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ARTICLE *in* FEBS LETTERS · FEBRUARY 2005

Impact Factor: 3.17 · DOI: 10.1016/j.febslet.2004.12.057 · Source: PubMed

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FEBS Letters 579 (2005) 728–732 FEBS 29212

5-Enolpyruvylshikimate-3-phosphate synthase from *Staphylococcus aureus* is insensitive to glyphosate

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Received 15 November 2004; revised 17 December 2004; accepted 17 December 2004

Available online 11 January 2005

Edited by Judit Ovádi

Abstract The enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) catalyzes the penultimate step of the shikimate pathway, and is the target of the broad-spectrum herbicide glyphosate. Kinetic analysis of the cloned EPSPS from *Staphylococcus aureus* revealed that this enzyme exerts a high tolerance to glyphosate, while maintaining a high affinity for its substrate phosphoenolpyruvate. Enzymatic activity is markedly influenced by monovalent cations such as potassium or ammonium, which is due to an increase in catalytic turnover. However, insensitivity to glyphosate appears to be independent from the presence of cations. Therefore, we propose that the *Staphylococcus aureus* EPSPS should be classified as a class II EPSPS. This research illustrates a critical mechanism of glyphosate resistance naturally occurring in certain pathogenic bacteria.

Keywords: Herbicide resistance; Antibiotic; Pathogen; Enzyme kinetics

1. Introduction

The enzyme 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS; EC 2.5.1.19) is the sixth enzyme of the shikimate pathway, which is essential for the synthesis of aromatic amino acids and many aromatic metabolites in plants, fungi, and microorganisms [1–3], including apicomplexan parasites [4]. Because the shikimate pathway is absent from mammals, enzymes of the shikimate pathway have received considerable attention recently as potential antimicrobial targets [5–7]. The following findings in particular, indicate the importance of functional EPSPS for microbial survival: (i) deletion of the *aroA* gene, which encodes EPSPS, causes *Streptomyces pneumoniae* strains and *Bordetella bronchiseptica* to be attenuated for virulence [6,8], (ii) the growth of *Mycobacteria* depends on the functionality of the shikimate pathway [9] and (iii) glyphosate restricts the growth of *Thermotoga gondii* [4]. Recently, it has been suggested that the shikimate pathway also presents an attractive target for malaria chemotherapy because shikimate analogs have been shown to inhibit the growth of *Plasmodium falciparum* [10].

EPSPS catalyzes the transfer of the enolpyruvyl moiety of phosphoenol pyruvate (PEP) to the 5-hydroxyl of shikimate-3-phosphate (S3P) (Fig. 1). This chemically unusual reaction proceeds via a C–O bond cleavage of PEP rather than via a P–O bond cleavage as is the case in most PEP-utilizing enzymes. EPSPS has been extensively studied over the last three decades, since it was identified as the target of glyphosate, the active ingredient of Monsanto's broad-spectrum herbicide Roundup® [11]. Glyphosate is a competitive inhibitor with respect to PEP and binds adjacent to S3P in the active-site of *E. coli* EPSPS, thereby mimicking an intermediate state of the ternary enzyme substrates complex [12]. Recently, this molecular mode of action of glyphosate has been demonstrated for EPSPS from *Streptococcus pneumoniae*, too [13–16].

In recent years, a number of glyphosate resistant bacteria [14,17–25] and glyphosate tolerant plants [26–31] have been identified. Here, we describe the overexpression, purification and kinetic characterization of *S. aureus* EPSPS. The results demonstrate that *S. aureus* EPSPS is naturally insensitive to glyphosate and yet maintains a relatively high catalytic efficiency without a decrease in the K_m of PEP. Implications from these studies on the mechanism of glyphosate resistance are discussed.

2. Material and methods

2.1. Materials

The pET24d vector containing a T7 promoter was purchased from Novagen (Madison, WI). DNA of *S. aureus* EPSPS (Japan strain) in a pT7 Blue3 plasmid was provided by Scott Crupper (Emporia State University, Emporia, KS). All products for restriction digest and ligations were purchased from New England Biolabs (Beverly, MA). Competent *E. coli* strains used were BL21(DE3) (Stratagene, La Jolla, CA), Stbl2(DE3) (Invitrogen, Carlsbad, CA), C41(DE3) (Avidis, Saint-Beauzire, France), and C43(DE3) (Avidis). *E. coli* EPSPS was overexpressed and purified as described previously [32,33]. S3P (triethylammonium salt) was synthesized from shikimic acid using recombinant archaeal shikimate kinase [34], and purified via anion exchange chromatography on Q-sepharose resin. PEP (potassium salt) and all other chemicals were purchased from Sigma (St. Louis, MO) unless otherwise noted. The Pierce (Rockford, IL) comassie reagent with bovine serum albumin as a standard was used to determine protein concentrations.

2.2. Polymerase chain reaction

Primers were designed for ligation into pET24d using an *Nco*I restriction site (underlined) at the 5′ end and a *Not*I restriction site (underlined) at the 3′: *S. aureus* EPSPS *Nco*I-GCGCCATGGTAA ATGAACAAATCATT, *S. aureus* EPSPS *Not*I-GCGGCGGCCGC TTATCCCTCATTTTGTAAAAG. All primers were synthesized by MWG Biotech (High Point, NC). PCR was accomplished following the protocol from the Fail Safe PCR kit (Epicenter Technologies, Madison, WI) using 5 ng of *S. aureus* EPSPS in a pT7 Blue3 vector and 1 μM of each primer. The PCR reaction was cycled 18 times at 95 °C for 30 s, 60 °C for 1 min and 69 °C for 15 min.

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

0014-5793/$30.00
doi:10.1016/j.febslet.2004.12.057

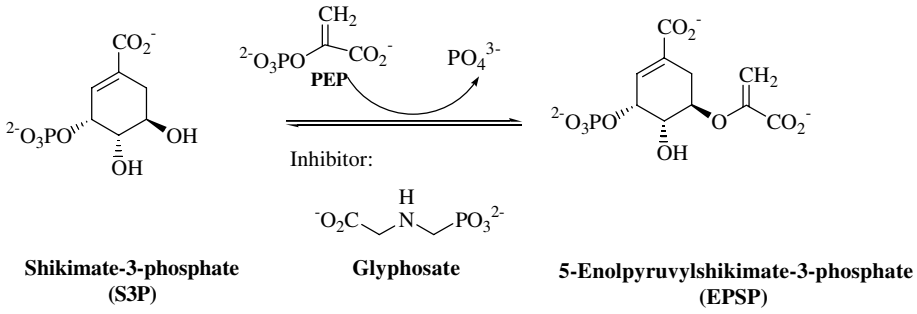

Fig. 1. Reaction catalyzed by EPSPS.

2.3. Restriction digest and ligations

Restriction digests of the PCR product and vector were accomplished by incubation of 2 μg of PCR product or vector with 20 U *Not*I and 20 U *Nco*I in NEB buffer3 at 37 °C for 2 h. The digested PCR product (300 ng) was ligated into the digested pET24d vector (200 ng) with 2 U of ligase at 19 °C for 16 h. The sequence of *S. aureus* EPSPS in the pET24d vector was confirmed by sequencing of the open reading frame at the University of Kansas Medical Center's biotech research support facility.

2.4. Overexpression of S. aureus *EPSPS*

The pET24d vector containing the *S. aureus* EPSPS gene was transformed into *E. coli* BL21(DE3), Stbl2(DE3), C41(DE3), and C43(DE3) competent cell lines. Clones from the four cell lines were tested for soluble overexpression of *S. aureus* EPSPS in LB-media at 37 °C by induction with 0.5 μM IPTG for 4 h after the cells reached an OD_{600} of 0.5. Further studies were carried out by lowering the temperature post-induction to 20 °C and growing the cultures overnight. The Stbl2 (DE3) cell line, growing at 20 °C after induction, was used for large-scale overexpression of *S. aureus* EPSPS.

2.5. Purification of S. aureus *EPSPS*

S. aureus EPSPS was purified using an ÄKTA FPLC system (GE Biosciences, Piscataway, NJ). Cells were resuspended in Buffer A (50 mM NaH_2PO_4/K_2HPO_4, 1 mM EDTA and 1 mM DTT, pH 6.8) plus lysozyme, sonicated, centrifuged, and the supernatant was loaded onto a Q-Sepharose (Q-seph) column (GE biosciences). The protein was eluted by increasing the salt concentration from 0% to 100% Buffer A + 0.4 M KCl. The fractions containing *S. aureus* EPSPS were then subjected to a 25% $(NH_4)_2SO_4$ precipitation, centrifuged and loaded onto a Phenyl Sepharose 26/10 (P-Seph) column (GE biosciences). The protein was eluted by decreasing the salt concentration from 100% to 0% Buffer A + $(NH_4)_2SO_4$. *S. aureus* EPSPS was desalted and loaded onto a resource-Q column and eluted using the same profile and buffers as the Q-Seph. The protein was desalted and concentrated to 10 mg/ml and stored at −80 °C.

2.6. Kinetics of S. aureus *EPSPS*

The effect of specific cations and anions on the activity of *E. coli* and *S. aureus* EPSPS was assayed in 100 μl of 150 mM HEPES-NaOH (pH 7.0), 2 mM DTT + 100 mM salt. The activity for each enzyme was calculated by determining the amount of inorganic phosphate produced in the reaction [35]. The reaction was initiated with the addition of enzyme and allowed to react for 3 min before the Lanzetta reagent (800 μl) was added; color development was stopped after 5 min by the addition of 100 μl 34% (w/v) sodium citrate. Change in optical density was measured at 660 nm and the amount of inorganic phosphate was determined by comparison to phosphate standards. Enzyme activity is expressed as [μmol product/min of reaction/mg of EPSPS] (U/mg). The final concentration of enzyme in the assay mixture was 69 nM and 660 nM for *E. coli* EPSPS and *S. aureus* EPSPS, respectively.

To determine the concentration of KCl required for optimal activity of *S. aureus* EPSP assays were carried out in 100 μl of 500 mM Tris pH 8.0 + 2 mM DTT with varied concentrations of KCl. All assays proceeded as described above. The kinetic parameters of *E. coli* EPSPS [32] and *S. aureus* EPSPS were assayed in 100 μl of 50 mM HEPES-NaOH (pH 7.0), 2 mM DTT ± 100 mM KCl as described above. The final concentration of enzyme in the assay mixture was 660 nM and 1.65 M for *S. aureus* EPSPS with and without KCl, respectively. The kinetic data were fit to the appropriate equations using the program SigmaPlot (SPSS Science, Chicago, IL). The kinetic data for determining the K_m values were fit to

$$v = \frac{V_{max}[S]}{K_m + [S]} \quad (1)$$

using the program SigmaPlot (SPSS Science, Chicago, IL, USA) where v is the initial velocity, V_{max} is the maximum velocity, K_m is the Michaelis constant and $[S]$ is the substrate (S3P or PEP) concentration.

The IC_{50} value for EPSPS inhibition by glyphosate was determined by fitting data to

$$v = V_{min} + \frac{V_{max} - V_{min}}{1 + \left(\frac{[I]}{IC_{50}}\right)^n} \quad (2)$$

where v is the initial velocity, V_{max} is the maximum velocity, V_{min} is the minimum velocity, $[I]$ is the concentration of glyphosate and n is the Hill slope.

3. Results

3.1. Cloning of S. aureus *EPSPS*

The gene encoding *S. aureus* EPSPS, *aroA*, was originally identified, cloned and sequenced by O'Connell et al. [36]. In order to characterize *S. aureus* EPSPS, *aroA* was cloned into the overexpression vector pET24d. The sequence of the ligation product was verified to be *aroA* from *S. aureus*.

3.2. Overexpression and purification of S. aureus *EPSPS*

The vector containing the open reading frame for *S. aureus* EPSPS was transformed into BL21(DE3), Stbl2(DE3), C41(DE3), and C43(DE3) cell lines for induction studies. All cell lines showed high levels of overexpression at 37 °C but the overexpression corresponded only to insoluble protein. In an attempt to obtain soluble protein, all induction studies were repeated by dropping the temperature to 20 °C post-induction. Using these conditions *S. aureus* EPSPS in Stbl2(DE3) showed 50% soluble overexpression (Fig. 2) but all of the other cell lines only showed insoluble overexpression. *S. aureus* EPSPS was purified to greater than 95% homogeneity (Fig. 2), yielding 75 mg of functional enzyme from a 3 l culture.

3.3. Kinetics analysis of S. aureus *EPSPS*

Steady-state kinetic characterization of *S. aureus* EPSPS was performed in parallel experiments with the enzyme from *E. coli* [32]. *S. aureus* EPSPS activity is sensitive to specific cations (Fig. 3) but not to anions (data not shown). Potassium and ammonium ions both provided the largest enhancement of activity compared to the activity observed with no cations present. This is in contrast to *E. coli* EPSPS, which is

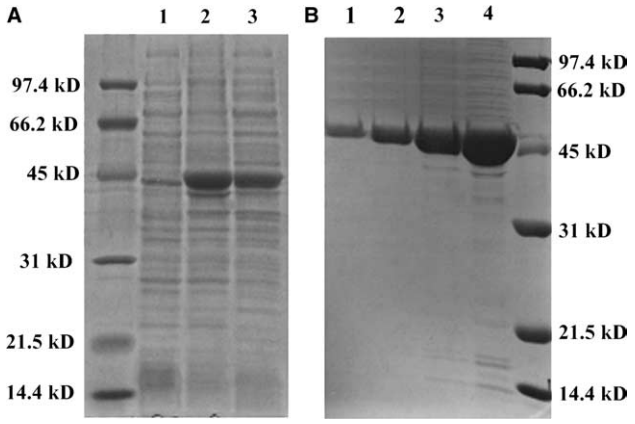

Fig. 2. Overexpression and purification of *S. aureus* EPSPS (SDS–PAGE). (A) Lane 1: molecular weight marker; lane 2: control before induction; lane 3: insoluble overexpression; and lane 4: soluble overexpression. (B) Lanes 1–4: increasing concentration (2.5, 5, 10, 25 μg) of purified *S. aureus* EPSPS; lane 5: molecular weight marker.

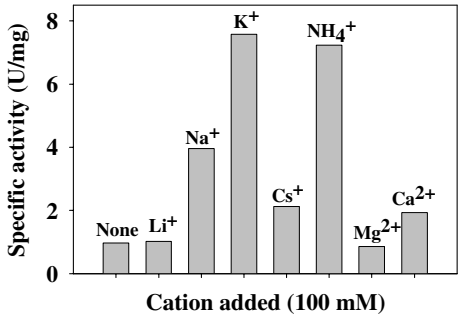

Fig. 3. Effect of cations on the activity of *S. aureus* EPSPS. Assays were performed with 1 mM PEP, 1 mM S3P and 100 mM cation as described in Section 2.

insensitive to both anions and cations (data not shown). The half-maximal concentration of potassium ions required for optimal activity of *S. aureus* EPSPS is ca. 50 mM (data not shown). Cations have also been reported to enhance the activities of EPSPS from *Bacillus subtilis* [22], *S. pneumoniae* [14] and *Klebsiella pneumonia* [37].

S. aureus EPSPS and *E. coli* EPSPS enzymes display normal saturation behavior when the specific activities (U mg^{-1}) are plotted as a function of substrate concentration (Fig. 4). The kinetic constants derived from these graphs (Table 1) demonstrate that the Michaelis constants for both substrates, S3P and PEP, are similar for *S. aureus* EPSPS and *E. coli* EPSPS, and appear to be unaltered by potassium ions. The K_m values for *E. coli* EPSPS are higher than those reported by Padgette et al. [38] (8.2 μM and 4.4 μM for PEP and S3P, respectively). Padgette et al. used radiolabeled PEP, making the determination of product more sensitive and accurate than the colorimetric assay employed here. However, the kinetic constants determined in this study agree with the values we have previously reported [32], and they serve here mainly for the direct comparison of the *S. aureus* and *E. coli* enzymes. The catalytic efficiency (k_{cat}/K_m) of *S. aureus* EPSPS with 100 mM KCl is one order of magnitude lower than that of the enzyme from *E. coli*, which is a direct effect of the smaller turnover number. The absence of potassium ions causes a 20-fold reduction in the k_{cat} value of *S. aureus* EPSPS.

Unlike *E. coli* EPSPS, which is very sensitive to glyphosate (IC_{50} = 0.009 mM), *S. aureus* EPSPS is less sensitive to inhibition by glyphosate both in the presence (IC_{50} = 0.9 mM) and absence of 100 mM potassium ions (IC_{50} = 1.6 mM) (Fig. 5). *B. subtilis* [22] and *S. pneumoniae* EPSPS enzymes [14] have also been reported to be insensitive to glyphosate. However, in the presence of 100 mM cations these enzymes are glyphosate sensitive (see also Section 4).

4. Discussion

The widespread global use of glyphosate has increased the importance of characterizing the mechanisms by which bacteria and plants are resistant to glyphosate. There are several mechanisms by which bacteria and plants are naturally resistant or become resistant to glyphosate: overproduction of EPSPS via gene amplification or increased mRNA biosynthesis, amino acid alterations of EPSPS, or natural resistance seen with class II EPSPS enzymes. This last mechanism of resistance has been utilized for the development of Monsanto's genetically modified Roundup Ready® crops, which have been successfully transformed with the naturally glyphosate resistant EPSPS from *Agrobacterium tumefaciens* sp strain CP4 [25,39].

Two single-site amino acid alterations have been shown to confer resistance to glyphosate: G96A [32,40] and P101S [31,40,41] (numbering according to *E. coli* EPSPS). The structure of the G96A mutation revealed that an alanine at position 96 sterically hinders the binding of glyphosate to the PEP binding site, which is accompanied by a corresponding increase in the K_m of PEP [32]. The mechanism by which the P101S mutation confers resistance is obscure. Position 101, which is located in the middle of the third helix, does not directly interact with glyphosate [12]. However, changes in this helix may cause small alterations in the active site, particularly around the region of G96, which constitutes part

Table 1
Kinetic parameters for *E. coli* and *S. aureus* EPSPS

	K_m (PEP) (mM)	k_{cat} (PEP) (s^{-1})	k_{cat}/K_m (PEP) (M^{-1} s^{-1})	K_m (S3P) (mM)	k_{cat} (S3P) (s^{-1})	k_{cat}/K_m (S3P) (M^{-1} s^{-1})	IC50 (Glp) (mM)
E. coli EPSPS	0.14 ± 0.02	59 ± 1.9	$4.2 \times 10^5 \pm 0.5 \times 10^5$	0.14 ± 0.01	51 ± 1.5	$3.7 \times 10^5 \pm 0.4 \times 10^5$	0.0086 ± 0.0003
S. aureus EPSPS + KCl	0.18 ± 0.02	4.3 ± 0.02	$2.4 \times 10^4 \pm 0.3 \times 10^4$	0.14 ± 0.02	4.2 ± 0.02	$3.0 \times 10^4 \pm 0.3 \times 10^4$	0.9 ± 0.006
S. aureus EPSPS no KCl	0.17 ± 0.02	0.22 ± 0.006	$1.3 \times 10^3 \pm 0.2 \times 10^3$	0.18 ± 0.02	0.20 ± 0.008	$1.1 \times 10^3 \pm 0.1 \times 10^3$	1.6 ± 0.005

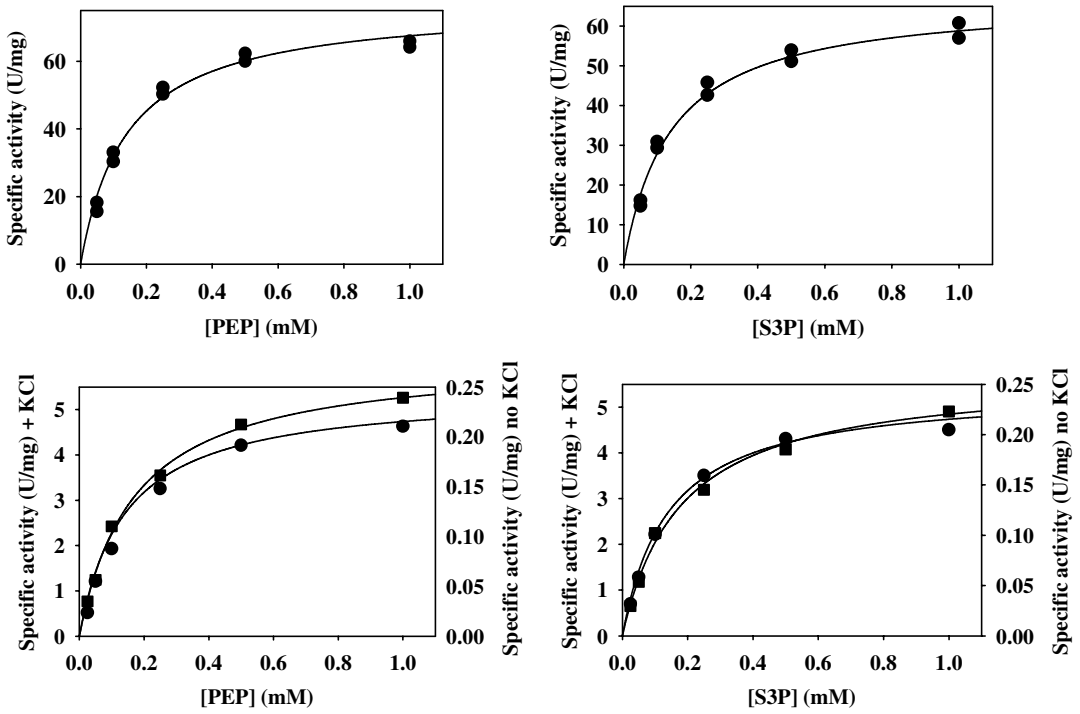

Fig. 4. Comparison of the steady-state kinetics of *E. coli* and *S. aureus* EPSPS. (Top) *E. coli* EPSPS assayed with 1 mM S3P (left) or 1 mM PEP (right) and increasing concentrations of the second substrate. (Bottom) *S. aureus* EPSPS assayed in the presence of 100 mM KCl (●) or in the absence of KCl (■) using 1 mM S3P (left) or 1 mM PEP (right) and increasing concentrations of the second substrate. The Y-scale on the left of each graph denotes the values for 100 mM KCl, the Y-scale to the right without KCL. All assays were run as described in Section 2, and the data were fit to Eq. (1) yielding the kinetics parameters shown in Table 1.

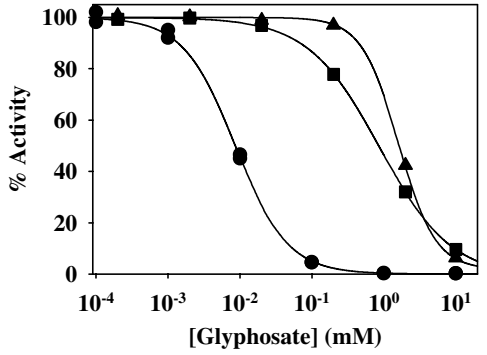

Fig. 5. Inhibition of EPSPS by glyphosate. IC_{50} values of glyphosate inhibition were determined for *E. coli* (●), *S. aureus* EPSPS with 100 mM KCl (■) and *S. aureus* EPSPS without KCl (▲) using 1 mM S3P, 1 mM PEP and increasing concentrations of glyphosate as described under Section 2. Data were fit to Eq. (2) yielding the IC_{50} values shown in Table 1.

of the binding pocket for the phosphonate moiety of glyphosate [32]. Sequencing alignments reveal a leucine at position 101 in *S. aureus* EPSPS instead of proline as is seen with *E. coli* EPSPS. Thus, this altered amino acid may play a key role in the mechanism of glyphosate resistance for the *S. aureus* enzyme. The structural analysis of *S. aureus* EPSPS is required to deduce if the leucine at position 101 is indeed responsible for glyphosate resistance, and/or if the overall three-dimensional structure of the class II EPSPS enzymes is the major contributor.

Type II EPSPS enzymes, of which *A. tumefaciens* CP4 is a member, are classified based on their high tolerance for glyphosate as well as their low K_m of PEP [25]. Both *B. subtilis* EPSPS and *S. pneumoniae* EPSPS may be considered class II enzymes; however, their sensitivity to glyphosate increases significantly in the presence of cations, as does their affinity for PEP [14,22]. In contrast, for *S. aureus* EPSPS the K_m of PEP and the insensitivity to glyphosate appears to be independent of cations (Figs. 4 and 5), indicating distinct differences within the enzymes from gram-positive organisms. Thus, EPSPS from *S. aureus* may be classified as a true class II EPSPS, since it has a high glyphosate tolerance and a high affinity for PEP regardless of the presence of cations.

It is unknown how the tertiary structures of class I and class II enzymes differ and how these changes relate to glyphosate resistance. Optimal global sequence alignments of *S. aureus* EPSPS with *E. coli* EPSPS reveal that the two enzymes are only 26% identical with 40% similarity. These large differences in the primary structure may contribute to the differences in catalytic turnover. The reaction of EPSPS follows an induced-fit mechanism with large conformational changes from an unliganded, open state to a substrate-liganded, closed state [12]. The rates at which EPSPS undergoes this open-closed transition may be altered in the *S. aureus* enzyme. This could cause changes in the catalytic efficiency of EPSPS, however, without altering the K_m values for S3P or PEP (Fig. 4). Cations may facilitate the open-closed transition of the *S. aureus* enzyme, and perhaps of EPSPS from other gram-positive organisms as well, thereby primarily affecting the turnover number.

Acknowledgements: We would like to thank Martha L. Healy Fried for her help in preparing this manuscript as well as her thoughtful comments. This work was supported by the NIH Center of Biomedical Research Excellence (P20 RR16443-01).

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