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ARTICLES

Understanding Glyphosate's Molecular Mode of Action with EPSP Synthase: Evidence Favoring an Allosteric Inhibitor Model

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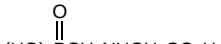
De novo design of potent enzyme inhibitors has increased dramatically, particularly as our knowledge of enzyme reaction mechanisms has improved. In a growing number of cases, such efforts have led to new compounds with useful biological properties. A complementary understanding of small molecule−enzyme interactions has also evolved from studies of substances with unusual biological activity. This Account summarizes recent efforts to more clearly define the molecular mode of action of glyphosate, the active ingredient in the popular herbicide Roundup (a registered trademark of Monsanto Co.), with its biological enzyme target EPSP (5-enolpyruvoylshikimate 3-phosphate) synthase (EPSPS; E.C. 2.5.1.19).

Glyphosate [(*N*-phosphonomethyl)glycine, **1**] is a nonselective, broad spectrum herbicide that effectively controls nearly all of the world's most problematic weeds while exhibiting many desirable environmental properties.[1] Glyphosate is essentially nontoxic to birds, fish, insects, and most bacteria and is readily broken down in soil by microbes which eventually produce ammonia, inorganic phosphate, and carbon dioxide.[2] With these favorable properties, glyphosate has become one of the most widely used and trusted herbicides in the world today.

Glyphosate is usually used in applications where total weed control is required. The phytotoxic symptoms observed with glyphosate are consistent with those of a metabolic plant poison and, in contrast to many contact herbicides, often develop relatively slowly. While glyphosate is phloem-mobile and rapidly translocated, actual plant death may take several days or even weeks. However, specific biochemical effects can often be observed within a few hours after its application.[3]



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Numerous physiological, biochemical, and genetic experiments have demonstrated that glyphosate controls weeds by inhibiting a single plant enzyme, EPSPS.[1,4,5] EPSPS is a key enzyme in the shikimic acid pathway which is found only in plants and certain microorganisms.[6] EPSP forms at the sixth step in this pathway and directly precedes the important branch-point intermediate chorsimate. Chorismate is required for the synthesis of nearly all aromatic plant metabolites, including the essential aromatic amino acids (phenylalanine, tyrosine, and tryptophan, Scheme 1). Approximately 35% or more of the ultimate plant mass in dry weight may be represented by aromatic molecules derived from this pathway.[7] Glyphosate's inhibition of EPSPS produces prodigious amounts of shikimate and severely disrupts carbon flow through this pathway, ultimately leading to plant death.

Glyphosate was one of the first commercially successful herbicides to have an identified enzymatic site of action in plants. This biochemical mode of action is unique for glyphosate-based herbicides. No other classes of com-

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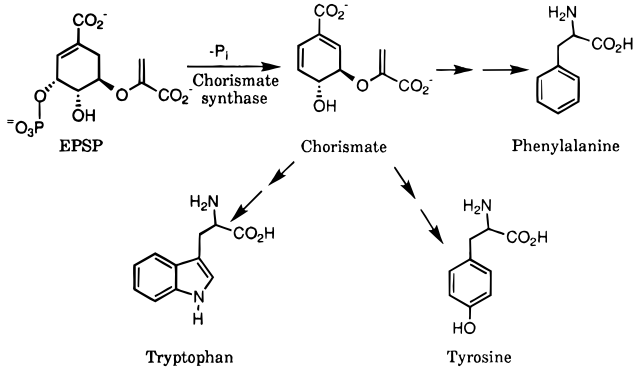
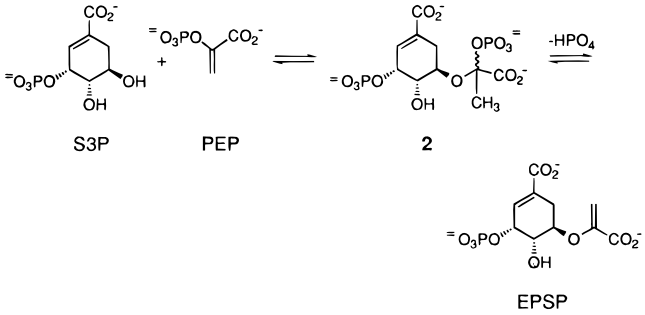
Scheme 1. Elements of the Shikimic Acid Pathway



Scheme 2. Reaction Catalyzed by EPSP Synthase

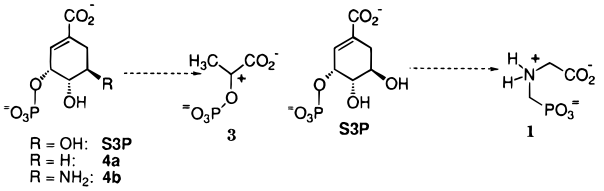


mercial herbicides are known that inhibit EPSPS or other enzymes in this important pathway. The selective and specific interaction of glyphosate with EPSPS accounts for its potent herbicidal properties and low toxicity to other life forms and led to the proposal that glyphosate functions as a transition-state (TS) inhibitor.[5b,8] This hypothesis has historically defined glyphosate's mode of action and has become well ingrained in the chemical literature.[9] This Account summarizes several recent synthetic and biochemical results that clarify glyphosate's interaction with EPSPS.

Evidence Supporting Glyphosate as a Transition-State Inhibitor

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).[1b,4] The *Escherichia coli* enzyme exhibits a random kinetic mechanism[10,11] through a single,[12] kinetically competent,[13] tightly bound (K_d = 50−250 pM),[14] tetrahedral intermediate, **2** (Scheme 2), as originally proposed by Sprinson et al.[15] The overall chemical equilibrium constant for this reaction has been measured ($K_{overall}$ = [EPSP][P_i]/[S3P][PEP] = 180).[4b] Thus, while the formation of EPSP and P_i is clearly favored,

Scheme 3



the reaction is completely reversible, and enzyme activity can be assayed in both directions. In the forward direction, the most reliable assay is an end point assay that measures the formation of [^{14}C]EPSP from either [^{14}C]S3P or [^{14}C]PEP by anion-exchange chromatography.[10] While an end point phosphate assay has been used for the forward reaction, it sometimes is problematic due to the hydrolytic lability of S3P and certain classes of inhibitors that can liberate P_i during sample preparation. The commonly used assay for the reverse reaction couples enzyme-produced PEP with pyruvate kinase and lactic dehydrogenase. The resulting loss of NADH can then be continuously monitored spectrophotometrically at 340 nm.[11]

Various studies using enzyme kinetics,[5b,16] fluorescence,[17] gel filtration,[18] solution[18] and solid-state NMR,[19] equilibrium titration,[20] and differential scanning calorimetry[21] demonstrate that glyphosate preferentially forms a stable ternary complex with enzyme and S3P (EPSPS·S3P·glyphosate). This ternary complex most likely represents the actual enzyme-bound form of glyphosate which is responsible for its herbicidal activity *in planta*.

Glyphosate has been postulated[5b,8] to act as a TS analog of the putative protonated PEP oxonium ion **3** (Scheme 3). The transient production of **3** during EPSPS catalysis is suggested from the observation that the combination of S3P and enzyme will induce deuterium exchange from D_2O into PEP.[15] A similar deuterium exchange into PEP has also been demonstrated with enzyme and S3P analogs lacking the 5-alcohol group required for turnover such as 4,5-dideoxy-S3P,[8] 5-deoxy-S3P (**4a**), and 5-amino-S3P (**4b**).[22] Alternatively, the tetrahedral geometry at the ketal phosphate center in **2** and its tight interaction with enzyme led to the proposal[4b] that the EPSPS·S3P·glyphosate ternary complex may actually mimic the enzyme-bound tetrahedral intermediate (EPSPS·**2**). Either scenario implies that glyphosate has direct interactions with those EPSPS amino acid residues intimately involved in PEP binding and catalysis.

Certain biochemical evidence has been accumulated since 1980 which suggests that there is substantial overlap between the PEP and glyphosate binding domains. In

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enzymes characterized from plant, fungal, and most bacterial sources,[23] glyphosate acts as a potent, competitive inhibitor versus PEP. For the *E. coli* enzyme the observed $K_{i(app)}$ for glyphosate versus PEP ranges between 0.2 and 0.9 μM.[17,23] Glyphosate typically exhibits uncompetitive behavior versus S3P.[16] The resulting EPSPS·S3P·glyphosate ternary complex was thought to resemble the EPSPS·S3P·PEP ternary complex required for catalysis. However, glyphosate binds significantly tighter than PEP,[13,17] yet does not inhibit any other PEP-dependent enzymes. Consequently, glyphosate cannot simply function as a PEP ground-state mimic.[5b] Indeed, a unique EPSPS binding interaction must exist for glyphosate and PEP, since other (aminoalkyl)phosphonates of similar size, shape, and functionality exhibit little significant interaction with this enzyme.[1,5b]

A number of active-site amino acid residues are protected from inactivation by either EPSP alone or S3P plus glyphosate, but not by glyphosate, PEP, or S3P alone.[24] These amino acids all appear at the proposed active site interface of the two globular domains in this protein.[25]

Comparing Glyphosate with PEP. To function optimally as a TS inhibitor, glyphosate should overlap as completely as possible with **3**, even though it contains one extra atom between the anionic centers. While glyphosate and PEP vary somewhat in their chemical and functional group composition, these two molecules are reasonably similar in three-dimensional space.[26] As shown in Figure 1, a three-dimensional representation of the known[27] extended conformation of glyphosate resembles the general overall shape of PEP quite well. However, optimum overlap between this extended glyphosate conformation and PEP at the critical anionic recognition centers is somewhat precluded by the extra atom in glyphosate.

The tetrahedral geometry at the glyphosate nitrogen atom might preclude an optimum fit with the planar configuration of **3**. In order to accommodate the configurational restrictions of this oxonium ion, the phosphonate and carboxylate functionalities in glyphosate should be bound in a more pinched conformation with each anionic group on the same side of the protonated nitrogen atom. As a result, the distance between the glyphosate phosphonate and carboxylate groups can be reduced considerably and more closely matches the distance between the carboxylate and phosphate groups in PEP.[26]

Precise three-dimensional details of the enzyme-bound glyphosate conformation are not yet available. Single-crystal X-ray structures of glyphosate alone have been reported in both extended[27] and pinched[28] conformations.

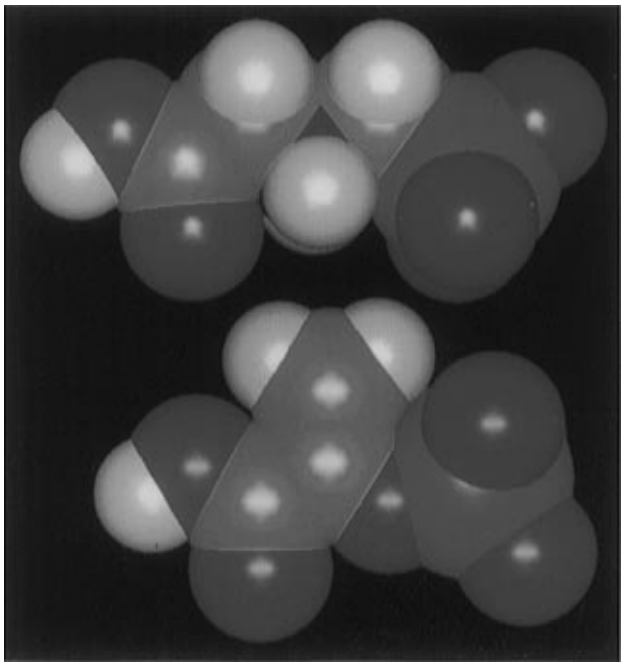

FIGURE 1. A three-dimensional comparison of the known extended conformation of glyphosate from X-ray crystallography (top) versus PEP (bottom) using space-filled models generated from INSIGHT. Atoms are color-coded as carbon = green, oxygen = red, nitrogen = blue, hydrogen = white, and phosphorus = magenta.

The X-ray structures of the bidentate glyphosate calcium complex[29] and the tridentate platinum[30] or cobalt[31] complexes of glyphosate also indicate that certain metals can induce a more compact glyphosate conformation. While no evidence has ever been presented implicating a metal requirement for EPSPS catalysis, it is reasonable to suggest that the enzyme might provide the appropriate interactions required to induce this more compact conformation.

Active Glyphosate Analogs. As an enzyme inhibitor, glyphosate displays a unique specificity for EPSPS. The ionic and steric requirements of the glyphosate binding site have been characterized[32] and are more restrictive than those associated with PEP.[33] While some modification of the PEP structure is tolerated, even minor structural changes in the glyphosate skeleton lead to a significant loss in inhibitor potency and reduced herbicidal activity. The addition of a single methyl group to any atom in glyphosate (e.g., **5**) dramatically reduces enzyme affinity.[34] As a result, glyphosate and its enzyme-active derivatives form a very narrow chemical class. Only two closely related analogs, *N*-hydroxyglyphosate (**6**) and *N*-aminoglyphosate (**7**), exhibit inhibition properties nearly comparable to glyphosate (Chart 1).[34] The protonated nitrogen atom in glyphosate also provides a critical recognition site, since the corresponding carbon, oxygen ether, sulfide, and

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Chart 1. EPSPS Inhibition Properties of Glyphosate and Its Analogs

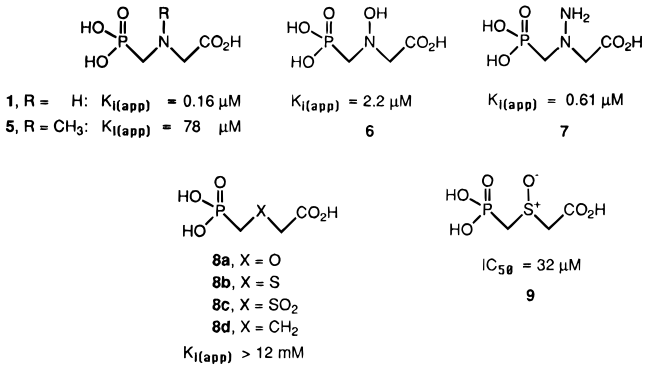
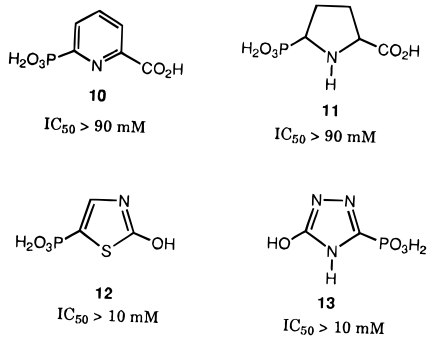


Chart 2. EPSPS Inhibition Properties of Cyclic Glyphosate Analogs



sulfone analogs **8a**−**d** are significantly less active. However, the dipolar sulfoxide analog **9** does exhibit some inhibitory activity.[35]

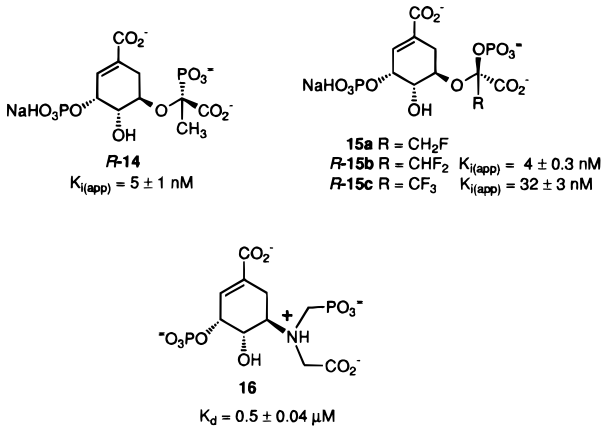
Titration calorimetry data demonstrate that the formation of the EPSPS·S3P·glyphosate ternary complex is enthalpy driven.[20] However, a fairly substantial negative entropy term partially offsets this effect. These data suggest that glyphosate fits snugly within the EPSPS·S3P·glyphosate ternary complex. Stronger binding synergism is observed between S3P and glyphosate [20] than exists between S3P and PEP.[10] These combined results appear consistent with the proposal that the EPSPS·S3P·glyphosate ternary complex mimics the transition state leading to **2**. However, as described below, more recent experiments demonstrate that the description for glyphosate in the EPSPS·S3P·glyphosate ternary complex is both more complex and subtle than either the TS analogy for **3** or the bound intermediate **2** proposal would indicate.

Synthetic and Biochemical Tests of the Transition-State Model

Planar Analogs of Glyphosate. As a TS analog inhibitor of the presumably planar **3**, one might expect that planar analogs of glyphosate would have some affinity for enzyme. However, none of the cyclic, planar glyphosate analogs (**10**, **11**) (Chart 2) evaluated to date display any significant activity versus EPSPS.[32]

In addition, more compact cyclic spatial mimics, such as the phosphonothiazole **12** and the phosphonotriazole **13**, which were designed to fit well within the spatial constraints of **3**, were both ineffective ($K_{i(app)} \geq 10$ mM) as EPSPS inhibitors.[36] The complete lack of any inhibitory activity observed with all cyclic glyphosate analogs strongly suggests that the conformational flexibility of the glyphosate backbone is a key component of its biological activity. To date no glyphosate analog or derivative has been identified that is more potent than glyphosate either as a herbicide or as an EPSPS inhibitor.[1b]

Chart 3



Analogs of the EPSPS·S3P·Glyphosate Ternary Complex.[26,37] Potent EPSPS bisubstrate inhibitors, such as **14** and **15**, have previously been identified which structurally resemble **2** and combine key features of S3P and PEP into a single molecule (Chart 3).[38,39] Inhibitors **14** and **15b**,**c** display potent, competitive inhibition versus EPSP and P_i in the EPSPS reverse reaction. The potency of these inhibitors is believed to arise through their effective interaction at the PEP−P_i subsite. Presumably, the glyphosate phosphonate must also interact with this site, if the TS comparison with **3** or the tetrahedral intermediate analogy is valid. Since glyphosate binds at least 75-fold more tightly to EPSPS·S3P than PEP, bisubstrate analogs which incorporate key structural features of S3P and glyphosate into a single molecule should be extremely potent EPSPS inhibitors.

The shortened hybrid **16** was designed as the first molecular probe of the EPSPS·S3P·glyphosate ternary complex for direct comparison with the shortened phosphonate inhibitor **14**,[26] one of the most potent known ($K_{i(app)} = 5 \pm 1$ nM) EPSPS inhibitors. Molecular modeling studies[37] predicted that **16** would fit well within a three-dimensional model of the EPSPS active site derived from **2** and **14** when a pinched conformation of the glyphosate side chains is utilized. A more compact conformation of **16** is needed to accommodate the extra atom in glyphosate within the spatial confines of the geminal anionic centers in **2** and **14**.

Steady-state kinetics demonstrated that **16** is a surprisingly modest inhibitor of *E. coli* EPSPS with an observed $K_{i(app)}$, competitive versus EPSP, of 7.4 ± 0.4 μM. Furthermore, in contrast to **14**, **16** displays mixed rather than

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competitive inhibition versus P_i.[37] This observed kinetic behavior suggests that **16** can occupy the S3P or EPSP site effectively, but does not completely overlap the PEP−P_i site.

Isothermal titration calorimetry demonstrates that **16** binds to free enzyme with an observed K_d of 0.53 ± 0.04 μM.[26] As such, **16** binds only 3-fold weaker than glyphosate and about 150-fold better than *N*-methylglyphosate (**5**). Consequently, **16** represents the most potent *N*-alkylglyphosate derivative identified to date. The measured thermodynamic binding parameters, however, clearly demonstrate that the formation of EPSPS·**16** is entropy driven like S3P recognition. The binding characteristics of **16** by ^{31}P NMR are also fully consistent with a primary interaction localized at the S3P subsite site with little, if any, recognition occurring at the PEP−P_i subsite.[26]

Thus, the correct spatial orientation between S3P and glyphosate is certainly not achieved in hybrid **16**. The interaction at the PEP−P_i subsite, which was so important for the observed potency of **14**, is lacking in **16**. Therefore, an EPSPS bisubstrate inhibitor linking S3P with PEP displays different potencies and inhibition patterns from one covalently combining S3P with glyphosate, even though both molecules fit well within the spatial constraints imposed by **2**. This difference implies that glyphosate and PEP are not superimposable in a shortened, compact conformation covalently bound to S3P and indicates that glyphosate is not exactly superimposable with PEP when S3P is present.

Solid-state NMR studies of the EPSPS·S3P·glyphosate ternary complex indicate that glyphosate is bound closely to S3P in a more extended conformation.[19a] Recent molecular dynamic simulations comparing **2**, **14**, and **16** with the EPSPS·S3P·glyphosate ternary complex suggest that glyphosate is unlikely to bind in the same manner as PEP.[19b]

Effect of S3P Analogs upon Glyphosate Binding. The first biochemical corroboration of these synthetic results came from the observation that no fluorescence change occurs upon adding glyphosate to EPSPS binary complexes containing analogs of S3P where the critical 5-alcohol group required for turnover is absent.[40] Thus, no glyphosate binding is detected in the presence of 4,5-dideoxy-S3P,[8] 5-deoxy-S3P[22] (**4a**), or 5-amino-S3P[22] (**4b**) although each will function as an effective S3P surrogate to promote deuterium exchange into PEP. Secondly, glyphosate is an extremely poor inhibitor when shikimate is used as an alternate substrate to form EPS.[10] Thus, **3** would appear very capable of forming in the presence of enzyme and these S3P mimics, yet glyphosate has little significant enzyme affinity when these S3P analogs are present.

EPSPS·EPSP·Glyphosate Ternary Complex. One would anticipate from the TS analogy that the carboxyvinyl group in EPSP should prevent glyphosate binding. The fluorescence changes induced by EPSP binding to free enzyme are similar, but smaller, to those induced by the addition of glyphosate to the EPSPS·S3P binary complex.[17] Since no comparable fluorescence changes are observed with S3P, PEP, or glyphosate alone, one might expect that there is at least partial overlap between glyphosate and the carboxyvinyl group of EPSP. Alternatively, some weak interaction between the glyphosate−phosphonate and the P_i site could happen instead. If this occurred, one would expect that glyphosate would exhibit a competitive inhibition pattern versus P_i in the EPSPS reverse reaction, on the basis of the known random kinetic mechanism.[10,11]

Glyphosate's inhibition of the EPSPS reverse reaction from *E. coli* has recently been reexamined and confirms that glyphosate is an uncompetitive inhibitor versus EPSP with an observed $K_{ii(app)}$ of 54 ± 2 μM.[41] The formation of an EPSPS·EPSP·glyphosate ternary complex was independently confirmed by rapid-gel-filtration methods and equilibrium fluorescence binding experiments. These techniques were previously used to characterize glyphosate binding in the EPSPS·S3P·glyphosate ternary complex. The observed changes in the EPSPS fluorescence spectrum indicated that glyphosate induces an additional conformational change in enzyme not accessible when EPSP alone is bound at the active site. The observed K_d is 56 ± 1 μM for glyphosate's affinity in this EPSPS·EPSP·glyphosate ternary complex.[41] The combined results demonstrate that glyphosate can bind directly to enzyme in the presence of EPSP. Clearly, the carboxyvinyl group in EPSP does not preclude glyphosate from binding.

Moreover, glyphosate exhibits mixed inhibition versus P_i,[41] as observed previously with enzymes from *Neurospora crassa*[16] and *Klebsiella pneumoniae*.[5b] In the context of a random addition of the substrates, as well as the uncompetitive inhibition versus EPSP, this result demonstrates that there is incomplete overlap between the phosphate and glyphosate binding sites in the EPSPS·EPSP binary complex. Thus, glyphosate's association with the EPSPS·EPSP binary complex does not preclude P_i binding and suggests that an EPSPS·EPSP·P_i·glyphosate quaternary complex can form. Since glyphosate can retain considerable interaction with EPSPS when the active site is filled by both substrates, these interactions must occur outside the defined EPSPS active site.

Glyphosate-Tolerant EPSP Synthase Variants. As a catalyst, the function of any enzyme is to make the reaction transition state more accessible to the substrates.[42] An experimental test to support TS behavior for any enzyme inhibitor is to demonstrate a correlation between potency (K_i) for a series of inhibitors and catalytic efficiency (k_{cat}/K_m) for a corresponding series of substrates.[43] Such a correlation has been established for the zinc endopeptidase thermolysin with a series of phosphorous-based inhibitors and their analogous substrates.[44] Unfortunately, such a correlation is impossible to establish between glyphosate and PEP with EPSPS, since PEP is the only compound that undergoes the carboxyvinyl transfer reaction. However, a complementary approach has been demonstrated comparing the potency of a single inhibitor

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Table 1. Kinetic Characteristics of Several EPSP Synthase Variants

EPSPS source	K_m(PEP) (μM)	K_i(glyphosate) (μM)	k_{cat} (s^{-1})	k_{cat}/K_m(PEP) ($s^{-1}\,\mu M^{-1}$)	ref
petunia (wt)[a]	5.0	0.40	36	7.2	51
petunia G101A	210	2000	21	0.10	51
petunia P106S	44	3.0	32	0.73	51
CP-4	12	2700	9.4	0.78	53
B. subtilis (wt)[a]	90	60	8.3	9.2×10^{-2}	52a
B. subtilis H385K	1000	40	0.018	1.8×10^{-5}	52a
B. subtilis R24D	200	50	0.064	3.2×10^{-4}	52a
B. subtilis P105S	590	210	5.8	9.8×10^{-3}	52a
B. subtilis R104K	300	5.0	0.080	2.7×10^{-4}	52b
B. subtilis R104Q	280	175	4.8	1.7×10^{-2}	52b

[a] wt = wild type.

with k_{cat}/K_m for a one-substrate system, such as carboxypeptidase A, using a series of enzyme mutants.[45] To the best of our knowledge, no analogous evaluation of enzyme mutants has been presented for two-substrate enzymes such as EPSPS.

If glyphosate functions in the EPSPS·S3P·glyphosate ternary complex as a TS analog for **3** or a mimic of intermediate **2**, one would expect that changes in any EPSPS active site amino acid residues which might reduce glyphosate's affinity would also have a corresponding dramatic effect in lowering EPSPS catalytic efficiency. Consequently, variant EPSP synthases with reduced glyphosate affinity would also be expected to have a correspondingly lower k_{cat}/K_m for PEP. Single- and multiple-site mutagenesis experiments in bacterial[46–49] and plant[50,51] enzymes have been reported in which glyphosate's affinity was selectively perturbed more dramatically than the k_{cat}/K_m for PEP. However, in these systems, no single-site amino acid change has yet been identified showing reduced glyphosate affinity without also exhibiting some reduced catalytic efficiency.

For example, replacing the conserved glycine-101 with alanine (G101A) in petunia EPSPS weakens the $K_{m(app)}$ for PEP by 40-fold, reduces k_{cat} by 2-fold, but perturbs glyphosate inhibition by nearly 5000-fold.[51] Thus, while the ratio of k_{cat}/K_m for PEP in this G101A variant drops by nearly a factor of 100 versus wild-type petunia enzyme, glyphosate's affinity is further reduced by an additional factor of nearly 50 (Table 1). A thorough kinetic, mechanistic, and biophysical characterization of this G101A variant petunia enzyme has not yet been completed. However, the observed kinetic constants for S3P and EPSP in this system are similar to those observed previously for the wild-type petunia and native *E. coli* enzymes. Moreover, the G101A variant is as sensitive as wild-type petunia enzyme to potent inhibition by **14**.[51]

Figure 2 depicts a log plot of the glyphosate inhibition constants for wild-type enzyme and two known (G101A, P106S) petunia EPSPS mutants versus their corresponding values of K_m/k_{cat} for PEP. While some correlation may exist, the slope of the resulting line is clearly closer to 2 than to 1. Another set of EPSPS mutants from *Bacillus subtilis* has recently been cloned, expressed, purified to homogeneity, and characterized by steady-state kinetics.[52] When these enzymes are plotted in Figure 2, the fitted line has a negative slope. In many of these *B. subtilis* mutants, glyphosate's affinity is largely maintained, but the K_m and k_{cat} for PEP vary considerably. Interestingly, in one case (R104K), the enzyme actually becomes more sensitive to glyphosate, yet its catalytic efficiency drops dramatically (Table 1).[52b] It appears from these two sets of mutants that no direct correlation exists between glyphosate inhibition potencies and the catalytic efficiency at the PEP site.

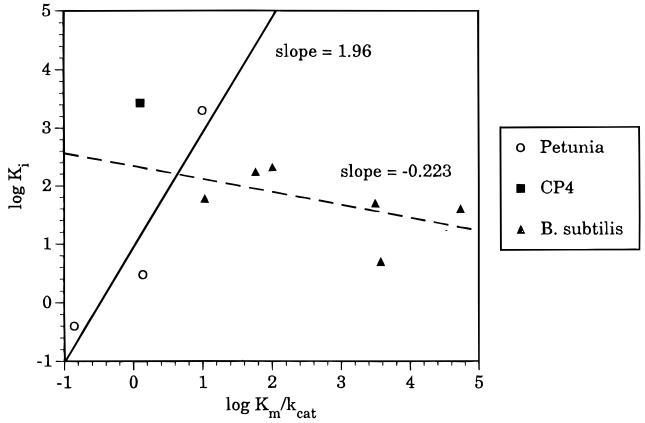

FIGURE 2. A log plot of kinetic inhibition constants (K_i) for glyphosate versus K_m/k_{cat} for PEP with EPSPS variants.

Even more striking evidence that disconnects glyphosate's binding efficiency from EPSPS catalysis is provided by recent reports[53] of a naturally occurring type-II EPSPS, from the soil bacterium *Agrobacterium* sp. strain CP4. This CP4 EPSPS has little significant affinity for glyphosate, and yet efficiently catalyzes the normal EPSPS reaction. For the CP4 enzyme, the ratio of k_{cat}/K_m for PEP is reduced by only a factor of 10 versus native petunia enzyme, but glyphosate's affinity decreases by nearly 7000 (Table 1, Figure 2).

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Summary and Conclusions

Numerous biochemical and physiological studies have confirmed that glyphosate functions as a potent inhibitor of EPSP synthase, a key enzyme in the aromatic amino acid biosynthetic pathway. The identification of EPSPS as the biological target for glyphosate has stimulated an enormous biochemical effort over the last decade to further define the molecular details of this enzyme and its interaction with glyphosate. The transition-state hypothesis, as originally proposed, seemed to provide an accurate interpretation of glyphosate's action based on the earlier biochemical characteristics of the EPSPS forward reaction. This proposal defined the paradigm for glyphosate's action for more than a decade and stimulated many additional studies.

However, the combined synthetic, kinetic, and biochemical evidence gathered to date indicates that glyphosate's affinity with S3P for the type-I EPSP synthases is not directly related to any transition state or intermediate involved in catalysis. In the EPSPS reverse reaction, glyphosate can still bind when the active site is filled by both substrates. Moreover, an analysis of the available kinetic data for various EPSPS mutant enzymes conclusively demonstrates that catalytic efficiency at the PEP site is not directly correlated with the potency of glyphosate inhibition. It would appear from these results that a substantial portion of the glyphosate binding site is separated from those amino acid residues intimately involved in substrate binding and catalysis. REDOR NMR experiments, however, clearly demonstrate that glyphosate binds very near to S3P.[19] Therefore, a significant part of the glyphosate molecule binds adventitiously near to, but outside, the EPSPS active site. Glyphosate is a rare example of an inhibitor whose binding domain differs from that of its competitive substrate. Thus, the competitive binding behavior of glyphosate versus PEP is only due to both molecules' specificity for the same enzyme form (i.e., EPSPS·S3P). The binding behavior of glyphosate with EPSPS is therefore better characterized as an adventitious allosteric interaction. It appears then that the known conformational change induced upon glyphosate binding simply makes the EPSPS active site unavailable to PEP. Consequently, it is difficult to imagine how glyphosate could be "designed", in retrospect, solely on the basis of a detailed knowledge of PEP catalysis and enzyme mechanism. More complete structural studies of the EPSPS·S3P·glyphosate and EPSPS·EPSP·glyphosate ternary complexes will be needed to assist inhibitor design for this allosteric glyphosate binding domain.

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