADH, 80 units/mL LDH, and 110 units/mL PK in addition to the buffer components described above. Due to the low K_m value for EPSP, a 10 cm path length cell was essential for these studies in order to obtain sufficient sensitivity. For reactions where EPSP was fixed at 50 μM and KP_i was varied, the cocktail was modified to 0.15 mM NADH, 22 units/mL LDH, and 30 units/mL PK in a total volume of 1 mL. The assay in this case was monitored using a 1-cm path length rectangular cell.

Kinetic data were fitted using the commercial software GraFit (Leatherbarrow, 1990) and the velocity equations given below. Equations 4 through 6 described competitive, uncompetitive, and mixed noncompetitive inhibition models respectively.

$$v = VA/[K_a(1 + I/K_{is}) + A] \quad (4)$$

$$v = VA/[K_a + A(1 + I/K_{ii})] \quad (5)$$

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

The individual terms define v, the velocity; V, the maximal velocity; K_a, the apparent Michaelis constant; K_{is} or K_{ii}, the apparent inhibitory constants; A, the variable substrate concentration; and I, the inhibitor concentration.

RESULTS AND DISCUSSION

To properly function as a transition state analog inhibitor in the forward direction, glyphosate should completely overlap with the presumably planar **3**, even though it contains one extra atom between the anionic centers. Since the EPSPS reaction is freely reversible, a transition-state analog should function as a potent inhibitor in both directions. In the case of glyphosate, however, in experiments that probe the reverse reaction, one would expect that the carboxyvinyl group present in EPSP would preclude glyphosate from binding. The alternative suggestion to the transition-state analogy, where it is proposed that the ternary EPSPS·S3P·glyphosate complex might mimic the enzyme-bound conformation stabilizing **1**, would lead one to the same expectation for effects of the EPSP carboxyvinyl group on glyphosate binding. Results from fluorescence titration experiments would appear to support either of these proposals. As described by Anderson et al. (1988c), the decrease in fluorescence emission induced by EPSP binding to free enzyme is similar to, but smaller than, that induced by the addition of glyphosate to the preformed EPSPS·S3P binary complex (9.6% versus 16%). Since no comparable fluorescence changes are observed with either S3P, PEP, or glyphosate alone with enzyme, it seemed reasonable to

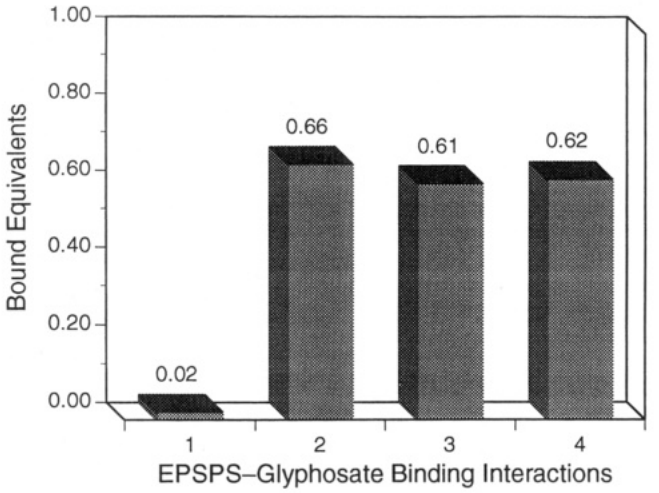


FIGURE 1: Glyphosate binding equivalents on EPSPS as determined by rapid gel filtration experiments. Refer to the Materials and Methods for experimental details. Reaction 1: EPSPS + [1-^{14}C]-glyphosate. Reaction 2: EPSPS + S3P + [1-^{14}C]glyphosate. Reaction 3: EPSPS + EPSP + [1-^{14}C]glyphosate. Reaction 4: EPSPS + [2′-^{14}C]EPSP + glyphosate.

conclude that there must be significant overlap at the enzyme active site between glyphosate and the carboxyvinyl group of EPSP.

On the basis of transition-state and tetrahedral intermediate binding models it is conceivable, however, that glyphosate binding could occur in the EPSPS reverse reaction due to an interaction of the glyphosate phosphonic acid moiety with the P_i binding domain on the enzyme. The P_i site is accessible with either free enzyme or the EPSPS·EPSP binary complex, although it is a low-affinity domain (i.e., K_ds of 14 and 4.4 mM, respectively, for P_i; Gruys et al., 1993). Indeed, we have shown that glyphosate can bind to free enzyme with a K_d of 12 mM and that this binding is competitive with PEP in the forward reaction when shikimate is substituted for S3P as cosubstrate (Ream et al., 1992; Gruys et al., 1992). Collectively this leads one to predict that, for the reverse reaction, glyphosate's effect would be limited to the weak binding to free enzyme and that such binding would compete with both EPSP and P_i since the inhibitor presumably spans all of the latter site and part of the former one.

Identification of a Ternary Complex of EPSPS·EPSP·Glyphosate by Rapid Gel Filtration. Our first observation that the above predictions were incorrect, and that the proposals for how glyphosate interacts with EPSPS would need to be modified, came from rapid gel filtration experiments. Figure 1 confirms that EPSPS and glyphosate alone do not significantly interact to form a complex that can pass through a rapid gel filtration column intact (reaction 1). Reactions 3 and 4, however, show that in the presence of EPSP a tight complex does form, presumably as a ternary EPSPS·EPSP·glyphosate complex. The fact that the bound equivalents are virtually identical regardless of whether the ^{14}C-label is in glyphosate or EPSP strongly supports the identity of this complex as EPSPS·EPSP·glyphosate.

Verification of the identity of ligands bound to EPSPS was confirmed by using Mono-Q HPLC separation of ligands following protein precipitation and then comparing the retention times to standards (not shown). This excludes the possibility that the complex formation indicated by Figure 1, column 3, is the ternary complex of EPSPS·S3P·[1-^{14}C]-glyphosate where the S3P was a result of EPSP hydrolysis. Figure 1, column 4, also excludes this possibility since hydrolysis of radiolabeled [2′-^{14}C]EPSP leads to no label in bound enzyme components (i.e., hydrolysis produces S3P and [2-^{14}C]pyruvate, the latter of which is trapped in the gel matrix as a small molecule).

Interestingly, when identical conditions are used for the trapping experiments, the bound equivalents for the ternary EPSPS·S3P·glyphosate complex is not significantly greater than that for the EPSPS·EPSP·glyphosate complex (i.e., reaction 2 versus 3 or 4). Combining this observation with a consideration of the concentrations of enzyme and ligands used in these experiments indicates that glyphosate has a reasonable affinity for the EPSPS·EPSP binary complex, certainly much greater than what has been demonstrated for glyphosate binding to free enzyme (Ream et al., 1992; Gruys et al., 1992). In addition, similar to the EPSPS·S3P·glyphosate complex, where the k_{off} for glyphosate is 0.12 s^{-1} (Anderson et al., 1988c), the successful trapping experiments in reactions 3 and 4 indicate that the dissociation of this new ternary complex must also be slow (i.e., k_{off} for glyphosate is $\leq$0.5 s^{-1}).

Quantitation of the EPSPS·EPSP·Glyphosate Ternary Complex by Fluorescence Titration. The formation of an EPSPS·EPSP·glyphosate ternary complex was independently confirmed and quantitated by equilibrium fluorescence binding experiments. This technique was previously used to characterize glyphosate binding in the EPSPS·S3P·glyphosate ternary complex (Anderson et al., 1988c; Shuttleworth & Evans, 1994). Binding of glyphosate to the preformed EPSPS·EPSP binary complex causes a slight blue shift in the emission λ_{max} and reduces the EPSPS fluorescence emission at 360 nm by 14.4% (not shown). Considering that the initial formation of the binary EPSPS·EPSP complex has a 9.6% reduction in fluorescence emission relative to native enzyme (Anderson et al., 1988), the total decrease in fluorescence from the native enzyme to the ternary EPSPS·EPSP·glyphosate complex is 24%. This compares to 16% for the formation of the EPSPS·S3P·glyphosate ternary complex (Anderson et al., 1988). Thus, glyphosate induces an additional conformational change in enzyme not accessible when EPSP alone is bound at the active site, and the conformational change accompanying formation of the EPSPS·EPSP·glyphosate ternary complex would appear to be greater in magnitude than that observed for the formation of EPSPS·S3P·glyphosate.

Figure 2a shows the titration of binary EPSPS·EPSP complex with glyphosate. Under established equilibrium conditions (Anderson et al., 1988c), the K_d for formation of the ternary EPSPS·EPSP·glyphosate complex was determined to be 56 $\pm$ 1 μM. This dissociation constant was independently corroborated by monitoring the fluorescence changes induced by adding EPSP to a mixture of enzyme and glyphosate where glyphosate was fixed at 350 μM. The binding curve (Figure 2b, $K_{d,appEPSP}$ = 0.307 μM) obtained from this experiment gave an indirect measurement of the binary EPSPS·EPSP complex with a calculated K_d of 1.9 $\pm$ 0.2 μM using eq 7 and the previously determined K_d of 56 μM for the EPSPS·EPSP·glyphosate complex.

$$K_{d,EPSP} = (K_{d,appEPSP})[\text{glyphosate}]/(K_{d,EPSP\cdot glyphosate}) \quad (7)$$

This equation is valid under the conditions of the experiment [refer to Anderson et al. (1988) for details]. The same approximate affinity for EPSP in a binary complex with enzyme has been independently confirmed in the absence

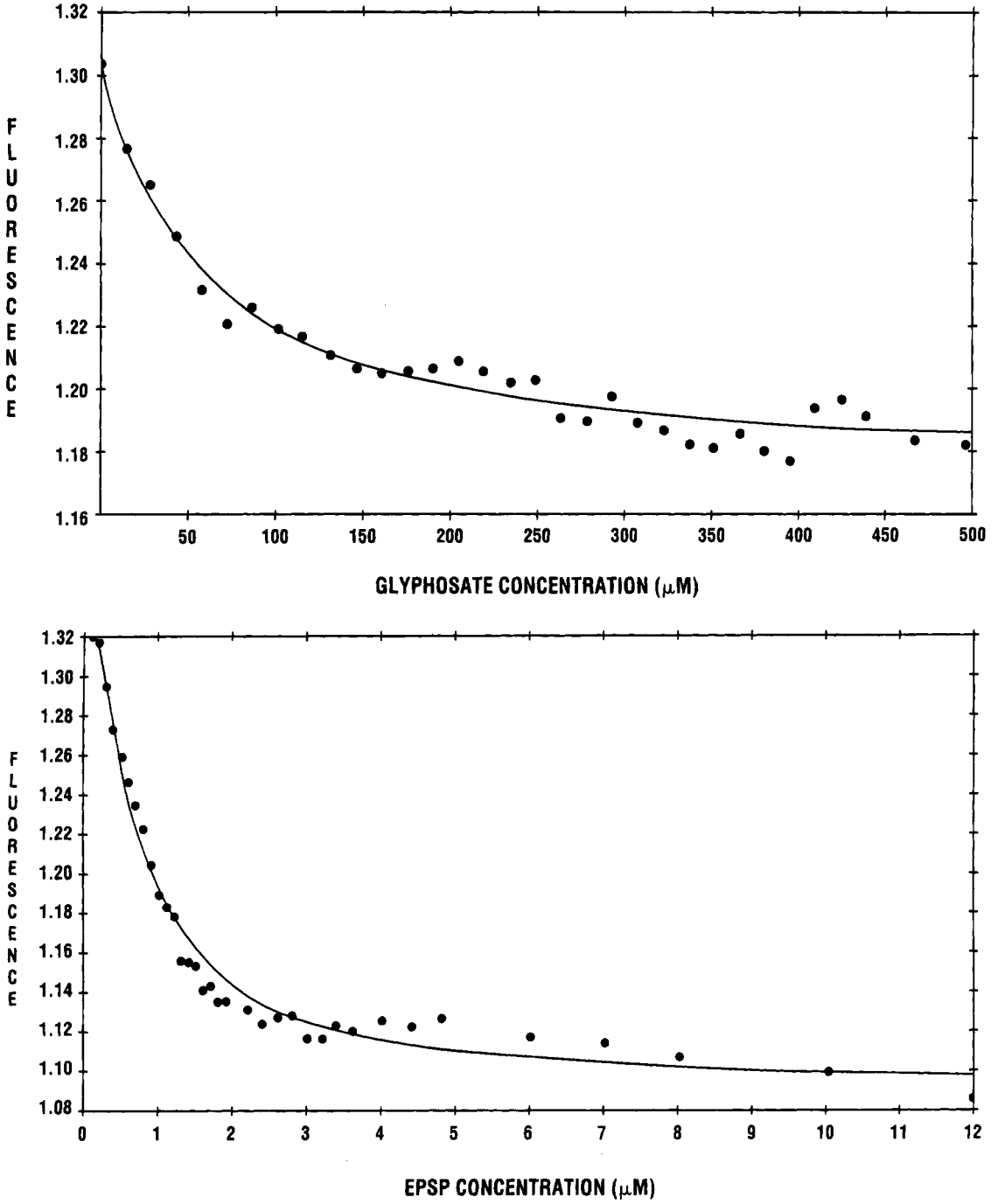


FIGURE 2: (A) Fluorescence titration of glyphosate binding to preformed EPSPS·EPSP. The decrease in fluorescence was monitored as glyphosate was added in small increments to a solution containing 0.5 μM enzyme and 100 μM EPSP. (B) Fluorescence titration of EPSP binding to EPSPS (0.5 μM) in the presence of 350 μM glyphosate.

of glyphosate by fluorescence binding measurements (Anderson et al., 1988c) and steady-state kinetics (Gruys et al., 1993).

Steady-State Kinetic Results on Glyphosate Inhibition of the Reverse Reaction. Identification of a ternary complex of EPSPS·EPSP·glyphosate led us to investigate the impact this complex would have on the kinetics of the EPSPS reverse reaction and to further corroborate its existence during enzymatic turnover of substrates. These kinetic experiments were also performed in the hopes that they would provide further insight into the nature of EPSPS·S3P·glyphosate ternary complex formation for the EPSPS forward reaction.

Prior to this investigation, Steinrücken and Amrhein (1984) reported that glyphosate was a mixed inhibitor versus EPSP using the enzyme from *Klebsiella pneumoniae*. No discussion of the mechanistic significance of glyphosate inhibition of the reverse reaction was presented, however. Glyphosate was suggested to be an uncompetitive inhibitor versus EPSP in the reverse reaction with the *Neurospora crassa* enzyme (Boocock, 1983; Boocock & Coggins, 1983), though no quantitative inhibition constants were reported. Boocock and Coggins rationalized that glyphosate's observed inhibitory behavior in the EPSPS reverse reaction was attributed to complications due to the steady-state formation of product S3P and the subsequent formation of EPSPS·S3P·P_i and EPSPS·S3P·glyphosate dead-end complexes. From this they concluded that it was not necessary to postulate binding of glyphosate to any kinetic intermediate other than the binary complex of EPSPS·S3P.

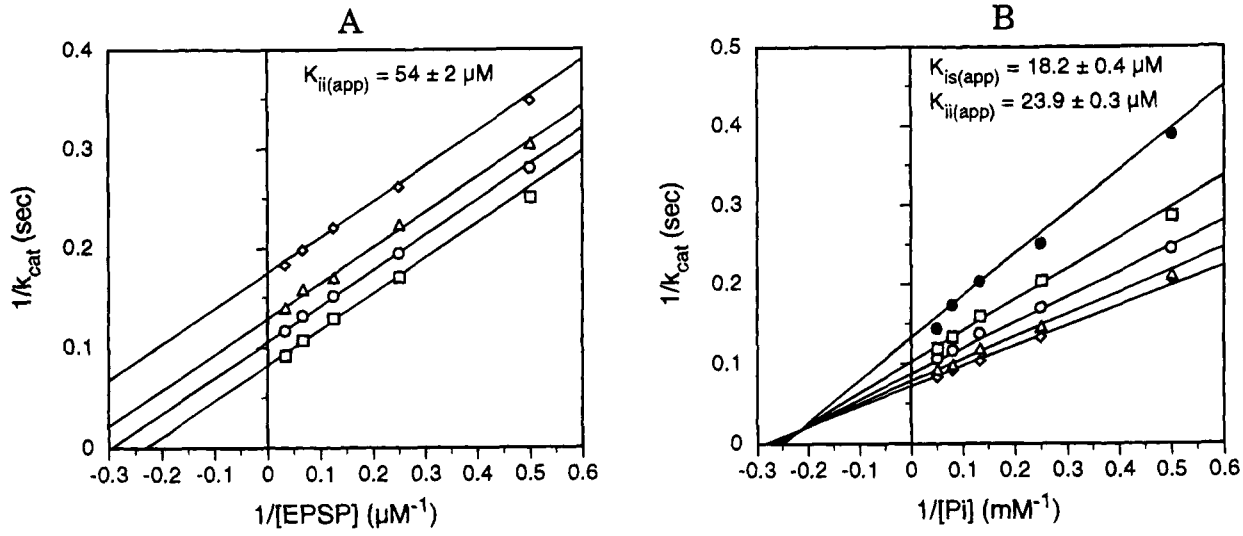


FIGURE 3: Lineweaver–Burke plots for inhibition of *E. coli* EPSP synthase by glyphosate. Experiments were performed in a 1-cm path length rectangular cell for variable phosphate (total volume = 1.0 mL) and a 10-cm path length cylindrical cell for variable EPSP (total volume = 4 mL). (A) Uncompetitive inhibition versus variable EPSP at fixed [P_i] = 50 mM; [glyphosate] = 0 (□), 15 (○), 30 (△), or 60 μM (◇). (B) Mixed inhibition versus variable phosphate at fixed [EPSP] = 50 μM; [glyphosate] = 0 (◇), 2 (△), 5 (○), 10 (□), or 20 μM (●).

The tightness of glyphosate's interaction in the EPSPS•S3P•glyphosate ternary complex does indeed require that some precautions be taken during kinetic studies of the reverse reaction where S3P is produced. First, EPSP must be pure and essentially free of extraneous S3P. This was true for all of our experiments, as addressed in the Materials and Methods. Second, enzyme-catalyzed EPSP hydrolysis to S3P, pyruvate, and P_i must be kept to a minimum. This is not a serious problem at catalytic concentrations of EPSPS since it is known that the enzyme only slowly catalyzes this reaction (Anderson et al., 1988b). In addition, this hydrolysis is effectively inhibited by glyphosate (Anderson et al, 1988a). Nevertheless, the enzyme-catalyzed EPSP hydrolysis rate was monitored under the conditions of our kinetic assays and was found to be insignificant or not detectable. Third, S3P produced as a normal consequence of EPSP turnover in the reverse reaction must be kept low. As such, all rates were calculated under conditions where ≤5% of starting EPSP was turned over to products. Since all three conditions were met for these experiments, the observed kinetic results cannot be attributed to the presence of S3P and the subsequent formation of the ternary dead-end complexes of EPSPS•S3P•glyphosate or EPSPS•S3P•P_i. This conclusion is based upon kinetic simulation results with KINSIM (Barshop et al., 1983), using previously published kinetic and dissociation constants (Anderson et al., 1988b,c; Ream et al., 1992; Gruys et al., 1992,1993), under conditions that mimicked the range of experiments in this investigation (not shown).

As shown in Figure 3a, we examined the EPSPS reverse reaction from *E. coli* and quantitatively confirmed that glyphosate is an uncompetitive inhibitor versus EPSP with $K_{ii(app)}$ = 54 ± 2 μM. This value is consistent with the fluorescence titration experiments and in combination with the gel filtration studies clearly demonstrates that glyphosate can bind directly to the enzyme in the presence of EPSP to form an EPSPS•EPSP•glyphosate ternary complex. Consequently, there is no need to invoke an interaction between glyphosate and other enzyme complexes to explain the observed inhibition pattern. Clearly, the carboxyvinyl group in EPSP does not preclude glyphosate from binding, and in fact, the addition of glyphosate to EPSPS requires strong synergism created by preformation of a binary EPSPS•EPSP complex, similar to that which occurs with enzyme, S3P, and glyphosate.

An interesting result was observed from the kinetics of glyphosate inhibition versus P_i for the *E. coli* EPSPS reverse reaction. As shown in Figure 3b, glyphosate clearly exhibits mixed inhibition versus P_i, with a $K_{is(app)}$ of 18.2 ± 0.4 μM and a $K_{ii(app)}$ of 23.9 ± 0.3 μM. This mixed inhibition versus P_i has also been observed with enzymes from *N. crassa* (Boocock & Coggins, 1983) and *K. pneumoniae* (Steinrücken & Amrhein, 1984). In the context of a random addition of EPSP and P_i (Gruys et al., 1993), as well as the uncompetitive inhibition versus EPSP, this result suggests that there is incomplete overlap between the phosphate and glyphosate binding sites in the EPSPS•EPSP binary complex. Therefore, it appears not only that the EPSP carboxyvinyl group does not preclude glyphosate from binding but that glyphosate's association with the EPSPS•EPSP binary complex also does not preclude P_i binding. This surprising result suggests the formation of an EPSPS•EPSP•glyphosate•P_i quaternary complex. An independent experimental corroboration would be beneficial in confirming the existence of this complex; however, a direct binding experiment is difficult to perform due to enzyme turnover of substrates to products.

Implications of Results on the Molecular Understanding of Glyphosate Binding to EPSPS. Previous studies using microcalorimetry established the K_d for glyphosate binding to free enzyme as 12 mM (Ream et al., 1992). Comparison of this K_d in a binary complex with that for glyphosate binding in the EPSPS•S3P•glyphosate ternary complex (K_d = 0.16 μM; Anderson et al., 1988c; Ream et al., 1992) indicates that S3P facilitates glyphosate binding by a factor of nearly 75 000. In comparison to S3P, the carboxyvinyl group in EPSP reduces glyphosate affinity by a factor of 350 (i.e., 56/0.16 = 350). This corresponds to ~3.5 kcal/mol and is consistent with the binding energy typical for one or more hydrogen-bonding interactions being lost between glyphosate and enzyme when EPSP is present.

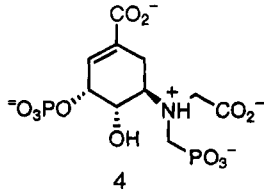
This loss in affinity might suggest that an altered conformation of enzyme-bound glyphosate is available with EPSP which is entirely different from that found in its ternary

complex with S3P. However, like the formation of ternary complex with S3P, strong synergism (≈200×) is still required for glyphosate binding with enzyme and EPSP. This is much greater than the observed synergism between S3P and PEP (10−20×) or between shikimic acid and glyphosate (7×), as we have previously reported (Gruys et al., 1992; Ream et al., 1992). The gel filtration (demonstrating slow dissociation of glyphosate from the ternary EPSPS•EPSP•glyphosate complex) and fluorescence titration (indicating a similar change in fluorescence properties upon binding of glyphosate to the enzyme binary complex with EPSP) experiments also allude to similarities in the two ternary complexes. Thus, it is unlikely that a completely new glyphosate binding domain is created with EPSP.

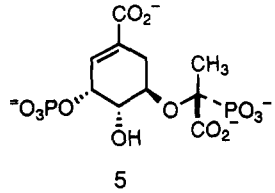
The carboxyvinyl group increases EPSP binding by less than a factor of 10 versus S3P (K_d = 1 vs 8 μM, respectively; Anderson et al., 1988c; Ream et al., 1992). Thus, one would not predict that a large electrostatic interaction occurs between enzyme and the side chain bearing the EPSP enol−carboxyl group. It is possible, then, that this weak enzyme interaction with the EPSP side chain might be displaced by glyphosate. Further structural characterization of this unexpected EPSPS•EPSP•glyphosate ternary complex will be necessary to define the exact orientation of the carboxyvinyl group in EPSP relative to the glyphosate carboxylate center and its relationship to the glyphosate conformation in the EPSPS•S3P•glyphosate ternary complex. In any event, EPSP synthase appears to have more structural flexibility at the glyphosate site than originally indicated from the evaluation of glyphosate analogs (Steinrücken & Amrhein, 1984; Knowles et al., 1993).

The exact spatial orientation between glyphosate and EPSP in either the ternary or the putative quaternary complex with P_i is obviously unknown. Recent results using solid-state REDOR NMR, however, have established that the glyphosate carboxylate carbon is positioned about 7.0 Å away from the S3P 3-phosphate center (Christensen & Schaefer, 1993). Thus, glyphosate binds very near to S3P in the EPSPS•S3P•glyphosate ternary complex. Molecular modeling experiments suggest that the EPSP enol−carboxylate group can be as close as 5.0 Å to the 3-phosphate center (J. A. Sikorski and J. L. Font, unpublished results). This suggests that there is ample room in 3D space to accommodate both carboxylate functionalities, even in a quaternary complex of EPSPS•EPSP•glyphosate•P_i.

We recently described steady-state kinetic results of the reverse reaction obtained from inhibition with **4** (Marzabadi et al., 1992; Gruys et al., 1993). This compound was



4



5

synthesized with the intent to incorporate and enhance the tight binding properties of the EPSPS•S3P•glyphosate ternary complex in a single molecule that combines S3P and glyphosate. The design for structure **4** was based on molecular modeling results (Marzabadi et al., 1992), which showed maximum overlap between it and the tight-binding tetrahedral intermediate analog **5**, synthesized by Alberg et al. (1992). The inhibition of the EPSPS reverse reaction by **4** was modest, and the kinetic patterns demonstrated that **4** and **5** display very different binding properties (Marzabadi et al., 1992; Gruys et al., 1993; Alberg et al., 1992). These results are consistent with the results presented here in that they indicate that glyphosate and PEP are not superimposable in a structure covalently linked to S3P, that is, as an analog of the tetrahedral intermediate, or as a transition-state analog.

In addition to **5**, several other potent bisubstrate inhibitors have been identified which closely mimic the tetrahedral intermediate **1** and clearly show competitive kinetic behavior versus EPSP and P_i for the EPSPS reverse reaction (Alberg et al., 1992). Thus, structural analogs of **1** show very good overlap with both substrate binding domains. In contrast, glyphosate exhibits a mixed inhibition pattern versus P_i and uncompetitive behavior versus EPSP. Therefore, glyphosate does not display the same key kinetic criteria previously demonstrated for EPSPS bisubstrate inhibitors which mimic **1**.

Very recent results on the UDP-GlcNAc enolpyruvyltransferase have bearing on the proposal for glyphosate as a transition-state analog of **3**. This enzyme constitutes the first committed step in bacterial peptidoglycan biosynthesis and is the only other enzyme known to catalyze an analogous enolpyruvoyl transfer like EPSPS. Of significance is the fact that UDP-GlcNAc enolpyruvyltransferase is not inhibited by glyphosate (Steinrücken & Amrhein, 1984). It had been previously proposed that the mechanism for this enzyme, contrary to EPSPS, proceeded through a carboxyvinyl or phosphoenolpyruvoyl covalent intermediate (Cassidy & Kahan, 1973; Zemell & Anwar, 1975). This divergence in mechanism for the two enzymes appeared to be consistent with the lack of inhibition of UDP-GlcNAc enolpyruvyltransferase by glyphosate. The recent isolation of an *O*-phosphothioketal of pyruvic acid covalently bound to the enzyme along with the demonstration of kinetic competence for this covalent intermediate agreed with this assessment (Wanke & Amrhein, 1993; Brown et al., 1994; Ramilo et al., 1994).

While the presence of the enzyme-bound covalent intermediate in UDP-GlcNAc enolpyruvyltransferase is not under dispute, recent results by Marquardt et al. (1993) strongly suggest that this enzyme utilizes a mechanism similar to that of EPSPS. These investigators isolated a kinetically competent, non-covalent tetrahedral intermediate that is analogous to **1** for EPSPS. To account for the covalently bound intermediate, the enzyme-bound *O*-phosphothioketal was proposed as the direct precursor of the non-covalent tetrahedral intermediate (Brown et al., 1994). The isolation of 3-fluoro-2-phospholactyl-UDP-GlcNAc produced by UDP-GlcNAc enolpyruvyltransferase using UDP-GlcNAc and the pseudosubstrate (*E*)- or (*Z*)-3-fluorophosphoenolpyruvate (Kim et al., 1994) further ties this transferase with EPSPS mechanistically. Nearly identical results (i.e., the slow formation of a fluoro tetrahedral intermediate and then lack of further processing to products) were described previously with EPSPS (Walker et al., 1992). These results with fluorophosphoenolpyruvate also support the formation of oxonium ion **3** for both enzymes since the inductive effect of fluorine would likely destabilize **3**, but would be expected to have little effect on a concerted mechanism where **3** is not formed transiently. An alternative interpretation of this data has recently been made (Seto & Bartlett, 1994). From all this it now appears that the substantial structural and functional homology (18.3% identical) between these two

enzymes (Marquardt et al., 1992; Wanke et al., 1992; Duncan et al., 1984) is not just coincidental but very likely related to mechanism. If this is true, then glyphosate as a putative transition-state analog of **3** should be a potent inhibitor of both enzymes. Glyphosate's selective and specific inhibition of only EPSPS (Steinrücken & Amrhein, 1984) casts doubts on this proposal.

Conclusions. The formation of a EPSPS•EPSP•glyphosate ternary complex has been independently corroborated by gel filtration, fluorescence binding measurements, and steady-state kinetics. Together these results demonstrate that the carboxyvinyl group in EPSP does not preclude glyphosate from binding, and as suggested by the P_i inhibition studies, glyphosate also does not preclude P_i binding in the EPSPS•EPSP binary complex. A simple interpretation of these latter results implicates the formation of a quaternary complex of EPSPS•EPSP•glyphosate•P_i. Consequently, glyphosate can exhibit a significant interaction with enzyme when both substrate sites are filled. The proposed model for glyphosate functioning as a transition-state analog of **3** (Anton et al., 1983; Steinrücken & Amrhein, 1984), which has defined the paradigm for glyphosate's molecular mode of action for more than a decade (Cromartie, 1986), is very difficult to reconcile with these results. Moreover, the alternative proposed model for glyphosate binding in the EPSPS•S3P•glyphosate ternary complex as an analog of enzyme-bound **1** (Anderson & Johnson, 1990a) also is not consistent with these results. Consequently, glyphosate does not fit the key kinetic criteria required for a transition-state analog or intermediate mimic inhibitor. Therefore, we conclude that glyphosate binding to either of the binary complexes of enzyme with S3P or EPSP must involve some substantial adventitious interactions with amino acid residues which are not intimately involved in substrate binding and catalysis. As such, part of the glyphosate molecule must bind near to, but outside, the EPSPS active site.

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