A T42M Substitution in Bacterial 5-Enolpyruvylshikimate-3-phosphate Synthase (EPSPS) Generates Enzymes with Increased Resistance to Glyphosate

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Mutants of class I enolpyruvylshikimate 3-phosphate synthase (EPSPS) with resistance to glyphosate were produced in a previous study using the staggered extension process with \textit{aroA} genes from \textit{S. typhimurium} and \textit{E. coli}. Two of these mutants shared a common amino acid substitution, T42M, near the hinge region between the large globular domains of EPSPS. Using site-directed mutagenesis, we produced the T42M mutants without the other amino acid changes of the original mutants. The T42M substitution alone produced enzymes with a 9- to 25-fold decreased $K_m$[PEP] and a 21- to 26-fold increased $K_i$[glyphosate] compared to the wild-type enzymes. These results provide more testimony for the powerful approach for protein engineering by the combination of directed evolution and rational design.

Key words: bacterial \textit{aroA}; glyphosate; mutagenesis; herbicide; 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS)

Glyphosate is one of the most potent broad-spectrum herbicides in use today. Glyphosate inhibits a key enzyme in the synthesis of aromatic amino acids, 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS), thereby depriving weeds of aromatic amino acids. Tolerance to glyphosate can be conferred in plants using the transgenic introduction of bacterial enzymes with engineered or naturally occurring tolerance. Two classes of enzyme, sharing less than 50% amino acid similarity, perform the reaction: shikimate-3-phosphate (S3P) + phosphoenolpyruvate (PEP) $\rightarrow\n$ EPSP + Pi. The class I enzymes include those found in plants and the bacteria \textit{E. coli} and \textit{S. typhimurium}. The class II enzymes are found in \textit{Agrobacterium tumefaciens} sp. CP4, \textit{Achromobacter} sp. LBAA, and \textit{Pseudomonas} sp. PG2982. Most of the reported studies of enzyme kinetics and active site analysis concern the class I enzymes.

Inhibition of EPSPS by glyphosate appears competitive with respect to PEP and is done by formation of a stable but non-covalent ternary complex of enzyme, S3P, and glyphosate. When the enzyme is bound to S3P and either PEP or glyphosate, a large conformational change alters the relationship between the two globular domains. A similar conformational change was first shown to occur in the structurally related enzyme, MurA. The free enzyme adopts an “open” conformation but in an inhibitor-enzyme complex the conformation is “closed”. Similarly, EPSPS adopts an “open” conformation as the free-enzyme or a “closed” conformation in the ternary complex of enzyme-S3P- and either PEP or glyphosate, as shown in X-ray crystallography studies. The conformational changes in these enzymes can occur because of flexibility in a hinged region between the two globular domains. As the globular domains close in a screw-like fashion, the active site is formed. In the closed conformation, specific amino acids are protected from chemical modification, and a trypsin site is hidden. Furthermore, chemical modification and site-directed mutagenesis studies indicate that amino acids important for binding substrates and inhibitor are present on both globular domains. Lastly, the presence of S3P and PEP or glyphosate in the “closed” conformation confirms that the active site is located in the cleft between the globular domains. These active site studies and the crystal structure indicate that the binding sites for glyphosate and PEP are overlapping but not superimposable, therefore it should be possible to alter the binding properties of the enzyme for PEP and glyphosate separately.

We have used the staggered extension process for random recombination and mutation of class I \textit{aroA} genes from \textit{S. typhimurium} and \textit{E. coli}. Several mutant enzymes with increased $K_i$[glyphosate] and lowered $K_m$[PEP] were isolated after selection for resistance to glyphosate in bacterial culture. These mutants contained multiple amino acid substitutions and crossovers between the parental sequences. Two of these mutants shared a T42M substitution, which

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*Abbreviations: S3P, shikimate-3-phosphate; PEP, phosphoenolpyruvate; EPSPS, 5-enolpyruvylshikimate-3-phosphate synthase
The T42M substitution in the *E. coli* and *S. typhimurium* EPSPS were introduced using overlap-PCR mutagenesis. The oligonucleotide primers used to generate the T42M mutant of *E. coli* EPSPS were: CATGCCATGGAATCCCTGACGTTACAA (primer 1), CACGTCATCGCTATCCAGCAGATTCATTAATAC (primer 2) and GGAATTCCTATGGCGCACGTCATCGCTATCCAGC (primer 3). The oligonucleotide primers used to generate the T42M mutant of *S. typhimurium* EPSPS were: primers 1 and 3, and GACGTCATCGCTATCCAGCAGATTCATCAGAGC (primer 4). NcoI and NdeI restriction sites (underline) were contained in primers 1 and 3. The mutagenic nucleotides (italic) used to convert codons ACC and ACG to ATG were incorporated in primer 2 and primer 4, respectively. Primers 2 and 4 overlapped primer 3 by 19 nucleotides.

Overlap-PCR mutagenesis was done in two rounds of PCR. The pET-EcaroA and pET-StaroA plasmids cloned previously were used as the templates in the first reaction to produce a 150-bp fragment using primers 1 and 2 or primes 1 and 4. Primer 1 corresponded to nucleotides 1–21 of EcaroA and StaroA. Primer 2 was complementary to nucleotides 118–150 of EcaroA, and primer 4 was complementary to nucleotides 118–150 of StaroA. Primers 2 and 4 contained the mutagenic oligonucleotides designed to introduce the T42M mutation. Primer 3 was complementary to nucleotides 128–159 of EcaroA and StaroA and included a 19-nucleotide overlapping fragment with primers 2 and 4. Primer 3 also contained an Ndel site at nucleotides 154 to 159. Primers 1 and 3 were used to produce the mutagenic fragments in the first round of PCR. These fragments were digested with NcoI and Ndel and ligated in place of the corresponding portion of the genes in pET-EcaroA and pET-StaroA (Fig. 1). The resulting constructs were designated pET-EcaroA-T42M and pET-StaroA-T42M.

The *E. coli* strain BL21 (DE3) (F*ompT hsdSde*rbbm*gal dcm* (DE3)) harboring the mutant or wild type aroA genes in pET-11d constructs were grown overnight in M9 media (6 g/l of Na2HPO4, 3 g/l of K2HPO4, 1 g/l of NH4Cl, 0.5 g/l of NaCl, 1 mM MgSO4, 0.4% [w/v] glucose, 100 μg/ml of thiamine, and 50 μg/ml of ampicillin) and 0.1 ml was used to inoculate fresh 20-ml cultures. Cells were grown at 37°C to a density of 0.1 A600, then protein expression was induced using 1 mM IPTG. Glyphosate was added at the same time as IPTG. Cells harboring pET-EcaroA-T42M and pET-StaroA-T42M were compared with those of the wild types in the presence of 30 and 60 mM glyphosate. Cells harboring the pET-StaroA-T42M grew better than cells harboring pET-EcaroA-T42M; but cells harboring either the wild type pET-EcaroA or pET-StaroA grew poorly (Fig. 2).

Cell-free extracts of expression bacteria were assayed for the kinetic properties of EPSPS activity, measured in the forward direction by the release of inorganic phosphate. BL21 (DE3) pLysS harboring the mutant or wild type aroA genes in pET-11d constructs were grown to 0.75 A600 in 200 ml of LB media containing 50 μg/ml ampicillin. Expression was induced with 1 mM IPTG. After 3 hours the cells were collected by centrifugation and resuspended in 20 ml of 50 mM Tris-HCl buffer, pH 7.0, containing 1 mM DTT. The cell suspension was frozen at −70°C then thawed at room temperature, and cells were lysed by sonication. The crude homogenate was clari-
Fig. 2. Growth Curves of E. coli Expression Cells Harboring pET-EcaroA, pET-StaroA, pET-EcaroA-T42M, and pET-StaroA-T42M Plasmids.

Cells were grown in liquid M9 minimal medium at 37°C. IPTG and glyphosate were added at time zero at a cell density of 0.1 A600. After 16 h of growth, 1-ml proteins were withdrawn from each culture, and measured for cell density at approximately 2-h intervals.

Fig. 3. SDS-PAGE of Crude Extracts Prepared from BL21 Cells Harboring Either Wild Types or Mutants pET Expression Plasmids.

M, molecular size marker; lane 1, cell containing no expression plasmid; lane 2, cell harboring pET-EcaroA; lane 3, cell harboring pET-StaroA; lane 4, cell harboring pET-EcaroA-T42M; and lane 5, cell harboring pET-StaroA-T42M. Equal amounts of total protein were separated on a 12% polyacrylamide gel then stained with Coomassie blue. The expressed proteins were indicated by an arrow.

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void using centrifugation at 12,000 × g for 30 min. The protein concentration of the mutant and wild type enzymes were essentially identical as estimated by the intensity of the 45 kDa bands in SDS-PAGE (Fig. 3) allowing the direct comparison of EPSPS kinetic properties. EPSPS activity was carried out at 30°C in a final volume of 100 μl containing 50 mM Hepes, pH 7.0, 2 mM S3P (prepared from the culture broth of Klebsiella pneumoniae ATCC 25597 according the method described by Lanzetta et al.,13) 1 mM PEP (Sigma, USA), 0.1 mM (NH₄)₆Mo₇O₂₄·2H₂O, and crude extracts. After incubation for 20 min, 1 ml of malachite green-ammonium molybdate colorimetric solution was added, and 1 min later, 0.1 ml of 34% sodium citrate solution was added. After 15 min of incubation at room temperature, samples were measured at 660 nm. Glyphosate inhibition was found to be competitive in the kinetic plots (data not shown). Compared to the wild types, the EcaroA-T42M and StaroA-T42M enzymes had, respectively, a 9- to 25-fold lower Km[PEP], a 21- to 26-fold higher Ki[glyphosate], and a 4.7- to 23.5-fold increased specific activity (Table 1).

A structural model of E. coli EPSPS shows the position of the T42M substitution (Fig. 4). The protein comprises two equally sized and structurally similar globular domains connected by a two-stranded hinge.14) In the EcaroA-T42M and StaroA-T42M mutants, the hydrophilic side group of threonine was replaced with the bulkier, hydrophobic side group of methionine. The T42M substitution in EPSPS is close to both strands of the hinge and its modification could affect the screw-like closure of the hinge, altering the relative positions of the clusters of active site amino acids that reside on opposite globular domains. For example, mutations of R100A, D242A, and D384A show a drastic decrease in activity but none of these amino acids are involved in substrate binding, and instead may hinder domain closure.15) Similarly, we speculate that the T42M amino acid substitution alters the relationship of the two globular domains.

In a previous study, we generated a set of mutant class I EPSPS enzymes with significantly increased
Table 1. Kinetic Properties of Mutants and Wild Type EPSPS

<table>
<thead>
<tr>
<th>Enzymes</th>
<th>Specific activity* (u/mg protein × 10⁻³)</th>
<th>(K_m) (PEP) (µM)</th>
<th>(K_i) (Glyphosate) (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EcaroA</td>
<td>7.558 ± 0.468</td>
<td>22.28 ± 6.26</td>
<td>1.48 ± 0.459</td>
</tr>
<tr>
<td>EcaroA-T42M</td>
<td>35.704 ± 4.979</td>
<td>2.36 ± 0.39</td>
<td>30.49 ± 10.06</td>
</tr>
<tr>
<td>StaroA</td>
<td>5.759 ± 0.419</td>
<td>43.60 ± 13.26</td>
<td>0.49 ± 0.13</td>
</tr>
<tr>
<td>StaroA-T42M</td>
<td>135.573 ± 23.395</td>
<td>1.75 ± 0.23</td>
<td>12.81 ± 2.04</td>
</tr>
</tbody>
</table>

* 1 u/mg protein = 16.67 nkat/mg protein.

Fig. 4. Ribbon Diagram of the Crystal Structure of EPSPS in the “Open and Closed” Conformation.
Amino acids 1–42 are highlighted in yellow. An arrow indicates the site of the T42M substitution. Position 42 is near the hinge region and may affect the closure of the hinge.

resistance to glyphosate.11) Sequence analysis showed that the mutants resulted from a combination of point mutations and crossovers between the two parental genes, EcaroA and StaroA. The amino acid substitution, T42M, was common to two of the mutants, aroA-M4 and aroA-M11. In this study, we engineered this amino acid substitution into the EcaroA and StaroA enzymes and confirmed the contribution of this T42M substitution to glyphosate resistance using bacterial culture and kinetic analysis. This single amino acid substitution decreased the \(K_m\) (PEP), increased the \(K_i\) (glyphosate) and increased the specific activity. Our results demonstrate that enzymatic properties can be changed greatly by a single mutation at a position distant from the active site, a result that sometimes cannot be predicted.

It is also apparent from the study that the rate of cell growth does not simply and solely depend on the \(K_i\) (glyphosate); it has very much to do with \(K_m\) (PEP) and the activity of the enzyme as well, for example the mutant aroA-M1, which has similar \(K_i\) (glyphosate) to that of mutant aroA-M4 but has lower \(K_m\) (PEP) and higher specific activity than aroA-M4, grows faster in the presence of glyphosate.11) Similarly, StaroA-T42M, even with a lower \(K_i\) (glyphosate) than EcaroA-T42M, grows slightly better than EcaroA-T42M in 60 mM glyphosate, as its \(K_m\) (PEP) is 25% lower and its specific activity is 3.8-fold higher compared to that of EcaroA-T42M.

This work confirms that the T42M substitution was primarily responsible for the resistant phenotype in the aroA-M4 and aroA-M11 mutants11) and that the other amino acid substitutions in these mutants were either not relevant or actually detrimental to resistance. However, we also found several other mutants without the T42M substitution that also gave rise to glyphosate resistance. How any of these mutations, especially the T42M, alter the conformation of the enzyme remains to be answered using NMR and crystallography.

Acknowledgments

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References


