Site-directed Mutagenesis of a Conserved Region of the 5-Enolpyruvylshikimate-3-phosphate Synthase Active Site*

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The active site of the enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) has been probed using site-directed mutagenesis and inhibitor binding techniques. Replacement of a specific glycyl with an alanyl or a prolyl with a seryl residue in a highly conserved region confers glyphosate tolerance to several bacterial and plant EPSPS enzymes, suggesting a high degree of structural conservation between these enzymes. The glycine to alanine substitution corresponding to Escherichia coli EPSPS G96A increases the $K_{\text{mapp}}$(glyphosate) of petunia EPSPS 5000-fold while increasing the $K_{\text{mapp}}$(phosphoenolpyruvate) about 40-fold. Substitution of this glycine with serine, however, abolishes EPSPS activity but results in the elicitation of a novel EPSP hydrolase activity whereby EPSP is converted to shikimate 3-phosphate and pyruvate. This highly conserved region is critical for the interaction of the phosphate moiety of phosphoenolpyruvate with EPSPS.

Site-directed mutagenesis of enzymes is a powerful technique for investigating active site structure-function relationships (1). While the technique has been used to verify the roles of active site residues, there are no reports of its use in examining the relatedness of active sites of enzymes from different sources. In this paper, we have used site-directed mutagenesis to establish that the active sites of 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) enzymes from bacteria and plants are similar. The x-ray crystal structure of Escherichia coli EPSPS has recently been elucidated at 3 Å resolution (2). At the present time, however, the structure of the EPSPS complex with the inhibitor glyphosate is not available. In the absence of detailed structural information on the EPSPS binding site for glyphosate, we have used site-directed mutagenesis to probe the interaction between the enzyme and glyphosate as well as PEP.

The enzyme EPSPS (EC 2.5.1.19) catalyzes the formation of EPSP and phosphate (P) from PEP and shikimate 3-phosphate (S3P) (3), in an unusual carboxyvinyl transfer reaction. EPSPS has been extensively studied since it is the target for glyphosate (N-phosphonomethyl glycine) (4), the active ingredient of Roundup™ herbicide, widely used for weed and vegetation control (5). Glyphosate is a competitive inhibitor with respect to PEP of EPSPS, and interacts with the E-S3P complex (6). Of the several known PEP-dependent enzymatic reactions, EPSPS is the only enzyme inhibited by glyphosate. The PEP binding region of EPSPS therefore appears to be unique; indeed, EPSPS is the only enzyme which interacts with PEP as an enzyme-substrate complex (E-S3P) and not as the free enzyme. In this paper, we have probed the PEP binding region of EPSPS enzymes through site-directed mutagenesis and kinetic analysis.

Identification of the active site of EPSPS has been largely based on chemical modification studies. Lys32 and Lys340 of E. coli EPSPS can be modified by reaction with pyridoxal phosphate (7) and o-phthalaldehyde (8), respectively, resulting in inactivation of the enzyme. Arg28 and Arg331 of petunia EPSPS are modified by phenylglyoxal, the former being essential for enzyme activity (9). Cys606 of E. coli EPSPS is highly reactive, but not essential for enzymatic activity (10). This residue is proximal to the active site, since its modification with bulky reagents results in inactivation of the enzyme.

Another useful method of active site characterization is identification of substitutions which impact ligand binding and catalysis. Comai and co-workers have described a glyphosate-tolerant Salmonella typhimurium strain, wherein the tolerance to glyphosate results from a single amino acid substitution of P101S in the araA gene encoding EPSPS (11, 12). We recently reported the isolation of an E. coli B variant, containing a highly glyphosate-tolerant EPSPS (13). Isolation and sequencing of the araA gene encoding this glyphosate-tolerant EPSPS revealed that the altered affinity for glyphosate was the result of a single amino acid substitution of alanine for glycine at residue 96. As shown in Fig. 1, alignment of the amino acid sequences of EPSPS from petunia (14), tomato (15), Arabidopsis (15), Brassica napus, soybean, maize, E. coli (16), S. typhimurium (12), Aspergillus nidulans (17), Saccharomyces cerevisiae (18), and Bordetella pertussis (19) revealed that the E. coli Gly96 residue is located in a highly conserved region of EPSPS, with a consensus sequence L93XLNAG293XRXL (X represents nonconserved

2 G. M. Kishore, L. Brundage, D. Rochester, and D. Shah, unpublished data.
3 C. S. Gasser, E. B. Levine, D. M. Shah, C. M. Hironaka, S. Elmer, and H. J. Kies, unpublished data.
The underlined glycine residues correspond to Gly\textsuperscript{96} of \textit{E. coli} EPSPS.

| EPSPS source | Sequence | Reference |
|--------------|----------|-----------|
| \textit{E. coli} | L\textsuperscript{96} Y LGNAG\textsuperscript{96} TAMRP\textsuperscript{96} L\textsuperscript{96} | 16 |
| \textit{S. typhimurium} | L\textsuperscript{96} Y LGNAG\textsuperscript{96} TAMRP\textsuperscript{96} L\textsuperscript{96} | 12 |
| \textit{Pseudomonas putida} | L\textsuperscript{96} Y LGNAG\textsuperscript{96} TAMRP\textsuperscript{96} L\textsuperscript{96} | 14 |
| Tomato | L\textsuperscript{96} Y LGNAG\textsuperscript{96} TAMRP\textsuperscript{96} L\textsuperscript{96} | 14 |
| Arabidopsis | L\textsuperscript{96} Y LGNAG\textsuperscript{96} TAMRP\textsuperscript{96} L\textsuperscript{96} | 15 |
| B. napus | L\textsuperscript{96} Y LGNAG\textsuperscript{96} TAMRP\textsuperscript{96} L\textsuperscript{96} | Footnote 3 |
| Soybean | L\textsuperscript{96} F LGNAG\textsuperscript{96} TAMRP\textsuperscript{96} L\textsuperscript{96} | Footnote 3 |
| Maize\textsuperscript{a} | L\textsuperscript{96} F LGNAG\textsuperscript{96} TAMRP\textsuperscript{96} L\textsuperscript{96} | Footnote 3 |
| A. nidulans\textsuperscript{b} | L Y LGNAG TASRF L | 17 |
| S. cerevisiae\textsuperscript{a} | L Y LGNAG TASRF L | 18 |
| B. pertussis | L\textsuperscript{96} F LGNAG\textsuperscript{96} TAMRP\textsuperscript{96} L\textsuperscript{96} | 19 |
| Consensus\textsuperscript{a} | L X LGNAG TASRX L | |

\textsuperscript{a} Based on prediction on start of mature protein (see “Results”).

\textsuperscript{b} Part of the aromatic enzyme complex.

\textsuperscript{c} For the consensus sequence, \(X\) represents nonconserved amino acids.

Amino acids. We now show that the glycine to alanine substitution corresponding to \(\textit{E. coli}\) EPSPS G96A also imparts glyphosate tolerance to five additional EPSPS enzymes. The substitution corresponding to \textit{S. typhimurium} EPSPS P101S (see Table I) (11, 12), discussed above, also confers glyphosate tolerance to \textit{petunia} EPSPS. This region is therefore a critical part of an EPSPS active site highly conserved between plant and bacterial enzymes.

**EXPERIMENTAL PROCEDURES**

**Plasmid Construction**—All plasmid constructions were carried out by standard methods (20, 21). Oligonucleotide-directed mutagenesis was carried out according to standard methods (22, 23) with minor modifications. Vectors for expression of the petunia, \textit{E. coli}, soybean, and maize enzymes in bacteria utilized the \(P_s\) promoter of phage \(\lambda\) (24). The tomato EPSPS bacterial expression vector utilized the \textit{E. coli} RecA promoter (25, 26). In each case the success and fidelity of the mutagenesis was confirmed by sequencing the mutated region (27), using chemically synthesized sequencing primers for an adjacent region.

**Enzyme Extraction and Assay**—\textit{E. coli} SR481 cultures (aroA deficient) (28) harboring plasmids containing EPSPS genes were grown in Luria-Bertani broth to saturation at 37 °C, centrifuged, washed with 0.9% saline, and resuspended in extraction buffer consisting of 100 mM Tris-Cl, 1 mM EDTA, 10% glycerol, 5 mM dithiothreitol, 1 mM benzamidine HCl, pH 7.5, at 4 °C. The cell suspensions were passed through a French pressure cell (Aminco) twice (1000 p.s.i.), and the resulting lysate was centrifuged (10 min, 10,000 rpm in a Sorvall SS-20 rotor). The supernatant was then passed over a G-25 gel filtration column (Pharmacia LKB Biotechnology Inc., P-10) equilibrated with extraction buffer, and protein concentrations were determined by the method of Bradford (29) using the Bio-Rad micro- protein assay. EPSPS was assayed by HPLC radioassay or phosphate activities are reported as pmol of EPSP/min/mg of protein (units/mg). The S3P, \(1[\text{[C]}\text{S3P}, \text{EPSP}, \text{and} [1\text{-enol-}[\text{C}]\text{EPSP}\) (used for both EPSPS and \(K_m\) determinations) were synthesized and purified as previously described (28, 30).

**Enzyme Purification**—EPSPSs were purified as previously described (28), with minor modifications. The pG96S EPSPS\textsuperscript{1} was followed during purification by HPLC with a Vyduc C18 column, using 45–85% RP-B in 20 min (RP-A, 0.1% trifluoroacetic acid; RP-B, 0.1% trifluoroacetic acid in acetonitrile) at 1 ml/min flow rate; the EPSPS retention time was 14 min.

**Equilibrium Dialysis**—For binding studies, enzymes were dialyzed at 5 °C into dialysis buffer (150 mM HEPES, 50 mM KCl, 5 mM MgCl\(\text{2}\), pH 7.0) (2 × 2 liters, 4 h). After dialysis, the concentration was adjusted to 1.0–1.8 mg/ml with dialysis buffer (using \(A_{280} = 8.2\)). The experiments (duplicate) were performed by dialyzing a fixed amount of enzyme against increasing concentrations of \[\text{[C]S3P}\] in a microdialysis apparatus (Hoefler, 250-μL chambers). S3P solutions (15–260 μM) were prepared by mixing \([\text{33P}]\text{S3P}\) (19 mCi/mmol) with increasing amounts of unlabeled S3P. The enzyme (200 μl) was placed into one half-cell and an equal volume of S3P was placed into the other half-cell; dialysis cells were rotated at 20 rpm for approximately 16 h at 5 °C. Aliquots (100 μl) were removed from each half-cell, and the radioactivity present at equilibrium was determined by liquid scintillation counting (Beckman LS). The radioactivity present in the half-cell without protein was used to calculate the concentration of bound + free S3P. The radioactivity present in the half-cell without protein was used to calculate the concentration of free S3P. The data was then plotted as 1/[S3P] vs. [EPSPS]/[S3P] bound to determine \(K_m\), the equilibrium dissociation constant (31).

**RESULTS AND DISCUSSION**

**Construction of EPSPS Expression Vectors**—We have previously described a system for the production of a mature (minus chloroplast transit peptide) form of \textit{petunia} EPSPS in \textit{E. coli} (28). The codon for Lys\textsuperscript{12} of \textit{petunia} preEPSPS (14) that represents the first amino acid of the mature protein was replaced by oligonucleotide-mediated mutagenesis with two codons, Met and Glu, producing a convenient restriction enzyme recognition site that allowed for correct attachment of the coding sequence to a strong \textit{E. coli} promoter and ribosome binding site (24). For the current study, we have constructed similar vectors for bacterial expression of mature forms of EPSPS from tomato, soybean, and maize. In \textit{tomato} and \textit{soybean} the high degree of identity of the EPSPSs to \textit{petunia} EPSPS allowed for identification of the homologous Lys residue as the putative N terminus of the mature EPSPS, which was altered as described above and inserted into \textit{E. coli} expression vectors. Our best prediction of the maize CTP cleavage site was between Glu\textsuperscript{22} and Ala\textsuperscript{23} of the maize preEPSPS\textsuperscript{2}. In this case mutagenesis was performed to add a codon for a Met residue just upstream of the Ala\textsuperscript{23} codon. The altered maize sequence was then used to construct a vector for expression in \textit{E. coli}. The bacterial expression plasmids were introduced into an araoA-deficient \textit{E. coli} strain, SR481, which does not produce an active EPSPS, as previously described (28). A genomic clone for \textit{Arabidopsis} EPSPS has previously been used to engineer high level expression of EPSPS in transgenic \textit{Arabidopsis} plants (15). Plants produced using this system provided a source for the \textit{Arabidopsis} EPSPSs.

To determine the effect on the plant EPSPSs of the glycine to alanine substitution corresponding to \textit{E. coli} EPSPS G96A, we introduced the corresponding substitution by site-directed mutagenesis into the EPSPS DNA clones for bacterial expression of the \textit{petunia}, \textit{tomato}, \textit{soybean}, and \textit{maize} variants, and for plant expression of the \textit{Arabidopsis} variant (Table I). The number of differences among the amino acid sequence numbering, the \textit{E. coli} EPSPS G96A substitution corresponds to different Gly to Ala substitutions in plant EPSPSs (for instance, G101A in \textit{E. coli} EPSPS will be denoted pG96A in \textit{petunia} EPSPS. Similarly, specific residues are preceded by their origin code, for instance pGly\textsuperscript{96} of \textit{petunia} EPSPS. Note that the conserved region containing this residue has the same numbering in both \textit{E. coli} and \textit{S. typhimurium} EPSPS.

\textsuperscript{1}C. S. Gasser, unpublished data.
petunia EPSPS), although as shown in Table I, the residues are homologous. The amino acid numbering system used herein is based on the E. coli EPSPS numbering scheme.

**Analysis of Wild-type and Ala variant EPSPSs**—The wild-type and Ala variant EPSPSs were extracted from E. coli, petunia, or Arabidopsis leaf tissue and assayed for EPSPS activity. The wild-type plant EPSPSs had specific activities ranging from 0.18 to 1.6 units/mg, compared to 0.11–0.69 unit/mg for the corresponding Ala variants (Table II). There does not appear to be any significant alteration in EPSPS specific activity resulting from the engineering of the Ala substitution corresponding to E. coli EPSPS G96A, except perhaps in the tG96A variant, which only exhibits about 25% of the specific activity of the tomato wild-type enzyme. Manipulation of residues immediately around the chloroplast transit peptide cleavage site does not appear to significantly impact the enzyme activity of the mature EPSPSs, probably because this region folds into a domain separate from the mature region of preEPSPS (32). All of the plant EPSPS cDNAs complemented the arao mutation in E. coli SR481.

Crude extracts containing either the wild-type or Ala-substituted variant EPSPSs corresponding to G96A were also assayed for their glyphosate sensitivity at saturating substrate concentrations. All the wild-type EPSPSs tested were sensitive to inhibition by glyphosate, while the Ala-substituted variants were extremely resistant to inhibition by glyphosate (Table II). Thus, the substitution corresponding to G96A affects the glyphosate sensitivities of both E. coli and plant EPSPSs to similar degrees, with more than a 500-fold increase in IC50, being obtained in all cases. This newly introduced Ala residue therefore interferes with glyphosate binding to EPSPS by a mechanism common to all EPSPSs tested, and furthermore, these results indicate that the active site of EPSPS is highly conserved among the plant and bacterial enzymes.

**State-wide Kinetic Analysis of pG96A Petunia EPSPS**—In order to better quantify the effect of the pG96A substitution on petunia EPSPS, state-wide kinetic constants were determined for purified wild-type and pG96A petunia EPSPSs (Table III). For the pG96A variant, the Kapp(glyphosate) vs. PEP was found to be 2.0 ± 0.2 mM, which is 5000 times greater than that of the wild-type enzyme; the inhibition remained competitive vs. PEP. The Kapp(glyphosate) is elevated from 5.0 ± 1.3 μM in the wild-type enzyme to 210 ± 20 μM in the pG96A variant, and the Km(app)(phosphate) is elevated from 0.59 ± 0.09 mM for the wild-type enzyme to 6.7 ± 1.2 mM for the pG96A variant. These kinetic constants for the pG96A EPSPSs are similar to those reported for the G96A E. coli B EPSPS (Kapp(glyphosate) = 3.7 μM, Km(app)(PEP) = 16 μM, Km(app)(phosphate) = 8.4 mM, Kc(P) = 1.4 mM for E. coli EPSPS) (33), indicating that, for EPSPS, measurement of Kapp values is a valid approximation to the ligand dissociation constants. Taken together, these kinetic data are consistent with a perturbation of a binding interaction common for glyphosate, PEP, and P, but not S3P or EPSP. Since glyphosate is a competitive inhibitor of EPSPS with respect to PEP, it is not surprising that the pG96A variant (and the E. coli EPSPS G96A variant) also displays a loss of PEP binding. If one views the Kapp(glyphosate)/Kapp(PEP) as a selectivity factor for PEP over glyphosate binding, the large increase in its value from the petunia wild-type EPSPS (Kc(P)/Kc(PEP) = 0.09) to the pG96A variant (Kc(P)/Kc(PEP) = 9.5) indicates that the introduction of the alanine residue selectively destabilizes the interaction of the enzyme with glyphosate compared to PEP.

In addition to its glyphosate insensitivity, the catalytic efficiency for pG96A EPSPS is also altered; the kcat in the forward direction reaction is 58% of that of the wild-type enzyme. The kcat/Kapp(PEP) values for the wild-type and pG96A EPSPSs were 7.2 s⁻¹μM⁻¹ and 0.1 s⁻¹μM⁻¹, respectively, indicating that the pG96A variant is about 72 times less efficient than the wild-type enzyme. The relative catalytic efficiency in the presence of glyphosate, kcat/Kapp( glyphosate) (Kapp(PEP), indicates that the pG96A variant is about 69 times more efficient than the wild-type enzyme in the presence of glyphosate.

**Interaction of pG96A Petunia EPSPS with a Reaction Intermediate Analog**—Alberg and Bartlett (34) have synthesized a potent EPSPS inhibitor, 5-[(R)-1-carboxy-1-phosphonooethyl]shikimate 3-phosphate, which mimics the tetrahydrofolate reaction intermediate (35) and inhibits petunia EPSPS with a Ki of 15 nM (34). In order to determine if pG96A EPSPS efficiently interacts with the tetrahydrofolate intermediate analog, we determined the Kapp for the R-inhibitor for pG96A and wild-type petunia EPSPS in the forward reaction direction. Interestingly, the analog inhibited pG96A EPSPS with a Kapp of 35 ± 2 nM, compared to 38 ± 5 nM for the wild-type enzyme (S3P = 2 mM; PEP = 5 μM for wild-type and 40 μM for pG96A EPSPS). The petunia EPSPS pG96A substitution therefore does not adversely affect the interaction of the phosphonate moiety of the analog with EPSPS and is in agreement with our observation that the pG96A variant has a Vmax close to that of the wild-type enzyme, presumably due to its high affinity for the reaction intermediate.

**pG96S Petunia EPSPS**—The pAAlα substitution in the pG96A petunia EPSPS variant clearly diminishes both glyphosate and PEP binding to the enzyme, albeit to different degrees. If this perturbation was a direct result of the increased steric bulk of the pAAlα methyl group compared to the p Glyα hydrogen of the wild-type enzyme, one would...
Predict that increasing the size of the side chain of the amino acid residue at this position would affect both glyphosate and PEP binding to EPSP. Therefore, the pG96S EPSPS variant was constructed and assayed in E. coli SR481 for its ability to complement the araA deficiency; no complementation was observed. Using E. coli extracts containing the pG96S EPSPS (Western blot analysis), we were unable to detect any EPSPS activity. This result suggested that the pG96S substitution may have significantly affected the binding of PEP to the enzyme. If this were the case, SSP binding should remain intact, although the enzyme is catalytically inactive. Indeed, equilibrium dialysis binding experiments using purified pG96S EPSPS established that the $K_d(S3P)$ was 6.0 ± 0.6 µM, compared to 15 ± 3 µM for wild-type petunia EPSPS. Thus, S3P binding remains unaltered in the pG96S variant. However, the pG96S-S3P complex was unable to bind glyphosate, based on fluorescence emission and equilibrium dialysis analyses (data not shown). These features distinguish the pG96S variant from the wild-type enzyme.

The intensity of the fluorescence emission spectrum of pG96S EPSPS did not increase upon EPSP addition, whereas a similar treatment of wild-type enzyme did result in a fluorescence emission spectrum intensity increase (36). HPLC analysis of a pG96S/EPSP mixture, after the addition of 10 mM potassium phosphate, indicated that the added EPSP had been hydrolyzed to S3P and an unknown product, while an identical reaction with wild-type petunia EPSPS gave the expected S3P and PEP product peaks. Additional experiments confirmed these results and showed that phosphate was not required for pG96S-catalyzed EPSP hydrolysis (Fig. 1). The most likely fate of the EPSP carboxyvinyl carbon fragment was postulated to be its conversion to pyruvate; EPSPS has been known to form pyruvate from S3P and PEP as a by-product at an exceedingly low rate (0.00047 s⁻¹ for E. coli EPSPS) (35). By using lactate dehydrogenase/NADH, we found that pyruvate is indeed the second product of pG96S-mediated hydrolysis of EPSP. The EPSP hydrolytic activity is not sensitive to inhibition by glyphosate, but is highly sensitive to inhibition by SSP (Table IV). In addition, 0.2 mM of the $R$-isomer intermediate analog of the EPSPS reaction described above did not inhibit the pG96S hydrolysis reaction (at 57 µM EPSP), while inhibiting 94% of the wild-type petunia EPSPS activity at 57 µM EPSP and 50 mM KP. These results confirm our hypothesis that the pGly⁶ region of the petunia EPSPS active site participates in the recognition of the phosphate moiety of PEP. The phosphate recognition site in the pG96S variant is severely disrupted and results in a loss of binding of the intermediate analog, and by inference, the tetrahedral intermediate itself.

There are three possible mechanisms by which EPSP can be hydrolyzed by pG96S EPSPS. The first possibility (Scheme 1, mechanism 1) is that nucleophilic addition of water to the C-2 carbon of the carboxyvinyl moiety of EPSP generates a tetrahedral intermediate $I$, which by ketonization of the C2-OH bond yields pyruvate and S3P. The tetrahedral intermediate $I$ is the dephosphorylated form of tetrahedral intermediate $II$, an intermediate of the EPSPS reaction (35). A second possibility is that the phosphorylated tetrahedral intermediate $II$ is hydrolyzed by the pG96S enzyme to the intermediate $I$ which then follows the same route suggested for mechanism 1. A third possibility is the direct nucleophilic attack of the pG96S pSer⁶ hydroxyl group on the C-2 carbon of the carboxyvinyl moiety of EPSP (mechanism 3), generating an enzyme-bound intermediate which is hydrolyzed to S3P and pyruvate. It is known that the enzyme UDP-N-acetyl mur-
**Table IV**

| Number | Additions | Rate | Uninhibited rate |
|--------|-----------|------|-----------------|
| 1      | No additions | 0.52 | 100 |
| 2      | 2.0 mM S3P | 0.009 | 1.7 |
| 3      | 25 mM glyphosate | 0.44 | 80 |

**Scheme 1. Mechanisms for pG96S EPSP hydrolase activity.**

Together, these results clearly demonstrate that pSer96 of pG96S does not interfere with the S3P/EPSP binding site, but perturbs the PEP binding site. Since the carboxyvinyl moiety of EPSP interacts with the enzyme, the inability of pG96S EPSPS to bind PEP may be most likely due to the steric hindrance for interaction of phosphate moiety of PEP at the active site. It is tempting to speculate that the pSer96 hydroxymethyl group of pG96S petunia EPSPS displaces the phosphate of PEP and functions as a nucleophile, attacking the C-2 carbon of the carboxyvinyl moiety of EPSP as suggested in mechanism 3. If this were to be the case, we would predict that variants of EPSPS containing alanine or valine at Gly96 should be ineffective in catalyzing this reaction. In accord with this, purified G96A *E. coli* EPSPS did not have detectable EPSP hydrolyase activity. Additional experiments are in progress to determine the hydrolase reaction mechanism of pG96S EPSPS.

**pP101S EPSPSs**—Yet another substitution that has been described in the conserved region of EPSPS is that of P101S in *S. typhimurium* EPSPS (11, 12). Since this proline is not conserved in all EPSPSs (Table I), it was of interest to determine if the corresponding proline to serine substitution would result in a glyphosate-tolerant petunia EPSPS. The pP101S EPSPS cDNA was therefore constructed by site-directed mutagenesis and expressed in *E. coli* SR481. Kinetic analysis of the purified enzyme showed that the $K_{\text{inapp}}(\text{PEP})$ was 44 ± 3 µM, the $K_{\text{inapp}}(\text{S3P})$ was 12 ± 2 µM, the $K_{\text{inapp}}(\text{glyphosate})$ was 3.0 ± 0.8 µM, and the specific activity was 40 units/mg. These results show that for petunia EPSPS, the pP101S substitution results in a decrease in glyphosate binding, the magnitude of which is, however, significantly lower than for the pG96A substitution. The results of these mutagenesis experiments further support the view that the active sites of bacterial and plant EPSPSs are highly conserved.

A doubly substituted petunia EPSPS variant of pG96A and pP101S was constructed in order to determine the effect of combining the two single substitutions which independently confer glyphosate tolerance to EPSPS. The pG96A pP101S variant had an $K_{\text{inapp}}(\text{PEP})$ of 390 ± 40 µM and an $K_{\text{inapp}}(\text{glyphosate})$ of 8.2 ± 0.4 µM. Thus, the addition of the pP101S substitution to the pG96A variant increases the $K_{\text{inapp}}(\text{glyphosate})$ by a factor of 4.1 and the $K_{\text{inapp}}(\text{PEP})$ by a factor of 1.8. This increase in $K_{\text{inapp}}(\text{glyphosate})$ is similar to the increase observed for the pP101S single variant relative to wild-type EPSPS (6-fold) and suggests that the pG96A and pP101S substitutions impart glyphosate tolerance by affecting distinct interactions between glyphosate and the conserved region. The $K_{\text{inapp}}(\text{PEP})$ may be somewhat obscure since the absolute $K_{\text{m}}(\text{PEP})$ has not been obtained. Nevertheless, the results of these studies suggest multiple interactions between PEP/glyphosate and the conserved region of EPSPS. One of these interactions may involve the guanidinium side-chain of Arg100, a conserved residue in all EPSPS enzymes (Table I). Unpublished experiments from our laboratory based on mutagenesis of pArg100 support this hypothesis. A complete understanding of the nature of these interactions will have to await the x-ray structure elucidation of the enzyme-substrate/inhibitor complexes.

It is clear that considerable information about the active site of EPSPS has been obtained in site-directed mutagenesis and kinetic characterization of the variant enzymes. Recently, the x-ray structure of wild-type *E. coli* EPSPS has been elucidated at 3-Å resolution (2). The EPSPS polypeptide has 170 amino acid residues, with 270 electron densities, and 320 residues of hydrogen atoms.

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6 S. R. Padgette, D. B. Re, C. M. Hironaka, and G. M. Kishore, unpublished data.
a two-domain structure with a novel fold that appears to be formed by a 6-fold replication of a protein folding unit comprised of two parallel helices and four stranded sheets. In this structure, the active site appears to be in the interdomain region, held apart by the interdipole repulsions in the absence of the anionic ligands. Since significant conformational changes can be demonstrated upon interaction of EPSPS with either S3P and glyphosate or EPSP and glyphosate, the structure of the native enzyme is insufficient to explain the results obtained with the variant enzymes. However, our knowledge of the variant enzymes can be of great value toward building models for the structure of the enzyme complexes. For a model to be valid, it must account for the perturbations in the kinetic constants of the variants described herein as well as the EPSP hydrolase activity of the pG96A variant. It is interesting to note that the two primary substitutions that confer glyphosate tolerance to EPSPS are localized within the conserved region shown in Table I. Whether this is the only region of EPSPS that can provide differentiation between PEP and glyphosate binding needs to be determined either by elucidation of the x-ray structure of an EPSPS-glyphosate (or PEP) complex or isolating additional glyphosate-tolerant EPSPS variants.

In summary, we have utilized site-directed mutagenesis to identify the active site of EPSPS. These studies have established that the conserved region of EPSPS containing GlyW is critical for interaction with the phosphate moiety of PEP, inorganic phosphate, and the phosphonate of glyphosate. The levels of tolerance are significantly higher than that achieved using the wild-type, glyphosate-sensitive EPSPS achieved using the wild-type, glyphosate-sensitive EPSPS. Further facilitate new inhibitor design and the understanding of the catalytic mechanism of the enzyme.

Acknowledgements—We thank Prof. Paul Bartlett for supplying a sample of the EPSPS tetrahedral intermediate analog inhibitor, Drs. Bill Stalling and Henry Dayringer for information on the E. coli EPSPS x-ray structure, Dr. Khai Huynh for a sample of purified pG96A EPSPS, Scott Elmer for helpful discussions on the soybean EPSPS, Yvonne Muskopf for generating the trascgenic Arabidopsis plants, Leslie Brand for E. coli expression of the pFI01S petunia EPSPS clone, and Dr. Jim Sikorski for providing a sample of EPSP. We thank Dr. Ernie Jaworski for his support of this work.

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