Bioscience, Biotechnology, and Biochemistry
Publication details, including instructions for authors and subscription information:
http://www.tandfonline.com/loi/tbbb20

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

Ming HE[a], Yan-Fang NIE[a] & Peilin XU[a]
[a] Biotechnology Research Center, The Key Laboratory of Gene Engineering of Education Ministry, Zhongshan UniversityGuangzhou, 510275, The People's Republic of China
Published online: 22 May 2014.

Biosci. Biotechnol. Biochem., **67** (6), 1405–1409, 2003

Note

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

Ming HE, Yan-Fang NIE, and Peilin XU†

Biotechnology Research Center, The Key Laboratory of Gene Engineering of Education Ministry, Zhongshan University, Guangzhou, 510275, The People's Republic of China

Received November 26, 2002; Accepted February 12, 2003

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 *aroA* genes from *S. typhimurium* and *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 mutagenisis, 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 *aroA*; glyphosate; mutagenesis; herbicide; 5-enolpyruvylshkimate-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),[1,2] 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)↔EPSP + Pi. The class I enzymes include those found in plants and the bacteria *E. coli* and *S. typhimurium*. The class II enzymes are found in *Agrobacterium tumifaciens* sp. CP4, *Achromobacter* sp. LBAA, and *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.[3–6] 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.[7] The free enzyme adopts an ''open'' conformation but in an inhibitor-enzyme complex the conformation is ''closed''.[7] 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.[8,9] 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.[7] In the closed conformation, specific amino acids are protected from chemical modification, and a trypsin site is hidden.[7] 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.[9] 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[10] for random recombination and mutation of class I *aroA* genes from *S. typhimurium* and *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.[11] These mutants contained multiple amino acid substitutions and crossovers between the parental sequences. Two of these mutants shared a T42M substitution, which

† To whom correspondence should be addressed.

Abbreviations: S3P, shikimate-3-phosphate; PEP, phosphoenolpyruvate; EPSPS, 5-enolpyruvylshkimate-3-phosphate synthase

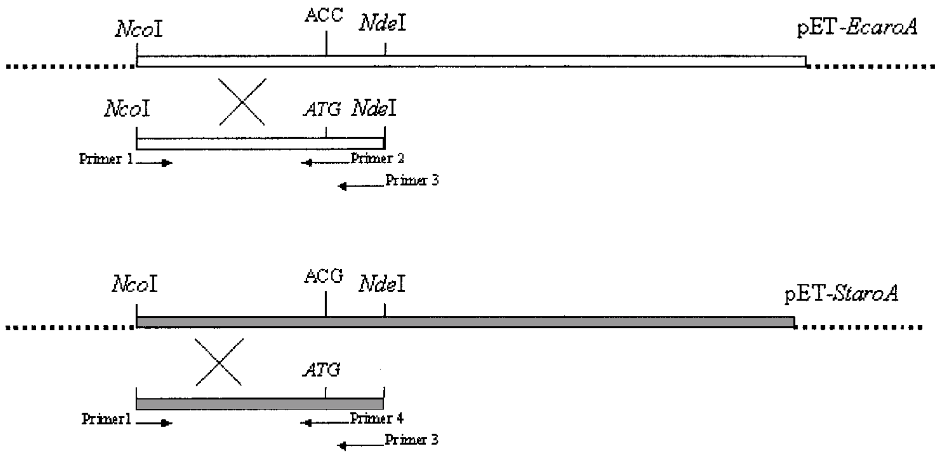


Fig. 1. Overview of the Site-directed Mutagenesis Strategy Used to Change Threonine to Methionine at Position 42 in *EcaroA* and *StaroA*.
The mutation codons were generated using two rounds of PCR and the mutagenic PCR fragments were placed into the corresponding restriction sites of pET-*EcaroA* and pET-*StaroA*.

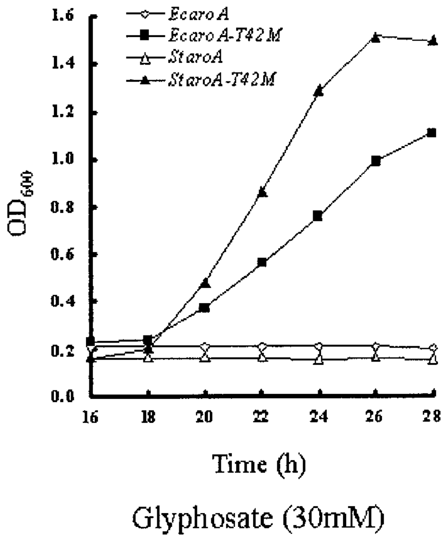
lies near the hinge between the two globular domains of EPSPS. In this communication, we report that the T42M substitution causes a large change in the K_i[glyphosate] and K_m[PEP] resulting in an EPSPS with high resistance to glyphosate.

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 GGAATTCCATATGGCGCACGTCATCGCTATCCAGC (primer 3). The oligonucleotide primers used to generate the T42M mutant of *S. typhimurium* EPSPS were: primers 1 and 3, and GACGTCATCGCTATCCAGCAGATT*CAT*CAGAGC (primer 4). *Nco*I and *Nde*I 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[11] 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 oligonuclotides 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 *Nde*I site at nucleotides 154 to159. Primers 1 and 3 were used to produce the mutagenic fragments in the first round of PCR. These fragments were digested with *Nco*I and *Nde*I 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 hsdS*$_D$*(*r_β^- m_β^-*) 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 Na_2HPO_4, 3 g/l of K_2HPO_4, 1 g/l of NH_4Cl, 0.5 g/l of NaCl, 1 mM $MgSO_4$, 0.4% [w/v] glucose, 100 μg/l 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 A_{600}, 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.[12] BL21 (DE3) pLysS harboring the mutant or wild type *aroA* genes in pET-11d constructs were grown to 0.75 A_{600} 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-



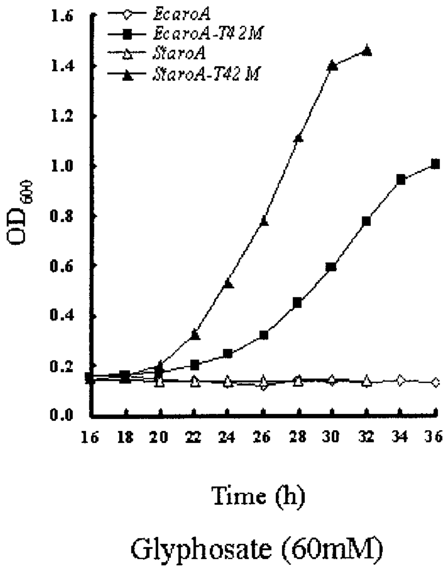


Fig. 2. Growth Curves of *E. coli* Expression Cells Harboring pET-*EcaroA*, pET-*StaroA*, pET-*EcaroA-T42M*, and pET-*StaroA-T42M* Plasmids.

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 A_{600}. After 16 h of growth, 1-ml proteins were withdrawn from each culture, and measured for cell density at approximately 2-h intervals.

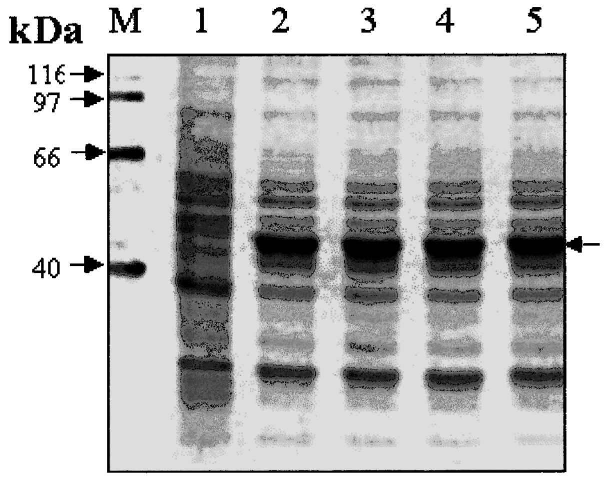


Fig. 3. SDS-PAGE of Crude Extracts Prepared from BL21 Cells Harboring Either Wild Types or Mutants pET Expression Plasmids.

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% polyacryamide gel then stained with Coomassie blue. The expressed proteins were indicated by an arrow.

fied 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 $(NH_4)_6Mo_7O_{24}\cdot 2H_2O$, 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 K_m[PEP], a 21- to 26-fold higher K_i[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] Simarily, 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

Enzymes	Specific activity* (u/mg protein $\times 10^{-3}$)	K_m(PEP) (μM)	K_i(Glyphosate) (μM)
EcaroA	7.558 ± 0.468	22.28 ± 6.26	1.48 ± 0.459
EcaroA-T42M	35.704 ± 4.979	2.36 ± 0.39	30.49 ± 10.06
StaroA	5.759 ± 0.419	43.60 ± 13.26	0.49 ± 0.13
StaroA-T42M	135.573 ± 23.395	1.75 ± 0.23	12.81 ± 2.04

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

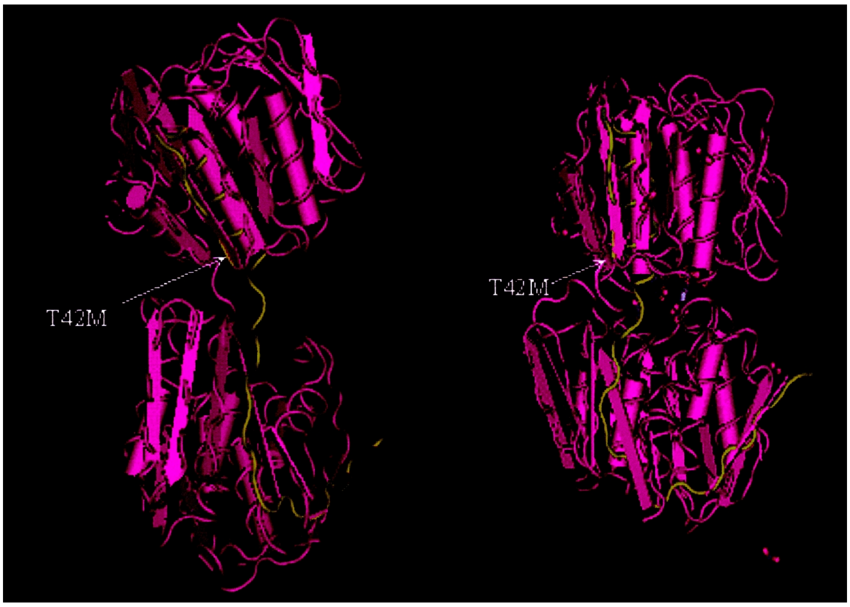


Fig. 4. Ribbon Diagram of the Crystal Structure of EPSPS in the "Open and Closed" Conformation.
Amino acids 1–42 are highlighted in yellow. An arrow indicates the site of the T42M substitution. Position 42 is near the hinge region and may affect the closure of the hinge.

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 depends upon 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* mutants[11)] 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

Financial support was received from the China National "863" grant 2001AA212191 and China National Science Foundation.

References

1) Schmid, J., and Amrhein, N., Molecular organization of the shikimate pathway in higher plants. *Phytochemistry*, **39**, 737–749 (1995).
2) Herrmann, K. M., and Weaver, L. M., The shikimate pathway. *Annu. Rev. Plant Physiol. Plant Mol. Biol.*, **50**, 473–503 (1999).
3) Ream, J. E., Yuen, H. K., Frazier, R. B., and Sikorski, J. A., EPSP syntase: binding studies using isothermal titration microcalorimetry and equilibrium dialysis and their implication for ligand recognition and kinetic mechanism. *Biochemistry*, **31**, 5528–5534 (1992).
4) Marzabadi, M. R., Gruys, K. J., Pansegrau, P. D., Walker, M. C., Yuen, H. K., and Sikorski, J. A., An EPSP synthase inhibitor joining shikimate 3-phosphate with glyphosate: synthesis and ligand binding studies. *Biochemistry*, **35**, 4199–4210 (1996).
5) McDowell, L. M., Schmidt, A., Cohen, E. R., Studelska, D. R., and Schaefer, J., Structural constraints on the ternary complex of 5-enolpyruvylshikimate 3-phosphate synthase from rotational-echo double-resonance NMR. *J. Mol. Biol.*, **256**, 160–171 (1996).
6) Lewis, J., Johnson, K. A., and Anderson, K. S., The catalytic mechanism of EPSP synthase revisited. *Biochemistry*, **38**, 7372–7379 (1999).
7) Krekel, F., Oecking, C., Amrhein, N., and Macheroux, P., Substrate and inhibitor-induced conformational changes in the structurally related enzymes UDP-N-acetylglucosamine enolpyruvyl transferase (MurA) and 5-enolpyruvylshikimate 3-phosphate synthase (EPSPS). *Biochemistry*, **38**, 8864–8878 (1999).
8) Stallings, W. C., Abdel-Meguid, S. S., Lim, L. W., Shieh, H., Dayringer, H. E., Leimgruber, N. K., Stegeman, R. A., Anderson, K. S., Sikorski, J. A., Padgette, S. R., and Kishore, G. M., Structure and topological symmetry of the glyphosate target 5-enopyruvylshikimate-3-phosphate synthase: a distinctive protein fold. *Proc. Natl. Acad. Sci. USA*, **88**, 5046–5050 (1991).
9) Schönbrunn, E., Eschenburg, S., Shuttleworth, W. A., Schloss, J. V., Amrhein, N., Evans, J. N. S., and Kabsch, W., Interaction of the herbicide glyphosate with its target enzyme 5-enolpyruvylshikimate 3-phosphate synthase in atomic detail. *Proc. Natl. Acad. Sci. USA*, **98**, 1376–1380 (2001).
10) Zhao, H., Giver, L., Shao, Z., Affholter, J. A., and Arnold, F. H., Molecular evolution by staggered extension process (StEP) *in vitro* recombination. *Nature Biotech.*, **16**, 258–261 (1998).
11) He, M., Yang, Z. Y., Nie, Y. F., Wang, J., and Xu, P., A new type of class I bacterial 5-enopyruvylshikimate-3-phosphate synthase mutants with enhanced tolerance to glyphosate. *Biochim. Biophys. Acta*, **1568**, 1–6 (2001).
12) Knowles, P. F., and Sprinson, D. B., Preparation of shikimate-5-phosphoate. *Methods Enzymol.*, **17A**, 351–354 (1997).
13) Lanzetta, P. A., Alvarez, L. J., Reinach, P. S., and Candia, O. A., An improved assay for nanomole amounts of inorganic phosphate. *Anal. Biochem.*, **100**, 95–97 (1979).
14) Stauffer, M. E., Young, J. K., Helms, G. L., and Evans, J. N. S., Chemical shift mapping of shikimate-3-phosphate binding to the isolated N terminal domain of 5-enolpyruvylshikimate 3-phosphate synthase. *FEBS Lett.*, **499**, 182–186 (2001).
15) Shuttleworth, W. A., Pohl, M. E., Helms, G. L., Jakeman, D. L., and Evans, J. N. S., Site-directed mutagenesis of putative active site residues of 5-enolpyruvylshikimate 3-phosphate synthase. *Biochemistry*, **38**, 296–302 (1999).