Glyphosate (N-phosphonomethylglycine) inhibits the shikimate pathway enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS); EC 2.5.1.19) (1), which is essential for the biosynthesis of aromatic compounds in plants, fungi, bacteria, and apicomplexan parasites (2-5). Glyphosate, the active ingredient in Roundup, exhibits broad-spectrum herbicidal activity, yet is essentially nontoxic to animals and does not persist in the environment. These characteristics have made it the world’s most popular herbicide, and usage continues to increase with the adoption of glyphosate-dependent technologies, including herbicide-tolerant crops and minimal tillage (no-till) agriculture. The enormous reliance on glyphosate and the absence of suitably safe alternative herbicides mean that the widespread emergence of glyphosate-tolerant weeds would have devastating agricultural and environmental consequences.

EPSPS, the molecular target of glyphosate, catalyzes the transfer of the enolpyruvyl moiety of phosphoenolpyruvate (P-enolpyruvate) to the 5-hydroxy position of shikimate 3-phosphate (S3P) (see Fig. 1). The structure of the glyphosate-inhibited complex shows that glyphosate binds to the P-enolpyruvate-binding site of EPSPS (6-8), corroborating early kinetic data demonstrating that glyphosate binding is competitive with respect to P-enolpyruvate (1, 9, 10). Before bacterial enzymes with innate glyphosate tolerance (class II EPSPS) were discovered and used to produce Roundup Ready crops, scientists described several mutations that decreased glyphosate sensitivity in the plant-like EPSPS from _Escherichia coli_ (11, 12). Typically, however, these mutant enzymes displayed an increased _K_m_ for P-enolpyruvate and a correspondingly decreased catalytic efficiency, indicative of decreased fitness in the absence of glyphosate, and were thus considered unsuitable for the development of glyphosate-tolerant crops.

Glyphosate-resistant plants have developed both through natural evolution _in situ_ and by directed evolution or mutagenesis _in vitro_, and glyphosate tolerance can be induced by either target-site or non-target-site mechanisms. Non-target-site tolerance mechanisms include overexpression of EPSPS (13) and decreased uptake or translocation of glyphosate (14). Target-site glyphosate tolerance can be induced by specific mutations of EPSPS (15, 16), including T42M (17); G96A (11, 18); T97I (19); P101L, P101T, P101A, and P101S (11, 20-24); and A183T (25, 26) (all numbering according to _E. coli_ EPSPS). Notably, field-evolved plants exhibiting target-site glyphosate tolerance invariably contain single-residue substitutions at the site corresponding to Pro101 of _E. coli_ EPSPS (20-24).

The structural basis for the Pro101 mutation-induced glyphosate tolerance was not known. Here, we describe the kinetic and structural basis for the Pro101 mutation-induced glyphosate tolerance resulting from mutations of Pro101 in _Escherichia coli_ 5-Enolpyruvylshikimate-3-phosphate Synthase*5

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*5 The atomic coordinates and structure factors (code 2qfs, 2qft, 2qaq, and 2qfu) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

1 The abbreviations used are: EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase; P-enolpyruvate, phosphoenolpyruvate; S3P, shikimate 3-phosphate; WT, wild-type; r.m.s.d., root mean square deviation.

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The kinetic characteristics of mutant and wild-type EPSPS enzymes were investigated. With bovine serum albumin as a standard, the single-site Pro101 mutations were introduced into wild-type EPSPS from E. coli using the QuikChange mutagenesis kit (Stratagene) and appropriate primers. All primers were synthesized by MWG Biotech (High Point, NC). The pET-24d vector (Novagen) containing the open reading frame of wild-type (WT) EPSPS was used as a template for the mutations. Pro101 mutant EPSPS enzymes were overexpressed in BL21(DE3) competent cells and purified as described previously (18). After the final purification step, the mutant enzymes were concentrated in 50 mM Tris and 2 mM dithiothreitol using Centricon-30 devices (Millipore Corp., Billerica, MA) at 4 °C.

The enzymatic activities of WT and Pro101 mutant EPSPS were measured spectrophotometrically at 25 °C with a SpectraMax 340PC plate reader (Molecular Devices, Sunnyvale, CA). The assay mixtures (60 µl) contained 50 mM HEPES (pH 7.5), 100 mM KCl, 2 mM dithiothreitol, 1 mM S3P, and varied concentrations of P-enolpyruvate and glyphosate. The reaction was initiated by the addition of enzyme (3.4 nM WT; 8.5 nM P101S, P101G, P101A, P101L, P101S, 105 70 1 3.9 0.5 3.9

### EXPERIMENTAL PROCEDURES

S3P was synthesized and purified as described previously (27). P-enolpyruvate was purchased from Sigma. Protein concentration was determined using Coomassie reagent (Pierce) (27). P-enolpyruvate was purchased from Sigma. Protein concentration was determined using Coomassie reagent (Pierce) with bovine serum albumin as a standard. The single-site Pro101 mutations were introduced into wild-type EPSPS from E. coli using the QuikChange mutagenesis kit (Stratagene) and appropriate primers. All primers were synthesized by MWG Biotech (High Point, NC). The pET-24d vector (Novagen) containing the open reading frame of wild-type (WT) EPSPS was used as a template for the mutations. Pro101 mutant EPSPS enzymes were overexpressed in BL21(DE3) competent cells and purified as described previously (18). After the final purification step, the mutant enzymes were concentrated in 50 mM Tris and 2 mM dithiothreitol using Centricon-30 devices (Millipore Corp., Billerica, MA) at 4 °C.

The enzymatic activities of WT and Pro101 mutant EPSPS were measured spectrophotometrically at 25 °C with a SpectraMax 340PC plate reader (Molecular Devices, Sunnyvale, CA). The assay mixtures (60 µl) contained 50 mM HEPES (pH 7.5), 100 mM KCl, 2 mM dithiothreitol, 1 mM S3P, and varied concentrations of P-enolpyruvate and glyphosate. The reaction was initiated by the addition of enzyme (3.4 nM WT; 8.5 nM P101S, P101G, P101A, P101L, P101S, 105 70 1 3.9 0.5 3.9

### TABLE 1

Summary of data collection and structure refinement

|                      | P101S-S3P | P101S-S3P/glyphosate | P101L-S3P | P101L-S3P/glyphosate |
|----------------------|-----------|----------------------|-----------|----------------------|
| **Data collection**  |           |                      |           |                      |
| Space group          | P2,2,2    | P2,2,2               | P2,2,2    | P2,2,2               |
| Resolution range     | 20–1.55 (1.6–1.55) | 20–1.55 (1.6–1.55) | 20–1.5 (1.55–1.5) | 20–1.6 (1.65–1.6) |
| Unique reflections   | 63,104 (5588) | 63,948 (5744) | 69,786 (6428) | 57,383 (4656) |
| Completeness (%)     | 98.8 (97.7) | 99.8 (99.9) | 99.7 (99.2) | 99.3 (92.9) |
| l/σ                  | 87.9 (87.6) | 88.3 (88.6) | 88.0 (88.3) | 88.0 (88.3) |
| Rmerge (%)           | 2.5 (6.5) | 4.4 (8.4) | 4.8 (17.1) | 2.8 (7.5) |

| **Structure refinement** |           |                      |           |                      |
| Protein atoms         | 3231      | 3231                 | 3233      | 3233                 |
| Average B-factor (Å²) | 11.6      | 9.8                  | 13.0      | 11.6                 |
| Ligand atoms          | 16        | 26                   | 16        | 26                   |
| Average B-factor (Å²) | 7.2 (S3P), 6.6 (glyphosate) | 13.9 | 10.0 (S3P), 9.1 (glyphosate) |
| Solvent molecules     | 596       | 669                  | 561       | 560                  |
| Average B-factor (Å²) | 25.0      | 24.4                 | 26.7      | 26.3                 |
| r.m.s.d. bonds (Å)    | 0.01      | 0.01                 | 0.01      | 0.01                 |
| r.m.s.d. angles       | 1.59°     | 1.59°                | 1.6°      | 1.6°                 |
| Rcryst (%)            | 15.9      | 15.6                 | 16.4      | 14.9                 |
| Rfree (%)             | 18.1      | 17.9                 | 18.2      | 16.9                 |

| **Cross-validated estimated coordinate error** |           |                      |           |                      |
| From Luzzati plot (Å) | 0.16      | 0.16                 | 0.16      | 0.15                 |
| From SigmaA (Å)       | 0.13      | 0.07                 | 0.11      | 0.05                 |

| **Ramachandran statistics** |           |                      |           |                      |
| Most favored regions  | 90.3      | 91.4                 | 91.6      | 92.2                 |
| Additionally allowed regions | 9.4 | 8.4 | 8.1 | 7.5 |
| Generously allowed regions | 0.3 | 0.3 | 0.3 | 0.3 |
| Disallowed regions    | 0.0       | 0.0                  | 0.0       | 0.0                  |

| **Fmerge = 100 × Σ|Fobs| - |Fcalc|/|Σ|Fobs|, where k and i are unique reflection indices. |
| r.m.s.d. from ideal values. |
| Rcryst = 100 × Σ|Fobs| - |Fcalc|/|Σ|Fobs|, where Fobs and Fcalc are observed and calculated structure factor amplitudes, respectively. |
| Rfree is Rcryst calculated for randomly chosen unique reflections, which were excluded from the refinement (1264 for P101S EPSPS-S3P, 1279 for P101S EPSPS-S3P/glyphosate, 1396 for P101L EPSPS-S3P, and 1149 for P101L-EPSPS-S3P/glyphosate). |

### TABLE 2

Kinetic characteristics of mutant and wild-type EPSPS enzymes

| EPSPS enzyme | K_cat/K_m(S3P) | V_max | k_cat/K_m(PEP) | K_cat(PEP) | K_cat/K_m(PEP) | K_i | K_cat/PEP/K_i |
|--------------|----------------|-------|----------------|------------|----------------|-----|---------------|
| WT           | 60 ± 6         | 50 ± 1| 6.4 × 10^4     | 60 ± 6     | 6.4 × 10^4     | 0.4 ± 0.06 | 150           |
| P101S        | 80 ± 6         | 22 ± 1| 2.0 × 10^5     | 70 ± 5     | 2.9 × 10^5     | 5.5 ± 0.3 | 13            |
| P101G        | 60 ± 10        | 28 ± 1| 3.9 × 10^5     | 90 ± 10    | 2.4 × 10^5     | 12 ± 4   | 7.5           |
| P101A        | 60 ± 4         | 31 ± 0| 3.9 × 10^5     | 80 ± 5     | 2.8 × 10^5     | 19 ± 3   | 4.2           |
| P101L        | 100 ± 7        | 8 ± 2 | 6.2 × 10^5     | 150 ± 20   | 4.1 × 10^4     | 66 ± 2   | 2.3           |

**PEP, phosphoenolpyruvate.**
P101G, and P101A; and 34 nM P101L) and allowed to proceed for 30 min at 25 °C before the addition of 140 μl of Lanzetta reagent. After an additional 10 min for color development, the change in absorbance at 650 nm was recorded, and the amount of inorganic phosphate produced was determined by comparison with phosphate standards. Enzymatic activity is expressed as micromoles of phosphate produced per min of reaction/mg of enzyme (units/mg).

The $K_m$ values were determined by fitting the data to the Michaelis-Menten equation using SigmaPlot (SPSS Inc., Chicago, IL). The $K_i$ values were derived by determining the $K_m(\text{obs})$ of P-enolpyruvate in the presence of increasing concentrations of glyphosate and fitting the data to the following equation:

$$
K_m(\text{obs}) = \frac{K_m}{K_i} \times [I] + K_m,
$$

where $K_m(\text{obs})$

FIGURE 2. Location of Pro$^{101}$ in the structure of WT EPSPS from E. coli (stereo view). EPSPS is composed of two globular domains that close upon binding of S3P and glyphosate (shown in yellow and green, respectively); the two ligands are located in the interdomain cleft of the closed enzyme state (Protein Data Bank code 1g6s) (6). Displayed in maroon is the helix in the upper (N-terminal) domain containing Pro$^{101}$. Glyphosate binds adjacent to S3P, its phosphonate moiety pointing toward the N-terminal end of the helix.

FIGURE 3. Structural differences between WT EPSPS and the Pro$^{101}$ mutant enzymes. Shown are the r.m.s.d. values of the main chain atoms of WT EPSPS (Protein Data Bank code 1g6s) (6) and the P101L (solid line) and P101S (dotted line) mutant enzymes complexed with S3P and glyphosate. The inset shows the r.m.s.d. values of residues 93–106 in multiples of the overall r.m.s.d.
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TABLE 3
Changes in backbone torsion angles of residues 96–98 as a result of Pro101 mutations.

| Torsion angle | Gly96 | Thr97 | Ala98 | Gly96 | Thr97 | Ala98 |
|--------------|-------|-------|-------|-------|-------|-------|
| $\phi$       | -63'  | -72'  | -104' | -60'  | -61'  | -88'  |
| $\psi$       | -34'  | -5.6' | -61'  | -38'  | -29'  | -45'  |
| $\omega$     | -178' | 170'  | -178' | -178' | 178'  | 180'  |
| Planarity    | 0.32' | -0.03' | 0.84' | 0.16' | 0.05' | -0.31' |

* The torsion angles were calculated with MOLEMAN2 (37), with $\phi = C(i-1) - N(i) - CA(i) - C(i)$, $\psi = N(i) - CA(i) - C(i) - N(i+1)$, $\omega = CA(i) - C(i) - N(i+1) - CA(i+1)$, and planarity = $C(i) - CA(i) - N(i+1) - O(i)$.

FIGURE 4. Structure determination of P101S and P101L mutant EPSPS enzymes complexed with S3P and glyphosate. A, electron density (contoured at 3$\sigma$) derived from $F_o - F_c$. Fourier syntheses, omitting residues 96–101 during refinement of the P101S enzyme (left) or the P101L enzyme (right). For clarity, only the density of Gly96, Thr97, and Ser98 is displayed. B, electron density (contoured at 4$\sigma$) derived from $F_o - F_c$ (left) or $F_c - F_o$ (right) Fourier syntheses using the coordinates of P101S (WT) or P101L (right) as reference. The highest peaks of 23$\sigma$ and 18$\sigma$ correspond to the position of the carbonyl oxygen of Thr97 in WT EPSPS, which shifts substantially as a result of the mutation. The difference density to the right of Thr97 corresponds to the carbon atoms of the Pro101 ring (see also Fig. 5). $F_o$ and $F_c$ are the calculated and observed structure factors, respectively.

The Michaelis constant for P-enolpyruvate in the presence of glyphosate, $[I]$ is the glyphosate concentration, and $K_m$ is the Michaelis constant for P-enolpyruvate in the absence of glyphosate.

P101S and P101L EPSPS enzymes were crystallized at 19 °C by the hanging-drop vapor diffusion method in the presence of 5 mM S3P with or without 5 mM glyphosate using the sodium formate crystallization conditions described previously (18). The protein concentration in each case was 37.5 mg/ml, or 810 units/mg, maintaining a ligand-to-receptor molar ratio of $\sim 6:1$. X-ray diffraction data were recorded at $-180$ °C using the rotation method on a single flash-frozen crystals of Pro101 mutant EPSPS enzymes (detector, R-AXIS IV++ imaging plate; x-rays, CuKα, focused by mirror optics; generator, Rigaku RU300 (MSC, The Woodlands, TX)). The collected data were reduced with XDS (28). The program package CNS (29) was employed for phasing and refinement, and model building was performed with program O (30). The structures were solved by molecular replacement using E. coli WT EPSPS (Protein Data Bank code 1g6s) (6) stripped of solvent molecules, ions, and ligands as the starting model. Refinement was performed using data to the highest resolution with no $\sigma$ cut-off applied. Solvent molecules were added to the models at reasonable positions, and S3P and glyphosate were modeled according to the clear electron density maps. Several rounds of minimization, simulated annealing (2500 K starting temperature), and restrained individual B-factor refinement were carried out. Data collection and refinement statistics are summarized in Table 1. Figs. 2, 4, and 5 were drawn with MolScript (31), BobScript (32), and Raster3D (33).

RESULTS AND DISCUSSION

Four single-site mutations were introduced into E. coli EPSPS, replacing Pro101 with glycine, alanine, serine, or leucine. The resulting mutant enzymes were characterized by steady-state kinetics using the forward reaction of EPSPS (Fig. 1). The P101S and P101L mutant enzymes were crystallized in the presence of S3P with or without glyphosate, and the crystal structures were determined to between 1.5- and 1.6-Å resolution (Table 1).

Enzyme Kinetics—All four mutant enzymes were found to be catalytically active, with specific activities ranging from 8 to 30 units/mg compared with 50 units/mg for WT EPSPS (Table 2 and supplemental Figs. S1–S5). The enzymatic reactions display normal saturation kinetics. Two distinct trends are discernible in the kinetic data. First, small residues may be substituted for Pro101 without substantially altering the affinity of the
enzyme for P-enolpyruvate. The $K_m$ values indicate that the binding affinities for P-enolpyruvate and S3P are unchanged or only slightly decreased for the P101G, P101A, and P101S mutant enzymes; P101L displays an 2–10-fold lower affinity for both S3P and P-enolpyruvate. Second, substitution of any residue for Pro101 decreases the binding affinity of glyphosate, reducing the potency of this inhibitor. Unlike WT EPSPS, with P101L EPSPS having the lowest catalytic efficiency. Overall, the substitution of residues smaller than leucine for Pro101 results in a glyphosate-tolerant, catalytically efficient enzyme.

Protein Crystallography—In WT EPSPS from E. coli, Pro101 is part of an internal helix (residues 97–105) in the N-terminal globular domain, ~9 Å distant from glyphosate (Fig. 2). Therefore, the effects of Pro101 mutations on the inhibition of EPSPS by glyphosate are likely to be due to long-range structural changes in the glyphosate-binding site. X-ray crystal structures at between 1.5- and 1.6-Å resolution revealed that the overall structures of the P101S and P101L mutant enzymes are nearly identical to that of WT EPSPS (Fig. 3). When comparing the glyphosate-bound forms of the mutant enzymes with that of WT EPSPS, the root mean square deviation (r.m.s.d.) values of all 427 C-α atoms are 0.066 and 0.097 Å for P101S and P101L, respectively. The largest differences occur in the backbone of Gly96, Thr97, and Ala98, with the C-α atom of Thr97 having 4–7 times larger r.m.s.d. values than the overall r.m.s.d. (Fig. 3). These structural alterations are also reflected by substantial changes in the backbone torsion angles of residues 96–98 (Table 3), and the changes are further evident from $F_o - F_c$ electron density maps with difference peak heights of 23 and 18 Å in the backbone around Thr97 for the P101S and P101L enzymes, respectively (Fig. 4).

The altered amino acids Ser101 and Leu101 are well defined in the respective electron density maps (Fig. 4). Notably, the side chain of Ser101 adopts two alternate conformations. Although the hydroxyl group of either Ser101 rotamer is in hydrogen-bonding distance (~3.2 Å) of the carbonyl oxygen of Thr97, its apparent flexibility makes a strong bonding interaction unlikely (Fig. 5). Neither the P101S nor P101L mutation disrupts the α-helix present in WT EPSPS. Rather, it seems that the loss of the Pro101 ring system causes a disruption of hydrophobic interactions that hold in place the carbonyl oxygen of Thr97. In the mutant enzymes, the backbone of Thr97 relaxes and reorients, rotating about its φ/ψ torsion angles (Table 3). As a result, Gly96 and Thr97 shift slightly toward the glyphosate-binding site, thereby causing repulsive forces (~3.2 Å).
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between the C-α atom of Gly\(^96\) and the phosphonate moiety of glyphosate. It appears that the altered orientation of Thr\(^97\) and Gly\(^96\) causes a slight narrowing of the binding site for glyphosate. This negatively impacts glyphosate inhibitory potency because this inhibitor interacts with EPSPS efficiently only in its extended conformation (7). Moreover, the binding of the substrate P-enolpyruvate would remain unaltered because the P-enolpyruvate molecule is significantly shorter than glyphosate (18).

Conclusion—The removal of undesired plant species (weeds) to increase nutrient availability and crop yields is an intrinsic component of agriculture. The current enormous (and growing) reliance on glyphosate indicates that the development and spread of glyphosate-resistant weeds would have far-reaching negative consequences (34). To date, reports of target-site mutations suggest that glyphosate-tolerant EPSPS enzymes typically have a substantial fitness cost, particularly in the absence of multiple (compensatory) mutations. Mutations at sites corresponding to Pro\(^{101}\) appear to incur the least fitness cost, and because point mutations are far more evolutionarily accessible than multiple mutations, target-site glyphosate tolerance seems most likely to arise via mutations of this residue. With continued selective pressure from glyphosate application, the evolution of weedy plants expressing such enzymes appears inevitable. Our study indicates that the structural basis for the glyphosate tolerance of such Pro\(^{101}\) mutant EPSPS enzymes is due to long-range alterations in the active site of the enzyme, in particular impacting the spatial orientation of Gly\(^{96}\) and Thr\(^{97}\). It has long been known that Gly\(^{96}\) is critical for the efficient binding of glyphosate. For \(E.\ coli\) EPSPS, mutation of Gly\(^{96}\) to alanine results in a complete loss of inhibitory potency because of the methyl group protruding into the glyphosate-binding site; however, this glyphosate tolerance comes at the expense of a drastically lowered affinity for P-enolpyruvate and poor catalytic efficiency (18). In general, because the degree of glyphosate tolerance depends on the extent to which the inhibitor-binding site is perturbed and because the catalytic efficiency depends on the extent to which the substrate-binding site is left intact, it appears that the Pro\(^{101}\) substitution is favorable precisely because the changes in the enzyme active-site structure are so slight. Residues other than proline substituted at position 101 reduce glyphosate binding, whereas residues smaller than leucine at this position essentially do not alter the S3P- and P-enolpyruvate-binding sites, retaining catalytic efficiency.

To prevent the expected deterioration of the herbicidal properties of glyphosate, aggressive strategies for combating glyphosate-tolerant weeds should be implemented. Our data indicate that plants with these target-site mutations likely remain susceptible to glyphosate at high concentrations, but non-target-site mutations may act synergistically (14, 22, 23), drastically decreasing the effectiveness of glyphosate. On the basis of the extensive structure-activity relationship studies performed with glyphosate analogs (15) and the subtlety of the structural changes observed in glyphosate-tolerant enzymes, glyphosate analogs may not represent suitable replacements for glyphosate. Because plants with target-site glyphosate tolerance mutations remain susceptible to herbicides targeting enzymes other than EPSPS, herbicide rotation practices may delay the development and spread of glyphosate-tolerant weeds, and integrated weed management programs should be encouraged (35). The engineering of crops with resistance to other herbicides, such as dicamba (36), holds some promise, but such crops are not yet commercially available, and glyphosate has unique advantages because of its very low toxicity to animals and its broad-spectrum activity against plants. In the long run, the development of completely new inhibitors of EPSPS or other shikimate pathway enzymes is desirable, as new nontoxic herbicides will be required.

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