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Molecular basis for the glyphosate-insensitivity of the reaction of 5-enolpyruvylshikimate 3-phosphate synthase with shikimate

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Abstract The shikimate pathway enzyme 5-enolpyruvyl shikimate-3-phosphate synthase (EPSP synthase) has received attention in the past because it is the target of the broad-spectrum herbicide glyphosate. The natural substrate of EPSP synthase is shikimate-3-phosphate. However, this enzyme can also utilize shikimate as substrate. Remarkably, this reaction is insensitive to inhibition by glyphosate. Crystallographic analysis of EPSP synthase from *Escherichia coli*, in complex with shikimate/glyphosate at 1.5 Å resolution, revealed that binding of shikimate induces changes around the backbone of the active site, which in turn impact the efficient binding of glyphosate. The implications from these findings with respect to the design of novel glyphosate-insensitive EPSP synthase enzymes are discussed.

Keywords: Herbicide resistance; Crystal structure; Induced-fit; Alternative substrate

1. Introduction

The shikimate pathway is found in all microorganisms, plants and fungi. This pathway is essential for the biosynthesis of chorismate, the precursor for aromatic amino acids and aromatic secondary metabolites [1–3]. Shikimate kinase catalyzes the fifth step in the pathway converting shikimate to shikimate-3-phosphate (S3P) (Fig. 1). The enolpyruvyl moiety of phosphoenolpyruvate (PEP) is then transferred to the 5-hydroxyl group of S3P, yielding 5-enolpyruvylshikimate-3-phosphate (EPSP). This reaction is catalyzed by 5-enolpyruvyl shikimate-3-phosphate synthase (EPSP synthase; EC 2.5.1.19). The final step in the shikimate pathway is the conversion of EPSP to chorismate by chorismate synthase. EPSP synthase is the target of glyphosate, the active ingredient of Monsanto's broad-spectrum herbicide Roundup® [4]. The shikimate pathway has attracted attention not only because it is vital for plants, but for pathogenic microorganisms such as *Mycobacteria* [5] and apicomplexan parasites [6,7] as well. In particular, it was reported that glyphosate inhibits the growth of *Plasmodium falciparum*, the causative agent of malaria [7].

The reaction of EPSP synthase with S3P and PEP and the inhibition of this reaction by glyphosate have been extensively studied during the past 30 years. Glyphosate is a reversible inhibitor of EPSP synthase that binds adjacent to S3P in the PEP-binding site, thereby mimicking an intermediate state of the ternary enzyme–substrates complex [8]. However, little is known about the molecular basis for the extremely selective action of this inhibitor on EPSP synthase, which has been responsible for its enormous success as an herbicide worldwide. Resistance to glyphosate can be achieved by mutations of the target enzyme, for example by mutating non-conserved residues around the glyphosate-binding site. The molecular mechanism of one such mutation causing resistance to glyphosate, Gly96Ala, was elucidated recently [9]. Other EPSP synthase mutant enzymes insensitive to glyphosate have been reported, either engineered or from natural sources, but have not been investigated in molecular detail [10].

Recently, it has been reported that shikimate serves as an alternative substrate for EPSP synthase [11,12], and that inhibition of this reaction by glyphosate was drastically reduced when compared with the potent inhibition of the reaction with S3P as substrate. This finding prompted us to study the reaction of EPSP synthase from *E. coli* with shikimate and glyphosate in more detail, using steady-state kinetics and protein crystallography. The implications from these studies on the molecular mechanism of inhibition and resistance of EPSP synthase with respect to glyphosate are discussed.

2. Materials and methods

E. coli EPSPS was overexpressed and purified as described previously [9,13]. S3P (triethylammonium salt) was synthesized from shikimic acid using recombinant archaeal shikimate kinase [14], 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) coomassie reagent with bovine serum albumin as a standard was used to determine protein concentrations.

2.1. Kinetic assays

The activity of EPSP synthase was calculated by determining the amount of inorganic phosphate produced in the reaction [15]. Enzyme activity is expressed as μmol phosphate produced/min of reaction time/mg of EPSP synthase (U/mg). The final concentration of EPSP synthase was 22 nM for all assays utilizing S3P and 1.1 μM for all assays utilizing shikimate, except when assayed with carbonate (220 nM). The reaction of EPSP synthase with either shikimate or S3P as substrate was assayed in 100 μl of 500 mM MES pH 5.5 including 2 mM DTT

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Abbreviations: PEP, phosphoenolpyruvate; S3P, shikimate-3-phosphate

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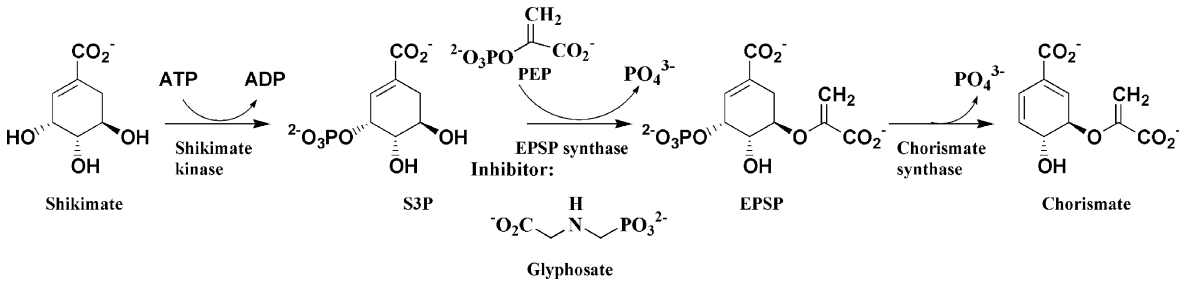

Fig. 1. Conversion of shikimate to chorismate in the shikimate pathway.

in the absence or presence of 100 mM $NaHCO_3$ or 100 mM KCl. pH 5.5 was chosen because this was the apparent optimum of the reaction with shikimate as substrate; the pH optimum with S3P as substrate is around 6.5 (data not shown). The high buffer concentration was needed to compensate for the acidity of the high shikimate concentrations used in the assays. To determine the concentration of carbonate required for optimal EPSP synthase activity with shikimate as a substrate, assays were carried out in 100 μl of 500 mM Tris, pH 8.0 + 2 mM DTT with increasing concentrations of carbonate to favor the reaction of carbonate and carbon dioxide towards the formation of the carbonate anion. To minimize the amount of carbonate converted to carbon dioxide, $NaHCO_3$ was added just prior to the reaction start. The range of substrate concentrations used to determine the steady-state kinetic parameters in the reaction of EPSP synthase with S3P was 0.01, 0.025, 0.050, 0.1, 0.25, 0.5, 1 and 2 mM for S3P and PEP; the shikimate concentration range was 1, 2.5, 5, 10, 25, 50, 100, and 250 mM, and the corresponding PEP concentration range was 0.1, 0.25, 0.5, 1, 5 and 10 mM. Data were fit to the Michaelis–Menten equation using the program SigmaPlot (SPSS Science, Chicago, IL, USA) The IC_{50} value for EPSP synthase inhibition by glyphosate was determined by fitting data to

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

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.

2.2. Crystallography

EPSP synthase in 50 mM Tris–HCl pH 8.0 and 2 mM DTT was concentrated to approximately 75 mg/ml using Centricon 30 devices (Amicon) at 4 °C and was crystallized in the presence of 10 mM shikimate, or with 10 mM shikimate and 30 mM glyphosate at 19 °C from 2 M sodium formate. Diffraction data were recorded at −180 °C using the rotation method on single flash-frozen crystals [Detector: R-axis IV^{++} image plate detector (MSC, The Woodlands, TX, USA) X-rays: Cu Kα, focused by mirror optics (MSC); generator: Rigaku RU300 (MSC)]. The data were reduced with XDS [16]. For phasing and refinement the program package CNS [17] was used. Model building was performed with O [18]. Data statistics are reported in Table 2.

3. Results and discussion

During their studies on the steady-state kinetics of the EPSP synthase reaction with S3P, Gruys et al. [11] observed that this enzyme could utilize shikimate as substrate, too. They also reported that this reaction is insensitive to glyphosate, a potent inhibitor of EPSP synthase when reacting with its natural substrate, S3P. With crystallization conditions for EPSP synthase in hand, we explored this reaction of EPSP synthase through a more thorough characterization.

3.1. Enzyme kinetics

First, we determined the kinetic parameters of the reaction of EPSP synthase with either shikimate or S3P as substrate (Table 1). The reaction of shikimate and PEP to enolpyruvyl shikimate has a pH optimum of 5.5–6.0, whereas the pH optimum for the conversion of S3P to enolpyruvyl shikimate-3-phosphate is between 6.0 and 6.5 under the high buffer concentrations used in this study (data not shown, see Section 2). The data display similar trends as those reported by Gruys et al. [11]. Larger differences were observed for the kinetic constants of the reaction with S3P and PEP, which are likely due to the different assay

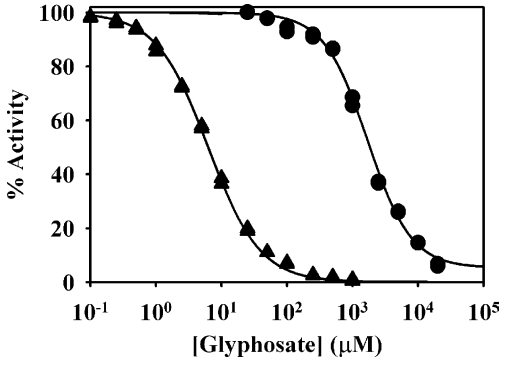

Fig. 2. Glyphosate-insensitivity of the EPSP synthase reaction with shikimate. Displayed are the IC_{50} value determinations for glyphosate when EPSP synthase was assayed with 250 mM shikimate (saturating), 1.0 mM PEP (=K_m) and 100 mM carbonate (●) or with 1 mM S3P (saturating) and 0.1 mM PEP (=K_m) (▲). Data were fit to Eq. (1) yielding the IC_{50} values shown in Table 1.

Table 1
Kinetic parameters for the reactions of EPSP synthase with S3P or shikimate

Substrate (S_1)	K_m (S_1) (mM)	V_{max} (S_1) (U/mg)	k_{cat}/K_m (S_1) (M^{-1} s^{-1})	K_m (PEP) (mM)	V_{max} (PEP) (U/mg)	k_{cat}/K_m (PEP) (M^{-1} s^{-1})	IC_{50} (Glp) (mM)
S3P	0.09 ± 0.005	53 ± 3.0	$4.5 \times 10^5 \pm 0.25 \times 10^5$	0.10 ± 0.004	58 ± 2.3	$4.5 \times 10^5 \pm 0.18 \times 10^S$	0.006 ± 0.0001
Shikimate + HCO_3^-	47 ± 2.9	6.8 ± 0.15	$1.1 \times 10^2 \pm 0.07 \times 10^2$	0.86 ± 0.05	6.0 ± 0.09	$5.4 \times 10^3 \pm 0.33 \times 10^3$	1.7 ± 0.11
Shikimate + Cl^-	31 ± 4.1	1.2 ± 0.04	$2.5 \times 10^1 \pm 0.28 \times 10^1$	1.1 ± 0.12	1.0 ± 0.03	$8.4 \times 10^2 \pm 1.2 \times 10^2$	1.2 ± 0.13

conditions employed. Gruys et al. assayed EPSP synthase in 200 mM HEPES pH 7 at 30 °C and determined the kinetic constants using a radioactive assay, whereas we assayed the enzyme in 500 mM MES pH 5.5 at 20 °C and determined the kinetic constants using a colorimetric assay.

The K_m for shikimate is over 500 times higher than the K_m for S3P, and the K_m for PEP is about 8 times higher with shikimate as substrate. Glyphosate action on the enzyme with shikimate as substrate displays significantly weaker inhibition, with an IC_{50} value 280 times higher than with S3P (Fig. 2). However, it was not recognized previously that the activity of EPSP synthase, when utilizing shikimate as a substrate, is greatly influenced by the presence of specific anions (Fig. 3), but is not affected by cations (data not shown). With shikimate as substrate, the activity was enhanced over 300-fold in the presence of carbonate (6.8 U/mg) versus no anion (0.02 U/mg). The binding of carbonate to the enzyme is saturable, with 17 mM carbonate required for half-maximal activity of the reaction with shikimate (Fig. 3). Formate and chloride ions also affected the enzymatic activity, although to a lesser degree (0.91 and 0.98 U/mg, respectively). Notably, with S3P as substrate, neither anions nor cations exerted any effect on the activity of EPSP synthase under the experimental conditions employed herein.

3.2. Crystallographic analysis

EPSP synthase crystallizes in the presence of shikimate using the same crystallization conditions as with S3P, in the same space group, with similar unit cell dimensions (Table 2). The structure at 1.5 Å resolution revealed that the en-

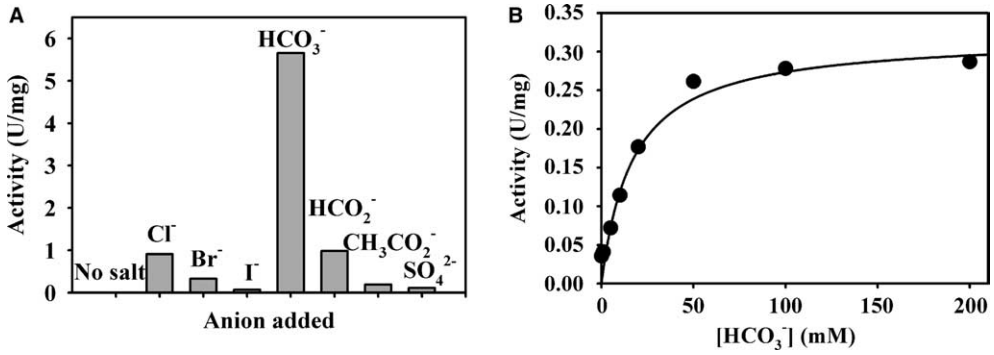

Fig. 3. Effect of anions on the reaction of EPSP synthase with shikimate. (A) Enzymatic activity as a function of anion tested; the enzyme was assayed with 10 mM PEP, 250 mM shikimate and 100 mM of the respective ion. (B) Enzymatic activity as a function of carbonate assayed with 10 mM PEP, 250 mM shikimate and increasing concentrations of carbonate. Data were fit to the Michaelis–Menten equation yielding a K_d value of 17 ± 3.4 mM for carbonate.

Table 2
Summary of data data set collection and structure refinement[a]

Data set	EPSPS–shikimate	EPSPS–shikimate–glyphosate
Space group	$P2_12_12_1$	$P2_12_12_1$
Unit cell dimensions (Å)	$a = 58.1$, $b = 85.0$, $c = 87.6$, $\alpha = \beta = \gamma = 90°$	$a = 57.7$, $b = 84.9$, $c = 87.5$, $\alpha = \beta = \gamma = 90°$
Molecules/asymmetric unit	1	1
Protein atoms	3232, average B-factor 14.5 (Å^2)	3232, average B-factor 13.7 (Å^2)
Alternate atom positions	67	68
Ligand atoms	12, average B-factor 11.2 (Å^2)	22, average B-factor 12.8 (Å^2)
Solvent molecules	498, average B-factor 25.7 (Å^2)	611, average B-factor 27.7 (Å^2)
Formate ions	17, average B-factor 27.0 (Å^2)	9, average B-factor 25.3 (Å^2)
Rmsd[b] bonds (Å)	0.01	0.01
Rmsd angles (°)	1.56	1.59
Resolution range (Å)	15.0–1.5 (1.6–1.5)	15.0–1.55 (1.6–1.55)
Measured reflections	448895 (72668)	414858 (27431)
Unique reflections	65778 (10915)	62861 (5490)
Completeness (%)	93.8 (89.7)	99.7 (97.4)
$I/\sigma I$	19.3 (8.4)	20.0 (6.5)
$R_{mrgd\text{-}F}$[c] (%)	4.4 (11.7)	4.9 (15.3)
R_{meas}[c] (%)	6.1 (23.7)	6.6 (29.1)
R_{cryst}[d] (%)	16.1	15.6
R_{free}[e] (%)	18.3	18.8

[a]Values in parentheses refer to the highest resolution shell.
[b]rmsd, root mean square deviation from ideal values.
[c]R_{meas} and $R_{mrgd\text{-}F}$ as defined by Diederichs and Karplus [22] are quality measures of the individual intensity observations and the reduced structure factor amplitudes, respectively.
[d]$R_{cryst} = 100 \times \sum|F_{obs} - F_{model}|/\sum F_{obs}$, where F_{obs} and F_{model} are observed and calculated structure factor amplitudes.
[e]R_{free}, R_{cryst} calculated for randomly chosen unique reflections, which were excluded from the refinement (1316 for EPSPS:shikimate and 1258 for EPSPS:shikimate:glyphosate, respectively).

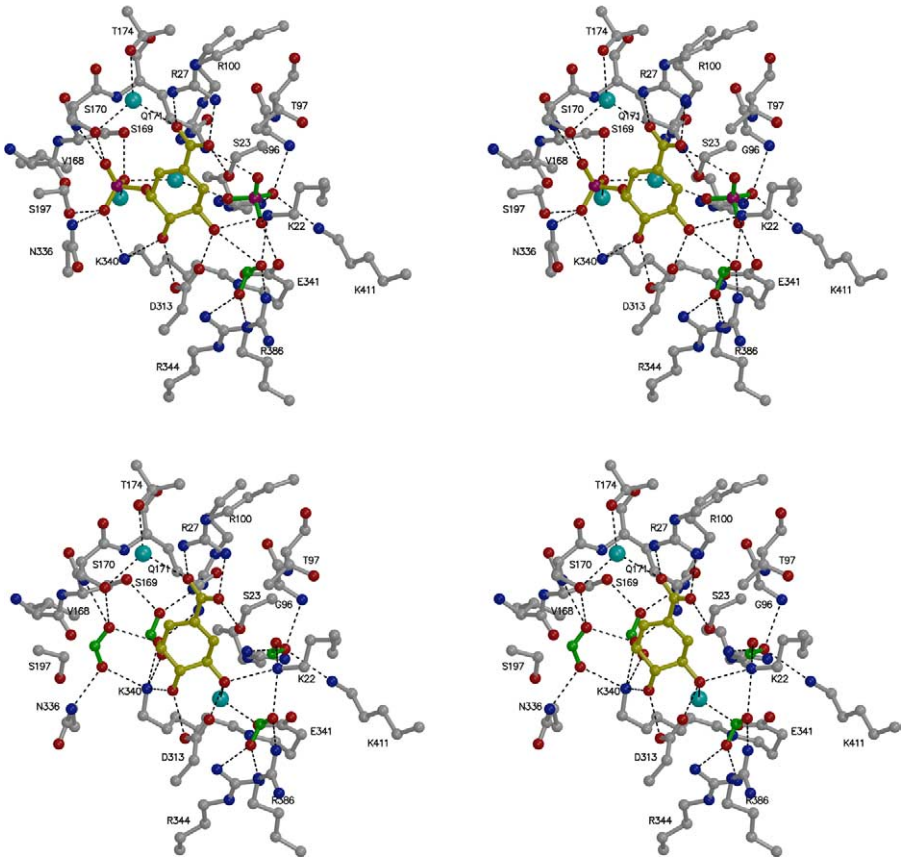

Fig. 4. The active site of EPSP synthase in its binary complex with S3P or shikimate (stereo views). (Top): S3P bound to EPSP synthase (PDB 1G6T). (Bottom): Shikimate bound to EPSP synthase (this work, PDB 2AA9). Shikimate and S3P are shown in yellow, formate ions in green, water molecules in cyan. Dashed lines indicate hydrogen bonding interactions.

zyme–shikimate complex exists in the closed state (Fig. 4). Thus, shikimate induces the same transition from an unliganded, open state to a liganded, closed state as seen with S3P [8]. This suggests that the phosphate group of S3P is not required per se for the induced-fit mechanism of the enzyme. At present, however, we cannot exclude changes in the rate constants of the open–closed transition upon binding of either substrate or anions. It should be noted that we have refined and evaluated three structures each of the binary and ternary complexes with shikimate/glyphosate bound to EPSP synthase to ensure the findings presented below. The structures with highest resolution were chosen as representative models. In addition, we have compared these structures not only with the published *E. coli* EPSP synthase binary and ternary complexes [8] but also with the corresponding structures from sub-atomic resolution data (Becker & Schonbrunn, unpublished).

Shikimate is bound to the active site in the same orientation as S3P, the target hydroxyl group (5-OH) pointing towards the glyphosate/PEP-binding site (Fig. 4). The space around the 3-phosphate group of S3P (opposite to the target hydroxyl group) is occupied by two formate ions that stem from the crystallization solution. Presumably, this is the site to which other anions like chloride or carbonate may bind under the conditions used for the kinetic studies (Fig. 3). Several attempts to clearly identify the nature of the anion binding site(s) by co-crystallization of the enzyme with shikimate in the presence of up to 100 mM carbonate or chloride failed, probably due to the much higher formate concentration (>2 M) present during crystallization. Notably, formate also stimulates the activity of this reaction (Fig. 3). Thus, ions like formate, chloride, or carbonate may compete for binding to the same site that is normally occupied by the phosphate group of S3P, when EPSP synthase is allowed to react with shikimate. This

Table 3
Bonding interactions between EPSP synthase and S3P or shikimate

Atom of S3P or shikimate	Enzyme residue	Distance (Å) S3P	Distance (Å) shikimate
Ol: ring position 3	Q171 NE2	3.83	2.94
	K340 NZ	4.02	3.00
O2: ring position 4	D313 OD2	2.77	2.73
	K340 NZ	3.02	2.66
O3: ring position 5	K22 NZ	3.02	3.10
	D313 OD1	2.61	2.65
O4: carbonyl	R27 NH1	2.77	2.74
O5: carbonyl	S23 OG	2.62	2.73
	R27 NH2	2.82	2.84
O6: phosphate	S170 N	2.73	na
	S170 OG	2.53	na
O7: phosphate	S197 OG	2.62	na
	N336 ND2	2.89	na
	K340 NZ	2.87	na
O8: phosphate	S169 OG	2.64	na

would explain why the reaction of EPSP synthase with S3P as substrate is independent of the anions studied herein. Presumably, anions bound to this site keep shikimate in place by bridging the backbone of the active site (Ser169, Ser170, Ser197) with the 3-hydroxyl group through multiple hydrogen bonding interactions (Fig. 4).

The hydrogen-bonding network between the functional groups common to shikimate and S3P with active site residues remains essentially unchanged in the respective binary complexes, as judged from the donor–acceptor distances (Table 3, Fig. 4). From this picture of a receptor (EPSP synthase binary complex) and a ligand (glyphosate), one intuitively would conclude that the inhibitory action of glyphosate should be similar in potency for the reactions with either S3P or shikimate, which contradicts the kinetic data (Table 1, Fig. 2). However, closer inspection of the shikimate–glyphosate complex reveals an increased number of clash distances between glyphosate and enzyme residues such as Gly96, Asp313 and Glu341, as compared to the S3P-glyphosate complex (Fig. 5, Table 4). Furthermore, glyphosate appears to bind less tightly in complex with shikimate, as is evident from the increased temperature factors (Fig. 6). The mid-region of the glyphosate molecule around the amide nitrogen is particularly flexible, with B-factors twice as high as seen in the dead-end complex with S3P. In addition, the entire glyphosate-binding site appears to be less rigid when compared with the genuine dead-end complex, as judged from the B-factor distribution among glyphosate's binding partners (data not shown).

The increased motion of the glyphosate molecule when reacting with the enzyme–shikimate complex can be attributed to a disruption of the precise interaction pattern between glyphosate and enzyme residues, as realized in the EPSP synthase complex with S3P. The positions of shikimate/glyphosate or S3P/glyphosate in the active site are essentially identical, as judged from F_o–F_o Fourier syntheses of the crystallographic data of the respective binary and ternary complexes, which were computed to eliminate potential model bias (data not shown). It appears that shikimate does not shift in the active site with respect to S3P, despite the missing phosphate group. Instead, the major difference electron density peaks (criterion $\geqslant 3\sigma$) of the two ternary complexes are present in the enzyme itself, around residues Val168 and Ser197, the region interacting with the phosphate group of S3P. Additional difference electron density is present around residue Glu341, which interacts with the amide nitrogen of glyphosate. This is also evident from the root mean square deviation of the respective main chain atoms (Fig. 7).

Glyphosate binding to the enzyme with S3P is accompanied by a slight shift of Glu341 away from S3P. However, when glyphosate binds to the enzyme–shikimate complex, the position of Glu341 remains unchanged, which appears to cause unfavorable steric interactions between the side chain carboxyl

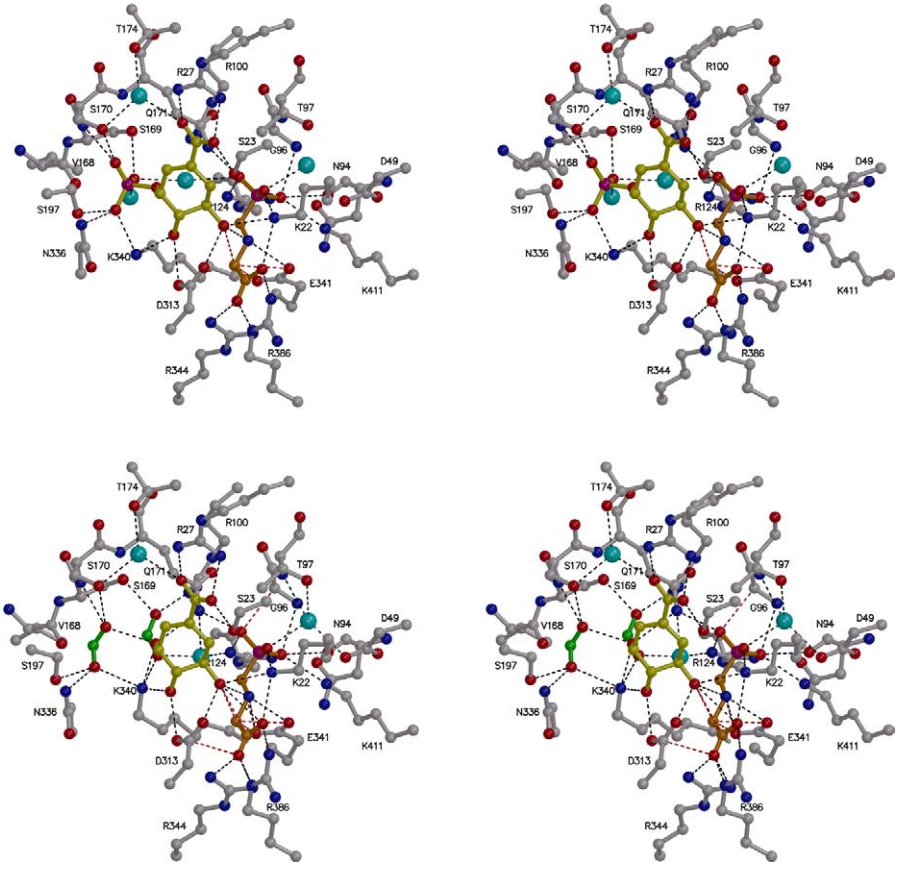

Fig. 5. The active site of EPSP synthase in its ternary complex with S3P/glyphosate or shikimate/glyphosate (stereo views). (Top): S3P and glyphosate bound to EPSP synthase (PDB 1G6S). (Bottom): Shikimate and glyphosate bound to EPSP synthase (this work, PDB 2AAY). Shikimate and S3P are shown in yellow, glyphosate in orange, formate ions are in green, water molecules in cyan. Black dashed lines indicate hydrogen bonding interactions; red dashed lines indicate clash distances.

Table 4
Bonding interactions between EPSP synthase and glyphosate[a]

Atom of glyphosate	Enzyme residue	S3P structure	Shikimate structure
O1: phosphate	K22 NH_2	2.90	2.80
O2: phosphate	R124 NH1	2.85	2.79
	Q171 NE2	2.88	2.68
	G96Cα	3.20	*3.08*
O3: phosphate	N94 O	3.11	3.09
	G96N	2.84	2.87
	R124 NH2	2.86	2.90
	K411 NZ	2.94	2.93
O4: carbonyl	K22NZ	2.92	2.90
	R386 NH1	3.10	3.09
	Ring position 5	3.08	3.08
O5: carbonyl	R344 NH2	2.97	2.89
	R344 NH2	2.84	2.84
	R386 NE	2.61	2.56
Nl	E341 OE1	3.28	3.02
	E341 OE2	2.87	2.74
	Ring position 5	2.82	2.72
Cl	E341 OE1	3.47	*3.18*
C2	E341 OE1	*2.96*	*2.70*
	E341 OE2	*3.18*	*3.01*
	Ring position 5	*3.11*	*2.95*
C3	Ring position 5	3.25	*3.15*

[a]Italicized values indicate clash distances.

group and carbon atoms of glyphosate (Table 4, Fig. 5). Apparently, the interaction of EPSP synthase with shikimate leaves the enzyme in a state not allowing the small conformational changes to accommodate the binding of glyphosate or PEP. As a consequence, it must be the region around the phosphate group of S3P, the backbone of the active site, located opposite to glyphosate and more than 11 Å distant from Glu341, which influences the glyphosate/PEP-binding site. The subtle changes of the glyphosate binding site in the enzyme–shikimate complex may be attributed to alterations within a hydrophobic pocket comprising the strictly conserved residue Pro125 and residues Val168/Val339, the latter two residues being semi-conserved, but hydrophobic in nature throughout the known EPSP synthase sequences (Fig. 7). In particular, the main chain around Val168 undergoes a substantial structural change upon binding of shikimate and glyphosate, causing the disruption of the hydrophobic forces within this pocket. This appears to result in a shift of the backbone around residues 339–344. Thus, this hydrophobic pocket may be important for the integrity of the PEP/glyphosate binding site, such that the small, but necessary, adjustments of key active site residues upon ligand binding can occur.

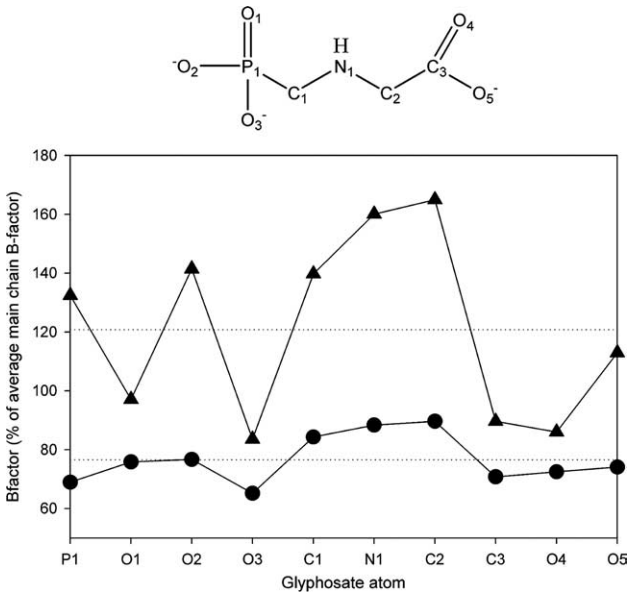

Fig. 6. Flexibility of glyphosate when bound to the EPSP synthase·shikimate complex. Displayed are the B-factor distributions of glyphosate atoms in the ternary complex of EPSP synthase with S3P (●) or shikimate (▲). For a more accurate comparison, the relative B-factor values of glyphosate atoms in the ternary complex of EPSP synthase with S3P (●) or shikimate (▲) are displayed with respect to the average B-factor of all enzyme main chain atoms. The average B-factor of all main chain atoms of EPSP synthase in complex with S3P and glyphosate is 10 $Å^2$, of all glyphosate atoms 7.7 $Å^2$. The average B-factor of all main chain atoms of EPSP synthase in complex with shikimate and glyphosate is 12.3 $Å^2$, of all glyphosate atoms 14.9 $Å^2$. The dotted lines denote the average B-factor of the respective glyphosate molecules.

4. Conclusions

Large structural changes in and around the active site have been reported previously for the potent inhibition of EPSP synthase by the (*R*)-phosphonate tetrahedral reaction intermediate analog, suggesting that this enzyme is capable of accommodating structurally diverse ligands [19]. On the other hand, the data presented herein demonstrate that even slight alterations within the active site render the enzyme insensitive to glyphosate. These differences lie within the nature of the respective enzyme–ligand interactions. While reaction intermediate analogs induce the transition from the open to the closed state of EPSP synthase to form a binary dead-end complex, glyphosate exerts its inhibitory action through binding to the binary substrate complex to form a ternary dead-end complex [8,19]. Thus, the major conformational changes in the enzyme have already occurred before glyphosate enters the active site, which leaves only the narrow PEP-binding site for structural adjustments. It seems that the necessary subtle structural changes to accommodate glyphosate are impaired when the enzyme reacts with shikimate instead of S3P.

This finding may be valuable for the design of EPSP synthase enzymes that are insensitive towards glyphosate. To date, only two sites in the enzyme have attracted major attention with respect to glyphosate resistance, Gly96 and Pro101. The molecular basis for the glyphosate insensitivity of the Gly96Ala mutant enzyme is mere steric hindrance, caused by the additional methyl group protruding into the glyphosate-binding site [9]. Not surprisingly, this mutation also severely impairs the utilization of PEP. Pro101 mutant enzymes have been reported to be less sensitive towards glyphosate, while the K_m for PEP is only slightly increased [20,21]. As is the case for the Gly96Ala mutant enzyme, mutations of Pro101 possibly impact the binding site around the phosphonate moiety of glyphosate. The data of this work suggest that mutations in the vicinity of the binding site for the phosphate group of S3P, in particular of the hydrophobic pocket built up by Pro125/Val168/Val339, may cause alterations of the glyphosate-binding site, such that the overall interaction pattern of enzyme residues with glyphosate is compromised. Clearly, however, the challenge remains to

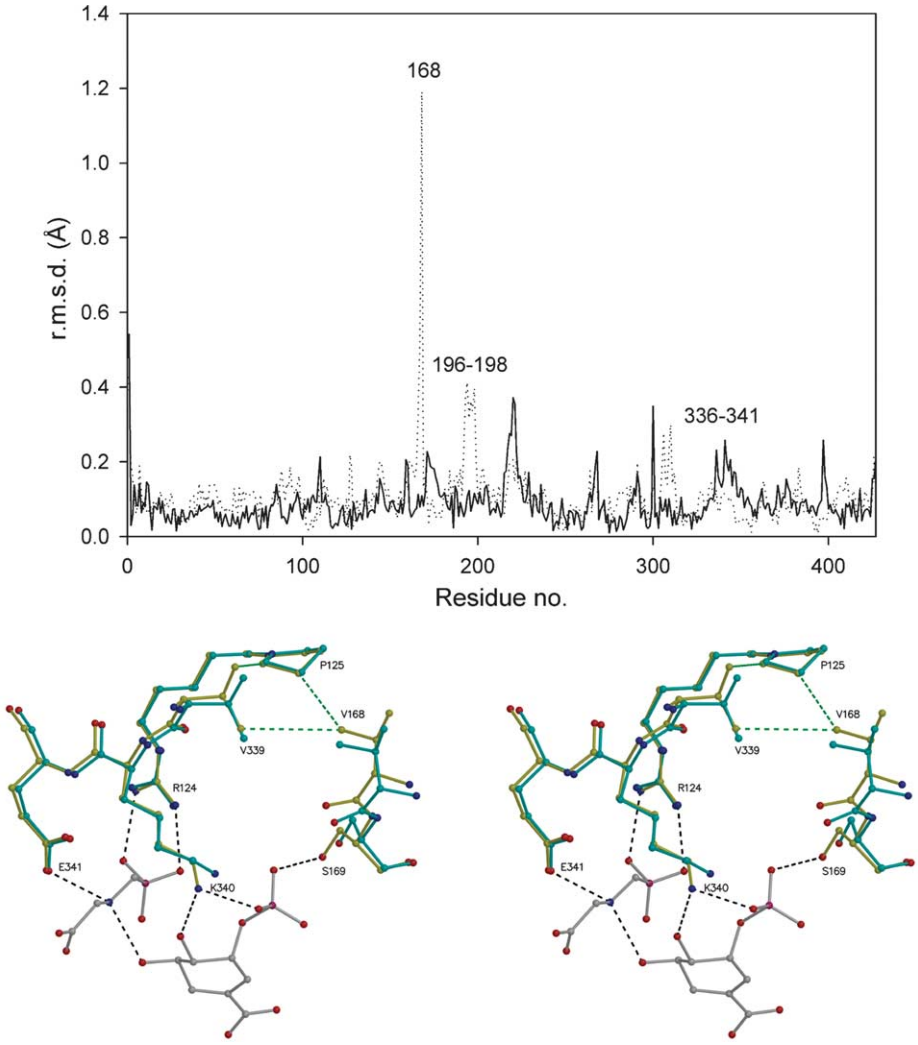

Fig. 7. Structural changes in EPSP synthase upon interaction with shikimate and glyphosate. (Top) Comparison of the root mean square deviations (rmsd) of the Cα-atoms of EPSP synthase liganded with S3P or S3P/glyphosate (black line; average rmsd = 0.1 Å) and that of EPSP synthase liganded with shikimate or shikimate/glyphosate (dotted line; average rmsd = 0.13 Å). Note the high rmsd around residues Val168 and Ser197, which are not present for the enzyme liganded with S3P. (Bottom) Stereo view of the hydrophobic pocket built up by Pro125/Val168/Val339 and its alteration upon binding of shikimate and glyphosate to the enzyme. EPSP synthase residues in the complex with S3P and glyphosate are shown in yellow; residues in the complex with shikimate and glyphosate in cyan. S3P and glyphosate are displayed in grey, hydrogen bonding interactions as black dotted lines and hydrophobic forces as green dotted lines.

design glyphosate-insensitive mutant enzymes that retain catalytic efficiency at the same time.

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References

[1] Bentley, R. (1990) The shikimate pathway – a metabolic tree with many branches. Crit. Rev. Biochem. Mol. Biol. 25, 307–384.
[2] Haslam, E. (1993) Shikimic Acid: Metabolism and Metabolites, John Wiley, Chichester, UK.
[3] Kishore, G.M. and Shah, D.M. (1988) Amino acid biosynthesis inhibitors as herbicides. Annu. Rev. Biochem. 57, 627–663.
[4] Steinrucken, H.C. and Amrhein, N. (1980) The herbicide glyphosate is a potent inhibitor of 5-enolpyruvyl-shikimic acid-3-phosphate synthase. Biochem. Biophys. Res. Commun. 94, 1207–1212.
[5] Parish, T. and Stoker, N.G. (2002) The common aromatic amino acid biosynthesis pathway is essential in *Mycobacterium tuberculosis*. Microbiology 148, 3069–3077.
[6] Roberts, C.W., Roberts, F., Lyons, R.E., Kirisits, M.J., Mui, E.J., Finnerty, J., Johnson, J.J., Ferguson, D.J., Coggins, J.R., Krell, T., Coombs, G.H., Milhous, W.K., Kyle, D.E., Tzipori, S., Barnwell, J., Dame, J.B., Carlton, J. and McLeod, R. (2002) The shikimate pathway and its branches in apicomplexan parasites. J. Infect. Dis. 185, S25–S36.
[7] Roberts, F., Roberts, C.W., Johnson, J.J., Kyle, D.E., Krell, T., Coggins, J.R., Coombs, G.H., Milhous, W.K., Tzipori, S., Ferguson, D.J., Chakrabarti, D. and McLeod, R. (1998) Evidence for the shikimate pathway in apicomplexan parasites. Nature 393, 801–805.
[8] Schonbrunn, E., Eschenburg, S., Shuttleworth, W.A., Schloss, J.V., Amrhein, N., Evans, J.N. and Kabsch, W. (2001) 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.

[9] Eschenburg, S., Healy, M.L., Priestman, M.A., Lushington, G.H. and Schonbrunn, E. (2002) How the mutation glycine96 to alanine confers glyphosate insensitivity to 5-enolpyruvyl shikimate-3-phosphate synthase from *Escherichia coli*. Planta 216, 129–135.

[10] Franz, J.E., Mao, M.K. and Sikorski, J.A. (1997) Glyphosate: A Unique Global Herbicide, Vol. 189, American Chemical Society, Washington, DC.

[11] Gruys, K.J., Walker, M.C. and Sikorski, J.A. (1992) Substrate synergism and the steady-state kinetic reaction mechanism of EPSP synthase from *Escherichia coli*. Biochemistry 31, 5534–5544.

[12] Miller, M.J., Anderson, K.S., Braccolino, D.S., Cleary, D.G., Gruys, K.J., Han, C.Y., Lin, K.-C., Pansegrau, P.D., Ream, J.E., Sammons, D. and Sikorski, J.A. (1993) EPSP synthase inhibitor design II. The importance of the 3-phosphate group for ligand binding at the shikimate-3-phosphate site and the identification of 3-malonate ethers as novel 3-phosphate mimics. Bioorg. Med. Chem. Lett. 3, 1435–1440.

[13] Eschenburg, S., Kabsch, W., Healy, M.L. and Schonbrunn, E. (2003) A new view of the mechanisms of UDP-*N*-acetylglucosamine enolpyruvyl transferase (MurA) and 5-enolpyruvylshikimate-3-phosphate synthase (AroA) derived from X-ray structures of their tetrahedral reaction intermediate states. J. Biol. Chem. 278, 49215–49222.

[14] Daugherty, M., Vonstein, V., Overbeek, R. and Osterman, A. (2001) Archaeal shikimate kinase, a new member of the GHMP-kinase family. J. Bacteriol. 183, 292–300.

[15] Lanzetta, P.A., Alvarez, L.J., Reinach, P.S. and Candia, O.A. (1979) An improved assay for nanomole amounts of inorganic phosphate. Anal. Biochem. 100, 95–97.

[16] Kabsch, W. (2001) XDS in: International Tables for Crystallography (Rossmann, M.G. and Arnold, E., Eds.), Kluwer Academic Publisher, Dordrecht, (Chapter 25.2.9).

[17] Brunger, A.T., Adams, P.D., Clore, G.M., DeLano, W.L., Gros, P., Grosse-Kunstleve, R.W., Jiang, J.S., Kuszewski, J., Nilges, M., Pannu, N.S., Read, R.J., Rice, L.M., Simonson, T. and Warren, G.L. (1998) Crystallography & NMR system: A new software suite for macromolecular structure determination. Acta Crystallogr. Sect. B Struct. Crystallogr. Cryst. Chem. 54, 905–921.

[18] Jones, T.A., Zou, J.Y., Cowan, S. and Kjeldgaard, M. (1991) Improved methods for building protein models in electron density maps and the location of errors in these models. Acta Crystallogr. B. 47, 110–119.

[19] Priestman, M.A., Healy, M.L., Becker, A., Alberg, D.G., Bartlett, P.A., Lushington, G.H. and Schonbrunn, E. (2005) The interaction of phosphonate analogs of the tetrahedral reaction intermediate with 5-enolpyruvylshikimate-3-phosphate synthase (EPSPS) in atomic detail. Biochemistry 44, 3241–3248.

[20] Baerson, S.R., Rodriguez, D.J., Tran, M., Feng, Y., Biest, N.A. and Dill, G.M. (2002) Glyphosate-resistant goosegrass. Identification of a mutation in the target enzyme 5-enolpyruvylshikimate-3-phosphate synthase. Plant Physiol. 129, 1265–1275.

[21] Padgette, S.R., Re, D.B., Gasser, C.S., Eichholtz, D.A., Frazier, R.B., Hironaka, C.M., Levine, E.B., Shah, D.M., Fraley, R.T. and Kishore, G.M. (1991) Site-directed mutagenesis of a conserved region of the 5-enolpyruvylshikimate-3-phosphate synthase active site. J. Biol. Chem. 266, 22364–22369.

[22] Diederichs, K. and Karplus, P.A. (1997) Improved R-factors for diffraction data analysis in macromolecular crystallography. Nat. Struct. Biol. 4, 269–275.