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Molecular basis for the herbicide resistance of Roundup Ready crops

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The engineering of transgenic crops resistant to the broad-spectrum herbicide glyphosate has greatly improved agricultural efficiency worldwide. Glyphosate-based herbicides, such as Roundup, target the shikimate pathway enzyme 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, the functionality of which is absolutely required for the survival of plants. Roundup Ready plants carry the gene coding for a glyphosate-insensitive form of this enzyme, obtained from Agrobacterium sp. strain CP4. Once incorporated into the plant genome, the gene product, CP4 EPSP synthase, confers crop resistance to glyphosate. Although widely used, the molecular basis for this glyphosate-resistance has remained obscure. We generated a synthetic gene coding for CP4 EPSP synthase and characterized the enzyme using kinetics and crystallography. The CP4 enzyme has unexpected kinetic and structural properties that render it unique among the known EPSP synthases. Glyphosate binds to the CP4 EPSP synthase in a condensed, noninhibitory conformation. Glyphosate sensitivity can be restored through a single-site mutation in the active site (Ala-100–Gly), allowing glyphosate to bind in its extended, inhibitory conformation.

Results and Discussion

Kinetically, the most intriguing feature of CP4 EPSP synthase is the strong dependence of the catalytic efficiency on monovalent cations, namely K+, Rb+, and NH4+. Whereas the Km for S3P appears to be independent of cations, the Km for PEP decreases from 3.5 mM to 0.2 mM in the presence of 100 mM KCl, resulting in an increase of kcat/Km by a factor of 58, from 1.9 × 105 M−1 s−1 to 1.1 × 106 M−1 s−1 (see the supporting information, which is published on the PNAS web site). The apparent dissociation constant for the interaction of K+ ions with the enzyme is ∼25 mM (Fig. 1B). It has been reported that potassium concentrations in planta are in fact sufficient to promote the enzyme’s interaction with PEP (13). In the absence of such cations, the low catalytic efficiency of CP4 EPSP synthase would render such engineered plants unsuitable. CP4 EPSP synthase...

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Abbreviations: EPSP, 5-enolpyruvylshikimate-3-phosphate; PEP, phosphoenolpyruvate; S3P, shikimate-3-phosphate.

Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 2GG4 (unliganded CP4 EPSP synthase), 2GG6 (S3P-ligated CP4 EPSP synthase), 2GG8 (S3P-glyphosate-ligated CP4 EPSP synthase), and 2GGD (Ala-100–Gly CP4 EPSP synthase-ligated with S3P and glyphosate)).

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coli to the enzyme from the overall structure of the CP4 EPSP synthase is more similar as a prerequisite for the enzyme's interaction with PEP (Fig. 2). State, suggesting an induced-fit mechanism with binding of S3P exists in an open, unliganded state and a closed, S3P-liganded state, which share the same binding site in EPSP synthase (14, 15). Thus, the CP4 EPSP synthase maintains activity over a broad pH range and for prolonged periods at elevated temperatures (supporting information). As expected, the CP4 EPSP synthase is insensitive to inhibition by glyphosate, exhibiting $K_i$ and IC$_{50}$ values of 6 and 11 mM, respectively (Fig. 1C; see also the supporting information). Moreover, the enzyme's interaction with glyphosate appears to be independent of cations present: The IC$_{50}$ values for glyphosate inhibition are ~10–20 mM with or without salt added (data not shown). This finding is surprising, because PEP and glyphosate share the same binding site in EPSP synthase (14, 15). Thus, one would intuitively expect cations to modulate the binding of glyphosate; however, the effect of potassium ions on the enzyme's activity appears to be selective toward PEP utilization. Indeed, the CP4 EPSP synthase is the prototypic class II EPSP synthase (Fig. 1A). The reaction catalyzed by EPSP synthase. (A) The activity of CP4 EPSP synthase depends strongly on the presence of cations, such as NH$_4^+$, Rb$^+$, and K$^-$. (Inset) Activation by K$^-$ is saturable with an apparent dissociation constant of 25 mM. (C) IC$_{50}$ studies with wild-type CP4 EPSP synthase (○), Ala-100–Gly CP4 EPSP synthase (▲), and wild-type E. coli EPSP synthase (●) reveal that CP4 EPSP synthase is inhibited only by high millimolar concentrations of glyphosate (IC$_{50}$ ~11 mM). The Ala-100–Gly mutant CP4 EPSP synthase is approximately two orders of magnitude more sensitive to glyphosate than to the enzyme from E. coli (IC$_{50}$ = 160 μM). The E. coli enzyme is inhibited by even lower glyphosate concentrations (IC$_{50}$ = ~2.5 μM).

Fig. 1. Key kinetic properties of CP4 EPSP synthase. (A) The reaction catalyzed by EPSP synthase. (B) The activity of CP4 EPSP synthase is insensitive to inhibition by glyphosate, exhibiting $K_i$ and IC$_{50}$ values of 6 and 11 mM, respectively (Fig. 1C; see also the supporting information). Moreover, the enzyme's interaction with glyphosate appears to be independent of cations present: The IC$_{50}$ values for glyphosate inhibition are ~10–20 mM with or without salt added (data not shown). This finding is surprising, because PEP and glyphosate share the same binding site in EPSP synthase (14, 15). Thus, one would intuitively expect cations to modulate the binding of glyphosate; however, the effect of potassium ions on the enzyme's activity appears to be selective toward PEP utilization. Indeed, the CP4 EPSP synthase is the prototypic class II EPSP synthase, because the catalytic efficiency remains essentially unaltered in the presence of high glyphosate concentrations. Notably, other known class II EPSP synthases display greater glyphosate sensitivity and, with the exception of the Staphylococcus aureus enzyme, ions typically affect binding of both PEP and glyphosate (12, 16, 17).

The three-dimensional structures of EPSP synthase from *E. coli* and *Streptococcus pneumoniae* are known, but both of these enzymes are sensitive to glyphosate (15, 18, 19). As observed in the *E. coli* and *Streptococcus pneumoniae* EPSP synthases, the CP4 EPSP synthase exists in an open, unliganded state and a closed, S3P-liganded state, suggesting an induced-fit mechanism with binding of S3P as a prerequisite for the enzyme's interaction with PEP (Fig. 2). The overall structure of the CP4 EPSP synthase is more similar to the enzyme from *Streptococcus pneumoniae* than to the enzyme from *E. coli*. In the binary complex with S3P, the active site architecture is highly conserved, even between the CP4 and *E. coli* enzymes. Strictly conserved residues from both globular domains of the enzyme constitute the PEP-binding site (Figs. 2B and 3). In the unliganded state, the CP4 EPSP synthase contains highly flexible regions, particularly around the strictly conserved residue Glu-354, which is part of a 12-residue loop of the C-terminal domain (the bottom globular domain of Fig. 2A) that is barely visible in electron density maps. In the enzyme's binary complex with S3P, this loop becomes structured and interacts with the N-terminal domain to constitute the active site. Remarkably, this loop also adopts a regular structure when the unliganded enzyme is crystallized with 100 mM KCl or RbCl. Several of our attempts to identify the cation binding site(s) by cocrySTALLization of the unliganded or S3P-liganded states of the enzyme with the more electron-dense Rb$^+$ ion failed. It is conceivable, however, that monovalent cations may bind transiently to the enzyme, acting as chaperones in restructuring the loop, enabling CP4 EPSP synthase to interact more efficiently with PEP during a catalytic cycle (20). Once the loop has adopted a regular structure, the cation-binding site(s) may be lost.

The weak action of glyphosate on CP4 EPSP synthase can be primarily attributed to an Ala residue in position 100, which is a Gly in the *E. coli* and *Streptococcus pneumoniae* EPSP synthases. The methyl group of Ala-100 protrudes into the glyphosate-binding site, clashing with one of the oxygen atoms of glyphosate's phosphonate group. As a result, the glyphosate molecule adopts an extended conformation (15, 18), whereas in the CP4 enzyme it adopts a shortened state, which is achieved through rotation about the N—C bond adjacent to glyphosate's carboxyl group.
group (Fig. 4). This rotation shortens the glyphosate molecule by \( \approx 0.6 \) Å. In its condensed conformation, the glyphosate molecule clashes with the side chain of Glu-354 (Fig. 3A). \textit{Ab initio} energy calculations of the two distinct glyphosate conformations revealed that the condensed conformation has an \( \approx 17 \) kcal/mol higher energy than the extended conformation (see \textit{Materials and Methods}). Thus, only the extended, low-energy conformation of glyphosate appears to be inhibitory.

We hypothesized that the mutation of this Ala-100 to Gly should restore the CP4 enzyme’s sensitivity toward glyphosate. The kinetic properties of the Ala-100–Gly CP4 EPSP synthase with respect to PEP utilization, cation dependence, and catalytic efficiency remain essentially unchanged (supporting information). However, this mutant enzyme is sensitive to inhibition by glyphosate with an IC\(_{50}\) value of 150 \( \mu \)M (Fig. 1C) and a \( K_i \) of 93 \( \mu \)M (supporting information). As expected, glyphosate binds to this mutant enzyme in its extended conformation (Fig. 3B). For EPSP synthase from \textit{E. coli}, the residue equivalent to Ala-100 is Gly-96. Mutation of this Gly residue to Ala confers insensitivity to glyphosate but results in a large decrease in affinity for PEP (6, 7). Distinct differences in the space provided for PEP may explain the kinetic differences between the \textit{E. coli} and the CP4 EPSP synthases. In particular, the clash between glyphosate’s phosphonate moiety and the Ala side chain is less pronounced in the CP4 enzyme than in the \textit{E. coli} EPSP synthase: The distance between the C of Ala-100 and the nearest phosphonate oxygen of glyphosate is 2.4 Å in the CP4 enzyme versus 2.1 Å in the \textit{E. coli} EPSP synthase. Although these clash distances are sufficient to hinder glyphosate from binding to either enzyme in its inhibitory conformation, the active site of CP4 EPSP synthase is expected to accommodate the shorter PEP molecule more efficiently than the Gly-96–Ala \textit{E. coli} EPSP synthase, rendering the CP4 enzyme more catalytically efficient.

Another prominent mutation-causing glyphosate insensitivity is Pro-101–Ser in EPSP synthases from \textit{Salmonella typhimurium} and goosegrass (5, 9, 21) and Pro-101–Leu in EPSP synthase...
from *Sta. aureus* (12). In the structure of the CP4 enzyme, the equivalent residue is a Leu residue in position 105, which is part of a helix in the core of the N-terminal domain. This substitution of Leu for Pro may additionally contribute to the distinct kinetic properties of the CP4 enzyme.

**Conclusions**

The data presented herein explain the agricultural success of Roundup Ready crops at the molecular level. In particular, a single residue in the active site (Ala-100) renders the CP4 EPSP synthase insensitive to glyphosate, whereas a highly conserved Gly residue is found at this position in known natural plant and bacterial enzymes. The continued presence of glyphosate is likely to favor mutations that reduce glyphosate sensitivity while still maintaining catalytic efficiency. It is therefore not surprising that the gene coding for CP4 EPSP synthase was isolated from a microorganism found in an extremely glyphosate-rich environment (refs. 1 and 10 and p. 632 of ref. 22). The speed at which glyphosate resistance develops depends on the organisms' generation time and fidelity of gene replication. The current low rate of appearance of plants with naturally acquired resistance to glyphosate may be attributed to the relatively high-fidelity replication and long generation times of most plants. However, extensive use of glyphosate increases the likelihood that more glyphosate-resistant organisms will emerge on a large scale. It is conceivably that a single Ala for Gly substitution in the active site of other class II EPSP synthases will confer resistance to glyphosate.

It appears that the confined space of the active site of EPSP synthase prohibits even slight alterations of the glyphosate molecule. From our structural studies, it is evident that even conformational changes within the glyphosate molecule result in loss of inhibitory activity, which is in accordance with extensive structure–activity relationship studies on glyphosate that have been conducted in the past. More than 1,000 analogs of glyphosate have been produced and tested for inhibition of EPSP synthase, but minor structural alterations typically resulted in dramatically reduced potency, and no compound superior to glyphosate was identified (ref. 22, pp. 441–519 and 569–578). Additionally, although EPSP synthase is considered a promising target for the treatment of diseases caused by pathogenic bacteria or eukaryotic parasites (23–25), glyphosate displays little antimicrobial activity. Taken together, these findings demonstrate the pressing need for the development of entirely new inhibitors that target sites different from the PEP/glyphosate binding site of this agriculturally and medicinally important enzyme.

**Materials and Methods**

S3P (triethylammonium salt) was synthesized from shikimic acid by using recombinant archaeal shikimate kinase (26) and purified via anion exchange chromatography on Q-Sepharose. PEP (potassium salt) and all other chemicals were purchased from...
optimized to implement the unique restriction site AsuII. The initial codon frequency A, base pairs 1–706, and part B, base pairs 701-1425) linked by amplifications in analogy to Fischer cloning of the synthetic gene sequence. Both segments (part A vector pNCO113). Finally, flanking sequences were added for around the C/H11015 the glyphosate molecule is E. coli with the Ala-100–Gly CP4 EPSP synthase is identical to the one observed in the synthase (ternary complexes of CP4 EPSP synthase (Left) With an Ala residue in position 100, the glyphosate molecule is ~0.6 Å shorter, mainly because of a rotation around the C—N bond next to the carboxyl group.

Sigma (St. Louis, MO) unless otherwise noted. Coomassie reagent (Pierce, Rockford, IL) with BSA as a standard was used to determine protein concentrations.

The amino acid sequence of CP4 EPSP synthase was obtained from U.S. Patent 5633435 (1). The sequence was initially optimized with the program package DNAWorks (27), which reverse-translated the protein sequence by using the codon frequency table for E. coli (Codon Usage Database available at www.kazusa.or.jp/codon) and divided into two segments (part A, base pairs 1–706, and part B, base pairs 701-1425) linked by the unique restriction site AsuII. The initial codon frequency threshold was set to 50%. The resulting sequence was further optimized to implement ~30 restriction sites (unique in the vector pNCO113). Finally, flanking sequences were added for cloning of the synthetic gene sequence. Both segments (part A and part B) were synthesized by a series of consecutive PCR amplifications in analogy to Fischer et al. (28). The final PCR product corresponding to segment A (715 bp) was digested with the restriction endonucleases EcoRI and AsuII. The final PCR product corresponding to segment B (734 bp) was digested with AsuII and HindIII. Both segments were combined, ligated into the plasmid pNCO113, which had been treated with the restriction enzymes EcoRI and HindIII, and transformed into E. coli strain XL-1 Blue, resulting in the recombinant strain XL-1-pNCO-EPSPS-syn. The sequence of the synthetic gene was monitored by DNA sequencing in both directions. The synthesized gene was then ligated into pET21a (Novagen, Darmstadt, Germany), and the resulting construct was transformed into E. coli BL21(DE3) (Invitrogen, Carlsbad, CA). The Ala-100–Gly mutant CP4 EPSP synthase was produced by site-directed mutagenesis with the QuiChangeII mutagenesis kit (Stratagene, La Jolla, CA). Overexpression of soluble CP4 EPSP synthase was induced at 37°C by addition of isopropyl β-D-thiogalactoside.

CP4 EPSP synthase was purified to homogeneity according to the protocol developed for Sta. aureus EPSP synthase (12).

Enzymatic activity was determined by the amount of inorganic phosphate produced in a 3-min reaction using malachite green (29). CP4 EPSP synthase activity assays were conducted at 25°C in 100 μl of 50 mM Hepes-NaOH, pH 7.5/2 mM DTT with or without KCl where indicated, in parallel with E. coli EPSP synthase. Enzyme activity is expressed as micromoles of phosphate produced per minute of reaction time per milligram of enzyme (units/mg). Data evaluation was performed with SigmaPlot (SPSS Science, Chicago, IL).

The $K_m$ values for S3P and PEP were determined by fitting the data to the equation

$$v = \frac{V_{max}[S]}{K_m + [S]}$$

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$_{50}$ values for glyphosate inhibition were obtained by fitting data to the equation

$$v = V_{min} + \frac{V_{max} - V_{min}}{1 + \left(\frac{I}{IC_{50}}\right)^\pi}$$

The values in parentheses refer to the highest resolution shell. rmsd calculations are from ideal values.

$^a$Resolution range, Å

$^b$Values in parentheses refer to the highest resolution shell. rmsd calculations are from ideal values.

$^c$Resolution range, Å

$^d$Rmerge = 100 × ΣI $h$ - $I_\text{obs}$ $/ \Sigma I_\text{obs}$, where $h$ are unique reflection indices.

$^e$Rmerge = 100 × ΣI $h$ = $F_\text{calc}$ - $F_\text{model}$ $/ \Sigma I_\text{calc}$, where $F_\text{calc}$ and $F_\text{model}$ are observed and calculated structure factor amplitudes, respectively.

$^f$Rmerge = Rmerge calculated for randomly chosen unique reflections, which were excluded from the refinement [1,174 for unliganded CP4, 1,419 for CP4:S3P, 1,290 for CP4:S3P:glyosphate, and 1,259 for CP4(A100G):S3P:glyosphate].
where \( v \) is the initial velocity, \( V_{\text{max}} \) is the maximum velocity, \( V_{\text{min}} \) is the minimum velocity, \( [I] \) is the concentration of glyphosate, and \( n \) is the Hill slope. The \( K_i \) was derived by determining the \( K_{m(\text{obs})} \) of PEP in the presence of increasing amounts of inhibitor and fitting the data to the equation

\[
K_{m(\text{obs})} = \frac{K_m}{K_i} + \frac{K_m}{V_{\text{max}}}.
\]  

where \( K_{m(\text{obs})} \) is the Michaelis constant for PEP in the presence of glyphosate, \( [I] \) is the glyphosate concentration, and \( K_m \) is the Michaelis constant for PEP in the absence of glyphosate.

CP4 EPSP synthase was concentrated to 40 mg/ml in 50 mM Tris-HCl, pH 8.0/2 mM DTT and crystallized by hanging drop vapor diffusion. The unliganded enzyme was crystallized in 12.5% (wt/vol) PEG 4000/50 mM KCl/2.5% DMSO/50 mM Tris-HCl, pH 8.5. The binary complex with S3P was crystallized in 1.0 M (NH₄)₂SO₄/0.1 M KCl/1% PEG 400/50 mM Tris-HCl, pH 8.5, in the presence of 5 mM S3P. The ternary complexes of the wild-type and Ala-100–Gly mutant enzymes were crystallized in 1.0 M (NH₄)₂SO₄/0.1 M KCl/1% PEG 400/50 mM Hepes-Na, pH 7.5, in the presence of 5 mM S3P and 40 mM glyphosate.

Diffraction data were recorded at -180°C by using the rotation method on single flash-frozen crystals of the enzyme in its unliganded or liganded states [detector: R-axis IV+ image plate; x-rays: CuKα, focused by mirror optics; generator: Rigaku RU300 (MSC, The Woodlands, TX)]. The structure of the unliganded CP4 EPSP synthase was determined with 25% PEG 200 for cryoprotection, and 25% glycerol was used for the binary and ternary complexes. The data were reduced with XDS (30) or HKL-2000 (31). The program package CNS (32) was used for phasing and refinement; model building was performed with O (33). The structures were solved by molecular replacement. For the unliganded Str. porteucytiae enzyme (Protein Data Bank ID code 1RF5) as search model, we first determined the structure of the CP4 enzyme in its unliganded state at 2.1-Å resolution. Subsequently, the S3P-liganded state was determined at 1.64-Å resolution with both globular domains of the unliganded CP4 enzyme as search models. This binary complex served as the search model for the determination of the ternary complexes of the wild-type and Ala-100–Gly mutant enzymes. Refinement was performed with the data at the highest resolution with no sigma cut-off applied. Several rounds of minimization, simulated annealing (2,500 K starting temperature) and restrained individual B-factor refinement were carried out. Data collection and refinement statistics are summarized in Table 1. Figs. 2 and 3 were drawn with Molscript (34) and Raster3D (35); Fig. 4 was drawn with Bobscrip (36) and Raster3D. Ab initio energy calculations of the two glyphosate conformations were performed with GAMESS using MP2/6–31+G* (37).

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References

3. National Agriculture Statistics Service (June 30, 2005) [Online].