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Molecular basis of glyphosate resistance: Different approaches through protein engineering

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Abstract

Glyphosate (N-phosphonomethyl-glycine) is the most-used herbicide in the world: glyphosate-based formulations exhibit broad-spectrum herbicidal activity with minimal human and environmental toxicity. The extraordinary success of this simple small molecule is mainly due to the high specificity of glyphosate towards the plant enzyme enolpyruvylshikimate-3-phosphate synthase in the shikimate pathway leading to biosynthesis of aromatic amino acids. Starting in 1996, transgenic glyphosate-resistant plants were introduced thus allowing the application of the herbicide to the crop (post-emergence) to remove emerged weeds without crop damage. This review focuses on the evolution of mechanisms of resistance to glyphosate as obtained through natural diversity, the gene shuffling approach to molecular evolution, and a rational, structure-based approach to protein engineering. In addition, we offer rationale for the means by which the modifications made have had their intended effect.

Keywords

Glyphosate; herbicide resistance; herbicide tolerance; transgenic crops; protein engineering

Introduction

Modern agricultural chemicals have greatly contributed to plentiful world food production by controlling crop pests such as yield-diminishing weed infestations. Among these molecules, the herbicide glyphosate (N-phosphonomethyl-glycine) has had the greatest positive impact. Developed by the Monsanto Co. and introduced to world agriculture in 1974, glyphosate is the number-one selling herbicide worldwide [1,2]. Glyphosate-based formulations exhibit broad-spectrum herbicidal activity with minimal human and environmental toxicity [3,4]. Glyphosate inhibits the enzyme enolpyruvylshikimate-3-phosphate synthase (EPSPS) in the plant chloroplast-localized pathway that leads to the biosynthesis of aromatic amino acids, see Fig. 1. Since its introduction, glyphosate has found a range of uses in agricultural, urban and natural ecosystems. Because it is a non-selective herbicide that controls a very wide range of plant species, it has been used for

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broad-spectrum weed control just before crop seeding (termed ‘burndown’) and in areas where total vegetation control is desired.

A revolutionary new glyphosate use pattern commenced in 1996 with the introduction of transgenic glyphosate-resistant soybean, launched and marketed under the Roundup Ready brand in the USA. In transgenic glyphosate-resistant crops, glyphosate can be applied to the crop (post-emergence) to remove emerged weeds without crop damage. Since introduction, herbicide-resistant soybeans have been quickly adopted. In 2010 93% of all soybeans grown in the USA were herbicide resistant, as well as 78% of all cotton and 70% of all maize (http://www.ers.usda.gov/Data/BiotechCrops/). As illustrated by genetically engineered maize, the current trend is toward varieties that have both herbicide and insect resistance traits. In 2010, 16% of maize varieties were only insect-resistant, 23% were only herbicide-resistant and 47% were stacked with both traits. “Glyphosate is a one in a 100-year discovery that is as important for reliable global food production as penicillin is for battling diseases” [5]. The popularity of glyphosate stems from its efficacy on a wide range of weed species, low cost, and low environmental impact [2,6]. Further impetus for adopting glyphosate resistance traits are lower prices brought about by the entry of generic producers following the expiration of the patent on the molecule itself in 2000.

There are two basic strategies that have been successful in introducing glyphosate resistance into crop species: i) expression of an insensitive form of the target enzyme, and ii) detoxification of the glyphosate molecule. The strategy used in existing commercial glyphosate-tolerant crops is the former, employing a microbial (CP4) or a mutated (TIPS) form of EPSPS that is not inhibited by glyphosate. The theoretical disadvantage of this approach is that glyphosate remains in the plant and accumulates in meristems, where it may interfere with reproductive development and may lower crop yield [7]. Resistance to herbicides is more commonly achieved through their metabolic detoxification by native plant or transgene-encoded enzymes. The advantage of glyphosate detoxification is the removal of herbicidal residue, which may result in more robust tolerance and allow spraying during reproductive development.

This review focuses on the evolution of mechanisms of resistance to glyphosate as obtained through natural diversity, the gene shuffling approach to molecular evolution, and a rational, structure-based approach to protein engineering. In addition, we offer rationale for the means by which the modifications made have had their intended effect.

EPSP Synthase insensitive to glyphosate

The discovery of EPSPS as the molecular target of glyphosate by Steinrücken and Amrhein in 1980 [8] prompted extensive studies on the catalytic mechanism and the structure-function relationship of this enzyme, performed by various laboratories over the past three decades. This review summarizes some of the key findings that led to our current understanding of the molecular mode of action of glyphosate and the molecular basis for glyphosate resistance.

Structure and function of EPSPS

EPSPS catalyzes the transfer of the enolpyruvyl moiety of phosphoenolpyruvate (PEP) to the 5-hydroxyl of shikimate-3-phosphate (S3P) to produce enolpyruvyl shikimate-3-phosphate (EPSP) and inorganic phosphate (Fig. 1). This reaction forms the sixth step in the shikimate pathway leading to the synthesis of aromatic amino acids and other aromatic compounds in plants, fungi, bacteria [9] and apicomplexan parasites [10]. The only enzyme known to catalyze a similar reaction is the bacterial enzyme MurA (**EC 2.5.1.7**), which catalyzes the first committed step in the synthesis of the bacterial cell wall. Early kinetic

characterization established that glyphosate is a reversible inhibitor of EPSPS by competing with PEP for binding to the active site [8,11,12]. Several studies on the reaction mechanism of EPSPS by different laboratories in the 1990s using chemical and spectroscopic methods provided evidence that the EPSPS reaction proceeds through a tetrahedral intermediate formed between S3P and the carbocation state of PEP, followed by elimination of inorganic phosphate, for a review see [13]. The first crystal structure of EPSPS was determined for the *E. coli* enzyme in its ligand-free state by a research group of Monsanto in 1991 [14], and revealed a unique protein fold (inside-out α/β barrel) with two globular domains, each composed of three identical folding units, connected to each other by a two-stranded hinge region (Fig. 2A). This structure, however, was void of substrate or inhibitor, and consequently did not unveil the nature of the active site or the mode of action of glyphosate. A decade later the crystal structure of EPSPS was determined in complex with S3P and glyphosate [15]. The compactness of the liganded EPSPS structure suggested that the EPSPS reaction follows an induced-fit mechanism, in which the two globular domains approach each other upon binding of S3P (Fig. 2A). This open-closed transition creates a confined and highly charged environment immediately adjacent to the target hydroxyl group of S3P, to which glyphosate or PEP bind (Figs. 2B, 2C). Another high-resolution crystal structure of EPSPS depicted the genuine tetrahedral reaction intermediate trapped in the active site, which established the absolute stereochemistry as *2S* and demonstrated that PEP and glyphosate share the identical binding site with similar binding interactions [16]. The same structural characteristics were later reported for EPSPS from *S. pneumoniae* [17] and *Agrobacterium sp. CP4* [18]. In addition, the crystal structures of EPSPS from *V. cholerae* and *M. tuberculosis* were deposited in the protein data bank (**PDB: 3nvs, 2o0d**). Notably, EPSPS shares with MurA the distinctive protein fold and the large conformational changes that occur upon substrate binding and catalysis [16,19,20].

Discovery and engineering of glyphosate-resistant EPSPS

The extraordinary success of glyphosate is due in large part to the high specificity of this simple small molecule towards EPSPS. No other enzyme, including MurA, has been reported to be inhibited by glyphosate to a considerable extent. Therefore, glyphosate cannot be regarded a mere analog of PEP, but it rather appears to mimic an intermediate state of PEP, presumably that of the elusive carbocation. More than a thousand analogs of glyphosate have been produced and tested for inhibition of EPSPS, but minor alterations in chemical structure typically resulted in dramatically reduced potency, and no compound superior to glyphosate was identified [21]. Beginning in the early 1980s, researchers sought to identify glyphosate-insensitive EPSP synthases that could be introduced into crops to provide herbicide resistance. A number of promising enzymes were identified by selective evolution, site-directed mutagenesis, and microbial screens [21,22]. However, as suggested by the fact that glyphosate and PEP bind to the same site, an increased tolerance for glyphosate is often accompanied by a concomitant decrease in the enzyme's affinity for PEP, resulting in a substantial fitness cost, particularly in the absence of multiple (compensatory) mutations. EPSPS from different organisms have been divided into two classes according to intrinsic glyphosate sensitivity. Class I enzymes, found in all plants and in many Gram-negative bacteria such as *E. coli* and *Salmonella typhimurium*, are inhibited at low micromolar glyphosate concentrations. Eventually, naturally occurring glyphosate-tolerant microbes were identified, including *Agrobacterium sp.* strain CP4, *Achromobacter sp.* strain LBAA, and *Pseudomonas sp.* strain PG2982 [23]. The enzymes isolated from these bacteria were designated as class II EPSP synthases on the basis of their catalytic efficiency in the presence of high glyphosate concentrations and their substantial sequence variation compared to EPSP synthases from plants or *E. coli* [24]. Other class II EPSP synthases have since been discovered, typically from Gram-positive bacteria including *Streptococcus pneumoniae* [25] and *Staphylococcus aureus* [26] to name a few.

The first single-site mutations reported to confer resistance to glyphosate were P101S in EPSPS from *Salmonella typhimurium* [27] and G96A in the enzyme from *K. pneumoniae* [28]. The G96A variant enzyme from *E. coli* is highly resistant to glyphosate due to the methyl group protruding into the glyphosate-binding site [29]; however, this comes at the expense of a drastically lowered affinity for PEP and poor catalytic efficiency. In contrast to G96, P101 is not an active site residue but is located approximately 9 Å distant from glyphosate as part of a helix (residues 97 to 105) of the N-terminal globular domain (Fig. 2C). Substitutions of P101 result in long-range structural changes of the active site by impacting the spatial orientation of G96 and T97 towards glyphosate [30]. Because these alterations are slight, P101 substitutions confer relatively low glyphosate tolerance while maintaining high catalytic efficiency, and therefore incur less fitness cost than mutations of active site residues. Notably, field-evolved plants exhibiting target-site glyphosate tolerance invariably contain single-residue substitutions at the site corresponding to Pro101 of *E. coli* EPSPS [31–35].

Multi-site mutations with more favorable properties were discovered for *P. hybrida* EPSPS G101A/G137D and G101A/P158S [36], *E. coli* G96A/A183T [37,38] and *Z. mays* T102I/P106S [37,39,40]. The T102I/P106S double mutant (corresponding to T97I/P101S in *E. coli*), abbreviated as TIPS EPSPS, had particularly favorable characteristics and was used to produce the first commercial varieties of glyphosate-resistant maize (field corn, GA21 event). The TIPS enzyme from *E. coli* is the only class I enzyme to date that is essentially insensitive to glyphosate (K_i > 2 mM) but maintains high affinity for PEP. The crystal structure of the TIPS enzyme revealed that the dual mutation causes G96 to shift towards glyphosate while the side chain of I97 points away from the substrate binding site thereby facilitating PEP utilization [41]. Remarkably, the single site T97I variant enzyme confers less resistance to glyphosate, and in the absence of the compensating P101S mutation, exhibits drastically decreased affinity for PEP. It appears that only the simultaneous mutation of T97 and P101 provides the conformational changes necessary for high catalytic efficiency and resistance to glyphosate.

The *Agrobacterium sp.* strain CP4, isolated from a waste-fed column at a glyphosate production facility, yielded a glyphosate-resistant, kinetically efficient EPSPS (so-called CP4 EPSPS) suitable for the production of transgenic, glyphosate-tolerant crops (Roundup Ready, NK603 corn event) [24]. The CP4 enzyme has unexpected kinetic and structural properties that render it unique amongst the known EPSP synthases and it is therefore considered the prototypic class II EPSPS [18]. An intriguing feature is the strong dependence of the catalytic activity on monovalent cations, namely K^+ and NH_4^+. The lack of inhibitory potential (K_i > 6 mM) is primarily attributed to residues A100 and L105 in place of the conserved *E. coli* and plant residues G96 and P101 (Fig. 2D). The presence of A100 in CP4 is of no consequence to the binding of PEP, but glyphosate can only bind in a condensed, high-energy and non-inhibitory conformation. Glyphosate sensitivity is partly restored by mutating A100 to glycine allowing glyphosate to bind in its extended, inhibitory conformation.

Detoxification of glyphosate

Detoxification of the glyphosate molecule is another strategy that has been employed to confer glyphosate resistance. Soil microorganisms can metabolizes glyphosate by two different routes (Fig. 3A): i) cleavage of the carbon-phosphorus bond, resulting in the formation of phosphate and sarcosine (the C-P lyase pathway) e.g., by *Pseudomonas* sp. PG2982; ii) oxidative cleavage of the C-N bond on the carboxyl side catalyzed by glyphosate oxidoreductase (GOX), resulting in the formation of aminomethylphosphonic acid (AMPA) and glyoxylate (the AMPA pathway). Neither of these mechanisms has been

shown to occur in higher plants to a significant degree. The C-P lyase pathway requires an unknown number of genes and the activity has not been reconstituted *in vitro*, casting doubt on the ability to create the activity in transgenic plants. The AMPA pathway appears to be the predominant route for degradation of glyphosate in soil by a number of Gram-positive and Gram-negative bacteria. Most recently, a glycine oxidase from *B. subtilis* was also demonstrated to metabolize glyphosate into AMPA and glyoxylate, but using a reaction mechanism different from GOX.

a. Oxidases

Glyphosate oxidoreductase (Monsanto)—Early on, Monsanto Co. isolated glyphosate-AMPA bacteria from a glyphosate waste stream treatment facility. The *Achromobacter* sp. LBAA was thus identified for its ability to use glyphosate as a phosphorous source [42]. By employing the ability of certain *E. coli* strains (Mpu^+, methylphosphonate utilizing) to utilize AMPA or other phosphonates as phosphorus sources through the activity of C-P lyase, a cosmid library of LBAA genomic DNA was screened for its ability to confer tolerance to glyphosate. An open reading frame (EMBL-Bank: GU214711.1) of 1690 bp was isolated that encodes glyphosate oxidoreductase, an FAD containing flavoprotein of 430 amino acids. GOX was over-expressed in *E. coli*, where activity in cell lysates reached 7.15 nmol/min mg protein [42]. Using oxygen as co-substrate, the recombinant enzyme catalyzes the cleavage of the C-N bond of glyphosate, yielding AMPA and glyoxylate without production of hydrogen peroxide (Fig. 3A). The authors proposed a mechanism that involves the reduction of FAD cofactor by the first molecule of glyphosate, yielding reduced FAD and a Schiff base of AMPA with glyoxylate that then hydrolyzes into the single components [42]. The reduced flavin is reoxidized by dioxygen yielding an oxygenated flavin intermediate. This intermediate catalyzes the oxygenation of a second molecule of glyphosate yielding AMPA and glyoxylate, again without hydrogen peroxide production. The activity (and kinetic efficiency) of wild-type GOX with glyphosate as substrate is quite low, mainly because of a high $K_{m,app}$ for the herbicide (27 mM, see Table 1).

Chemical mutagenesis and error-prone PCR were used to insert genetic variability in the sequence coding for GOX and enzyme variants were selected for their ability to grow at glyphosate concentrations which inhibit growth of the *E. coli* Mpu^+ control strain. As shown in Table 1, a substantially higher kinetic efficiency (the $V_{max,app}/K_{m,app}$ ratio) for glyphosate occurs because of a significantly lower $K_{m,app}$ [42]. Worthy of note, the best variants have a more basic residue at position 334. To facilitate the expression of GOX in plants, the gene sequence was re-designed to eliminate stretches of G and C of 5 or greater, A+T rich regions that could function as polyadenylation sites or potential RNA destabilizing regions, and codons not frequently found in plant genes. When this gene was modified and transformed into tobacco plants, expression of GOX resulted in glyphosate tolerance.

Evolved glycine oxidase—The flavoenzyme glycine oxidase (**EC 1.4.3.19**, GO) is a member of the oxidase class of flavoproteins that was discovered in 1997 following the complete sequencing of the *Bacillus subtilis* genome [43]. GO is a homotetrameric flavoenzyme that contains one molecule of non-covalently bound flavin adenine dinucleotide per 47 kDa protein monomer. GO catalyzes the O_2-dependent oxidative deamination of primary and secondary amines (sarcosine, *N*-ethylglycine, glycine) and D-amino acids (D-alanine, D-proline) yielding the corresponding α-keto acid, ammonia or primary amine and hydrogen peroxide [44–46]. This reaction resembles that of the prototypical flavooxidase D-amino acid oxidase [47]. In *B. subtilis* GO is involved in biosynthesis of the thiazole moiety of thiamin pyrophosphate (vitamin B1). This reaction requires the direct transfer of the imine product to the next enzyme in the pathway to avoid

non-productive hydrolysis, which would occur if it dissociates from the enzyme. Noteworthy, GO can be efficiently expressed as active and stable recombinant protein in *E. coli* at up to ≈ 4% of the total soluble protein content of the cell [48].

Wild-type GO shows broad substrate specificity [44,45,48] and also oxidizes the herbicide glyphosate, which can be viewed as a derivative of glycine. GO catalyzes the deaminative oxidation of glyphosate yielding glyoxylate, AMPA, and hydrogen peroxide, using 1 mol of dioxygen per 1 mol of herbicide (Fig. 3B). The efficient oxidation of glyphosate by wild-type GO is prevented by the low affinity for the herbicide ($K_{m,app}$ = 87 mM, a value 125-fold higher than for the physiological substrate glycine, see Table 2). An *in silico* docking analysis of glyphosate binding at the GO active site showed that the herbicide is bound in the same orientation inferred for glycine (with the phosphonate moiety pointing toward the entrance of the active site) and allowed identification of 11 positions of the active site potentially involved in glyphosate binding [49]. Site saturation mutagenesis at these positions and a simple screening procedure with glycine and glyphosate as substrates was used to identify single-point variants of GO with improved activity on glyphosate and decreased activity on glycine. The ratio of apparent specificity constants for glyphosate to glycine ($k_{cat}/K_{m\ glyph}$ / $k_{cat}/K_{m\ glycine}$) increased from 0.01 for wild-type GO up to 40 for G51R variant (Table 2). In the final stage, the information gathered from the first site saturation mutagenesis approach was combined by performing site saturation at position 51 on the A54R GO mutant, then introducing the A244H substitution into the G51S/A54R mutant by site-directed mutagenesis [49]. The G51S/A54R/H244A GO possesses a 200-fold increased kinetic efficiency (k_{cat}/K_m) with glyphosate and up to a 15,000-fold increase in the ratio $k_{cat}/K_{m\ glyph}$ / $k_{cat}/K_{m\ glycine}$ over that for the wild-type enzyme, mainly resulting from a 175-fold decrease in $K_{m,app}$ for glyphosate and a 150-fold increase in the same kinetic parameter for glycine (Table 2).

As made apparent by the resolution of the crystal structure of the evolved G51S/A54R/H244A variant in complex with glycolate, the substitutions introduced in GO appear to modify its substrate preference in different ways [49]. 1) The newly introduced arginines at the active site entrance (positions 51 and 54) favour the interaction with glyphosate and thus decrease the $K_{m,app}$ value up to 20-fold in the G51R/A54R variant. However, one or both of these substitutions negatively affects protein stability, as the G51R/A54R double variant shows a drastically lower stability than the wild-type GO (Table 2), see below. 2) Introduction of the bulky side chain of arginine at position 54, which appears to locate close to the phosphonate group of glyphosate and to electrostatically interact with it, allows tighter binding of the herbicide and optimal positioning for catalysis (Fig. 4). The dramatic decrease in kinetic efficiency with glycine observed for the best GO variants is largely due to a decrease in binding energy for this small-size substrate. Because of the introduction of an arginine at position 54, the α2–α3 loop (comprising residues 50–60) assumes a different conformation in the G51S/A54R/H244A variant as compared with wild-type GO (Fig. 4). 3) The presence of a smaller alanine residue at position 244 eliminates steric clashes with the side chain of Glu55, thus facilitating the interaction between Arg54 and glyphosate in the GO variant (Fig. 4).

Comparison between evolved GOX and GO—The observation that the same main products (*i.e.*, AMPA and glyoxylate) are produced by glyphosate oxidation using GO and GOX (Fig. 3A and B) might suggest a close similarity between these two FAD-containing flavoenzymes, but such is not the case. First, the two enzymes show a low sequence identity (18.1%); a Blast sequence analysis identifies D-amino acid dehydrogenases as the most related proteins for GOX [49]. Second, the reaction catalyzed by GO differs from that of GOX because with the latter enzyme two molecules of glyphosate are oxidized per molecule of oxygen and no hydrogen peroxide is produced [42,50]. Furthermore, the mechanism

proposed for GOX (i.e., the reduced flavin obtained by oxidation of the first molecule of glyphosate catalyzes the oxygenation of a second molecule of glyphosate) [42] profoundly differs from the hydride transfer mechanism proposed for GO [51,52]. A further main difference is related to the kinetic properties of the two oxidases on glyphosate: the G51S/A54R/H244A GO shows a 5-fold lower K_m for glyphosate and 10-fold higher kinetic efficiency than that of the best variant obtained for GOX (2.1 *vs.* 0.3 mM^{-1} s^{-1}, respectively). The low level of activity and heterologous expression observed for GOX might explain the limitations encountered to develop commercially available crops based on this enzyme. Noteworthy, the triple GO variant was recently expressed in *Medicago sativa*, which acquired resistance to the herbicide (D. Rosellini, unpublished results).

b. Glyphosate acetyltransferase

Another mechanism for detoxification of glyphosate was suggested by Nature, in its handling of phosphinothricin. Organisms that produce this cytotoxic inhibitor of glutamine synthetase have acetyltransferases that derivatize the molecule to a non-inhibitory acetylated form (Fig. 5) [53]. The paradigm set by Nature with phosphinothricin held true with glyphosate in that N-acetylglyphosate is not herbicidal and does not inhibit EPSP synthase [54]. A sensitive mass spectrometric screen to detect production of N-acetylglyphosate in a collection of environmental microbes yielded three alleles of closely related glyphosate acetyltransferase (GLYAT) enzymes from separate isolates of *Bacillus licheniformis* [54]. Shuffling these genes with additional diversity from related sequences resulted in many variants of GLYAT having catalytic proficiency appropriate for commercial levels of tolerance to glyphosate in crop plants [54, 55]. The first traits, in which GLYAT is deployed in soybean and canola, are in advanced stages of development (Pioneer Hi-Bred Technical Update).

The physiological substrate for native GLYAT is unknown but the enzyme acetylates D-2-amino-3-phosphonopropionic acid (D-AP3) with the highest efficiency among all compounds tested [55]. Glyphosate and D-AP3 have the same chemical composition and key recognition groups, but D-AP3 is a branched primary amine while glyphosate is a secondary amine with a linear structure and longer length (Fig. 5). Eleven iterative rounds of gene shuffling resulted in a large shift in the ratio of the specificity constants for glyphosate and D-AP3 ($k_{cat}/K_{m\ glyph}$ / $k_{cat}/K_{m\ D\text{-}AP3}$). For specific wild-type, 7^{th} round and 11^{th} round GLYAT variants the values are 0.00272, 39.4 and 55.7, respectively, representing 14,500- and 20,500-fold increases [54,55]. The specificity shift was driven purely by screening for improved $k_{cat}/K_{m\ glyph}$ without reference to a structural model. The three native proteins failed to produce crystals suitable for structure determination. However, among eight shuffled variants subjected to the same panel of conditions, two crystallized readily, and a structure was solved for one of those (**PDB: 2jdd**) [56]. Among the eleven variants in the experiment, 75% of the 50 positions containing amino acid diversity were at the surface, where they can affect crystal packing, including 50% of those present at the protein interfaces. Thus, shuffling efficiently sampled those positions that affect crystal packing and enabled discovery of several successful combinations.

Structure and mechanism of GLYAT—The **PDB 2jdd** structure of R7 GLYAT is a ternary complex with acetyl coenzyme A (AcCoA) and 3-phosphoglycerate (3PG), an inhibitor competitive with glyphosate [55] (Fig. 6). The overall fold with its signature V-shaped wedge formed by the splaying β4 and β5 strands identifies GLYAT as a member of the GCN5-related *N*-acetyltransferase (GNAT) superfamily [57]. The interactions between cofactor and GLYAT are similar to those observed throughout this latter superfamily [58], with the adenosine group of AcCoA being largely solvent-exposed, and the pantetheine moiety forming a pseudo-β sheet by inserting between the splaying β4 and β5 strands. 3-PG

(replaced with modeled glyphosate in Fig. 6) sits on a platform defined by the pseudo-β sheet, covered by two loops that join at their tips; loop20 connecting helices α1 and α2 and loop130, spanning strands β6 and β7. Eight amino acids interact directly (< 4 Å) with 3PG: the majority of contacts are made between charged groups, including side chain interactions with the phosphate end (Arg21, Arg111, and His138) and with the carboxylate end (Arg21 and Arg73) of 3PG. Of particular note is a short, 2.46-Å hydrogen bond between the N-ε of His138 and a phosphate oxygen of 3PG.

Alanine substitutions at selected positions allowed catalytic roles of several amino acids to be assigned (Table 3). His138, each of the three arginines, and Tyr118 all play significant roles in binding and/or catalysis. The 110-fold reduction in k_{cat} observed with the H138A mutant is consistent with the loss of a catalytic base, while the 17-fold drop in k_{cat} in the Y118F mutant implicates Tyr118 as a catalytic acid. The proposed reaction, based on a substrate-assigned proton transfer mechanism, and roles of particular amino acids are diagrammed in Figure 7.

Effect of optimization for glyphosate—The structures of D-AP3 and glyphosate suggest that effecting a shift in substrate specificity toward glyphosate may have required that loop20 and loop130, which embrace the substrate in the active site, be enabled to move further apart to allow access of the longer glyphosate. The K_i values with glyphosate as substrate obtained for a series of inhibitors of varying chain length support that idea by demonstrating that: 1) wild-type GLYAT accommodates shorter ligands (with 3 and 4 atoms in the main chain) more readily than longer ones, and 2) progressive optimization for glyphosate activity is accompanied by improved binding to longer ligands (up to 5 atoms in the main chain) and retained binding to shorter ligands [55].

Of the 21 changes in the evolution of R7 from native GLYAT (Fig. 6), none affect the residues that ligate 3PG or are implicated in catalysis. Only four changes (Y31F, V114A, I132T and I135V) occurred in residues within the perimeter of the active site; positions 31, 132 and 135 belong to loop20 or loop130. Of note is that all four substitutions in the active site reduce the size of the side chain, directly increasing the volume of the active site, and enhance the flexibility of loops 20 and 130, allowing them to open wider to accommodate longer ligands. When those four changes are individually changed back to the native amino acid, there was no negative impact on k_{cat}, and mostly minor impacts on K_m (Table 3). However, when all four R7 substitutions in the active site were reverted to the native amino acid, k_{cat} was reduced 30-fold and K_m was returned to the range of native GLYAT.

The quadruple revertant R7 variant had catalytic efficiency (k_{cat}/K_m) 5-fold greater than wild-type GLYAT, suggesting that mutations outside of the active site in some way created a context that is more favourable for activity with glyphosate. The remaining 17 substitutions are distributed throughout the sequence. The ten mutations at the surface are all hydrophilic substitutions that increased net positive charge by seven, and enabled protein-protein interactions favourable for crystal formation. Of the overall 11 interior mutations, four were isomer switches between leucine and isoleucine and the remaining seven were to amino acids of smaller size (Y31F, T33S, T89S, V114A, Y130F, I132T, and I135V). Those interior downsizing mutations may reduce the protein's overall packing strength, creating the flexibility to allow loops20 and 130 to open wider (Z. Hou, personal communication).

Conclusions

We have described three methods by which enzymes that endow glyphosate resistance have been discovered: 1) discovery within existing natural diversity, 2) rational modification of an existing enzyme as guided by a structural model, and 3) modification of an existing

enzyme by gene shuffling and selection. While each approach has its advantages, the choice of which to employ will largely depend on the available starting enzyme and the extent of existing structural and mechanistic characterization of it or its close homologues.

Following the advent of glyphosate-resistance crops mainly based on EPSPS insensitive to the herbicide, there are increasing instances of evolved glyphosate resistance in weed species [2,59]. In several cases, moderate resistance is imparted by mutations to the target enzyme (target-site mechanism of resistance) [60], but there is yet no documented case of a plant species having native or evolved tolerance to glyphosate by virtue of a metabolic enzyme. Instead, the most common resistance mechanism emerging in weed populations is reduced translocation of the herbicide from the sprayed leaf to the growing points of the plant, the root and apical meristems, i.e. non-target-site mechanisms might be the major cause for most glyphosate resistant biotypes. In the case of *Conyza canadensis*, glyphosate accumulates in vacuoles of resistant plants at a markedly faster rate than in sensitive plants [61]. Analysis of the transcriptome of resistant and sensitive lines revealed up-regulation of genes for tonoplast intrinsic proteins and ABC transporters, with the implication that the resistant lines acquired an increased capacity for sequestering glyphosate in the vacuole of the treated leaf, thereby reducing the amount translocated to meristems [62].

In order to preserve the utility of this valuable herbicide, growers must be equipped with effective and economical herbicide-trait combinations to use in rotation or in combination with glyphosate. In theory, the same methods described here can be applied to generate resistance traits for any target herbicide. In practice, a starting point, meaning an existing enzyme with detectable activity, may not be available. Fortunately, methods of computational enzyme design are advancing to the point that *de novo* design of an enzyme with a particular and novel catalytic function is a reasonable expectation [63]. As an example, computational design of an enzyme that catalyzes a Kemp elimination resulted in a variant with a k_{cat}/K_m of 1.4 $min^{-1}mM^{-1}$ [64], the same order of magnitude as native GLYAT for glyphosate. Gene shuffling improved the designed enzyme by 200- to 400-fold [65], illustrating the advantage of combining tools for enzyme optimization. With the increasing demand for food and biofuel, all available technologies should be explored to identify feasible options for delivery of genes conferring traits of novel value or efficacy.

Abbreviations

AcCoA	acetyl coenzyme A
AMPA	aminomethylphosphonic acid
EPSP	5-enolpyruvylshikimate-3-phosphate
EPSPS	enolpyruvylshikimate-3-phosphate synthase (**EC 2.5.1.19**)
GO	glycine oxidase (**EC 1.4.3.19)**
GOX	glyphosate oxidoreductase
PEP	phospoenolpyruvate
S3P	shikimic acid-3-phosphate
SSC	substrate specificity constant
GLYAT	glyphosate acetyltransferase
D-AP3	D-2-amino-3-phosphonopropionic acid
3PG	3-phosphoglycerate

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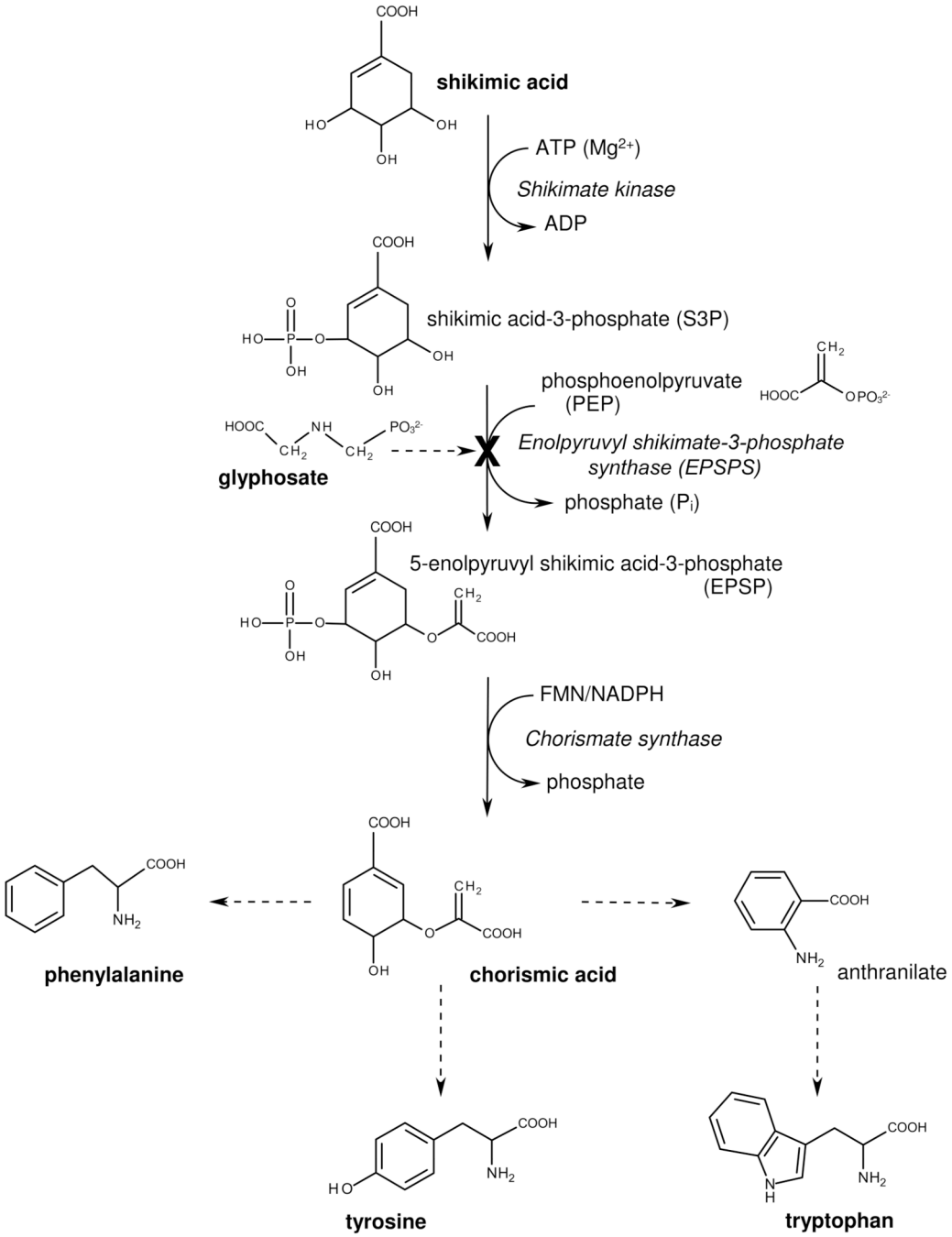

Fig. 1.

Shikimate pathway that leads to the biosynthesis of aromatic amino acids and mode of action of glyphosate on the reaction catalyzed by EPSPS.

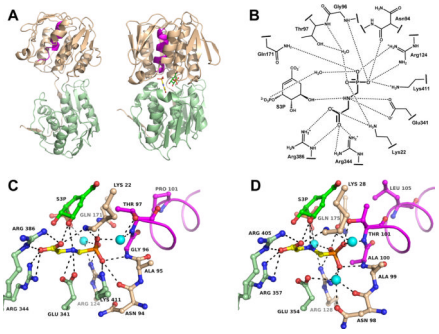

Fig. 2.

Molecular mode of action of glyphosate and the structural basis for glyphosate resistance. A) In its ligand-free state, EPSPS exists in the open conformation (left; **PDB: 1eps**). Binding of S3P induces large conformational in the enzyme to the closed state to which glyphosate or substrate PEP bind (**PDB: 1g6s**). Shown are the respective crystal structures of the *E. coli* enzyme, with the N-terminal globular domain colored in palegreen and the C-terminal domain colored in wheat. The helix containing P101 is indicated in magenta and the S3P and glyphosate molecules in green and yellow, respectively. B) Schematic representation of potential hydrogen bonding and electrostatic interactions between glyphosate and active site residues including bridging water molecules in EPSPS from *E. coli* (**PDB: 1g6s**). C) The glyphosate binding site in EPSPS from *E. coli* (**PDB: 1g6s**). Water molecules are shown as cyan spheres and the residues known to confer glyphosate resistance upon mutation are indicated in magenta. D) The glyphosate binding site in CP4 EPSPS (**PDB: 2gga**). The spatial arrangement of the highly conserved active site residues is almost identical for class I (*E. coli*) and class II (CP4) enzymes, with the exception of an alanine residue in position 100 (G96 in *E. coli*). Another significant difference is the replacement of P101 (*E. coli*) by a leucine (L105) in the CP4 enzyme. Note the markedly different, condensed conformation of glyphosate as a result of the reduced space provided for binding in the CP4 enzyme.

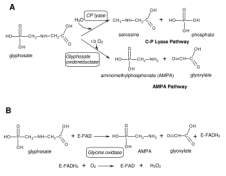

Fig. 3.

Microbial mechanisms of glyphosate degradation. A) Two principal pathways of glyphosate degradation are known: top) cleavage of the carbon-phosphorus bond yielding phosphate and sarcosine (the C-P lyase pathway); bottom) cleavage to yield the formation of aminomethylphosphonic acid (AMPA) and glyoxylate (the AMPA pathway), referred to as the glyphosate oxidase (GOX) pathway. B) Reaction catalyzed by GO on glyphosate [49], an alternative of the AMPA pathway as catalyzed by GOX (panel B, bottom).

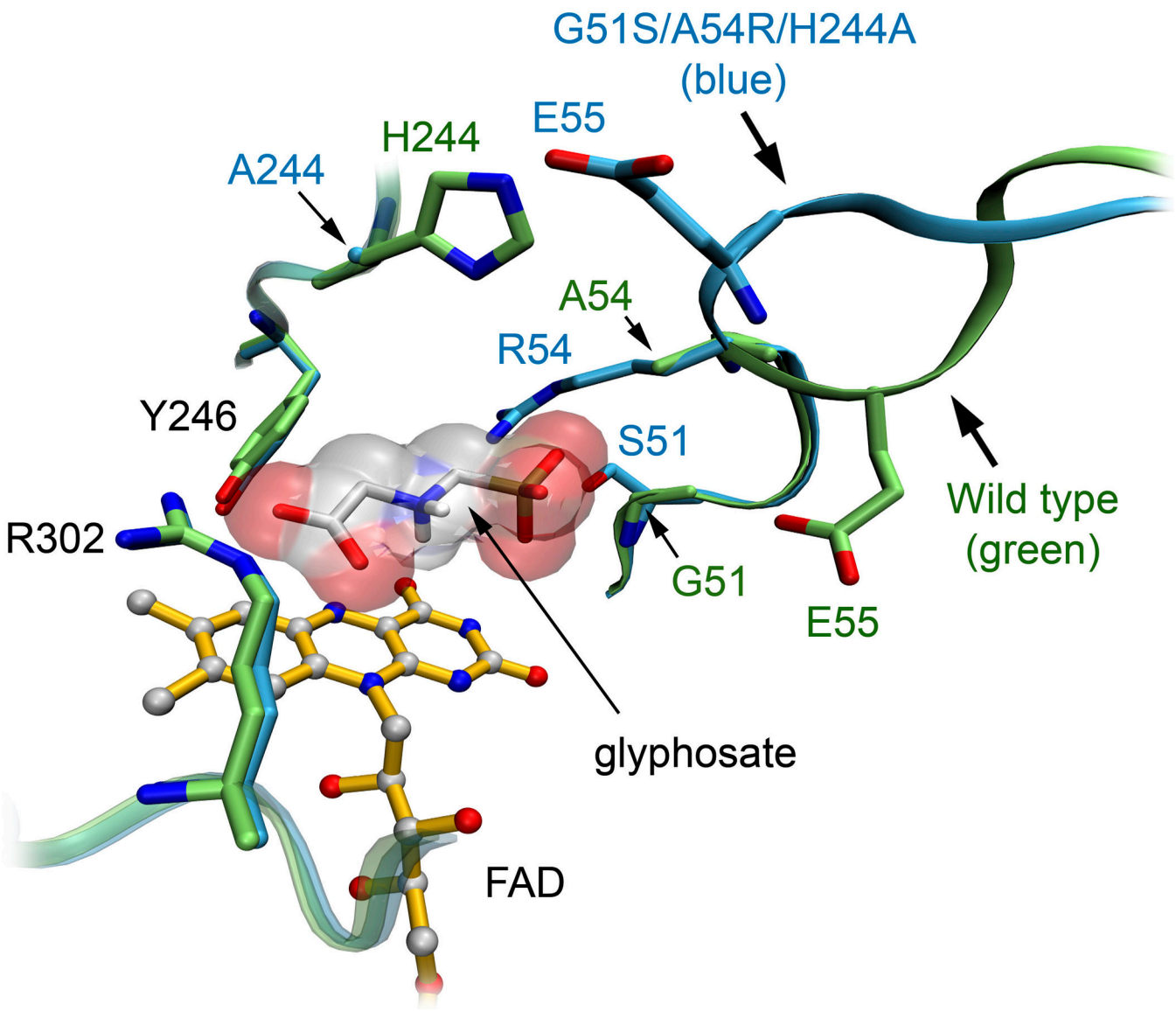

Fig. 4.

The superposition of wild-type (**PDB: 1rhl**, green) and G51S/A54R/H244A GO (**PDB: 3if9**, blue) structures shows the different conformation of the main chain of α2–α3 loop, see arrows [49]. For the sake of clarity, only the FAD and the ligand belonging to the wild-type GO structure are shown, and Arg329 was omitted.

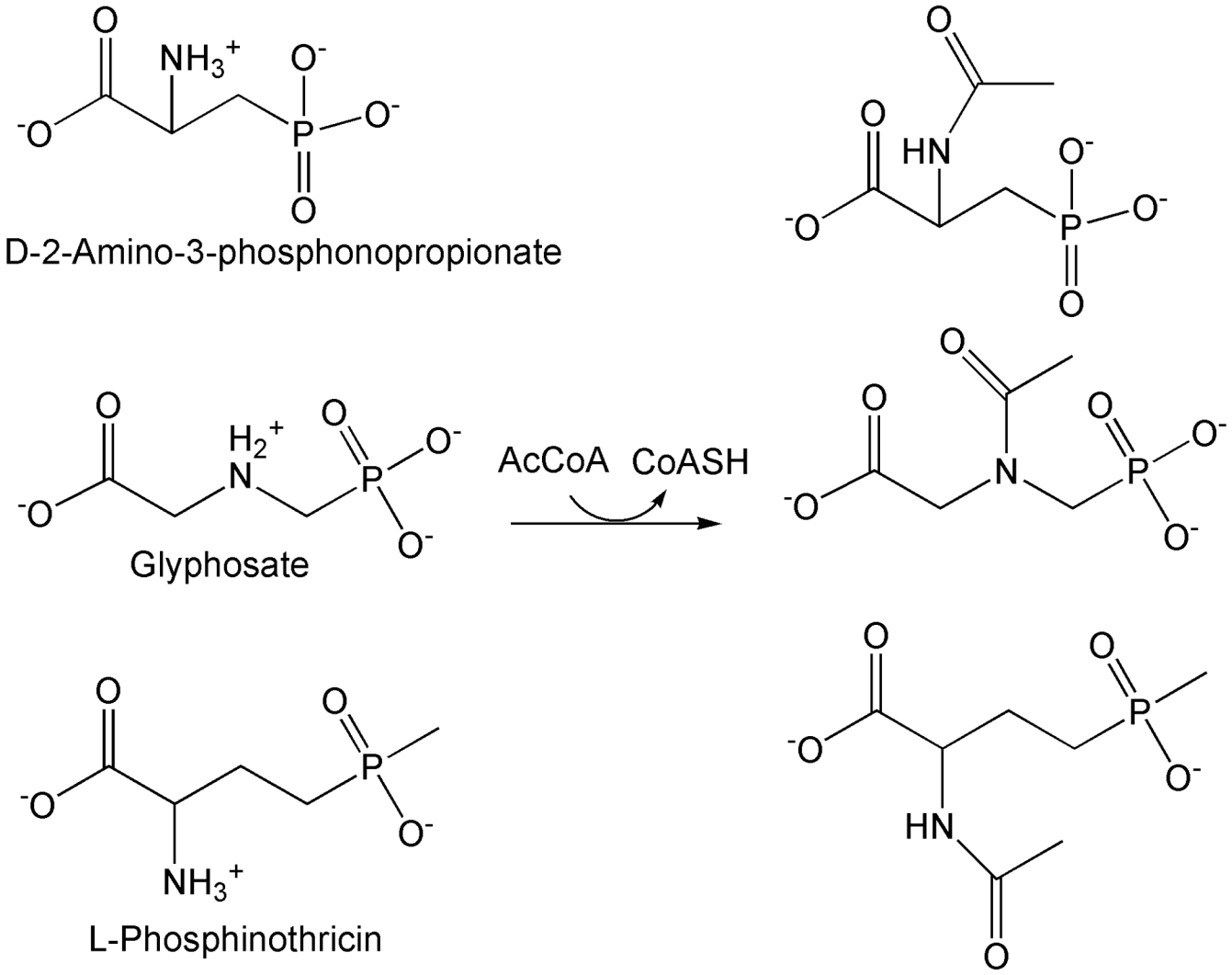

Fig. 5.
Substrates of acetyltransferase reactions mentioned in the text [53,55].

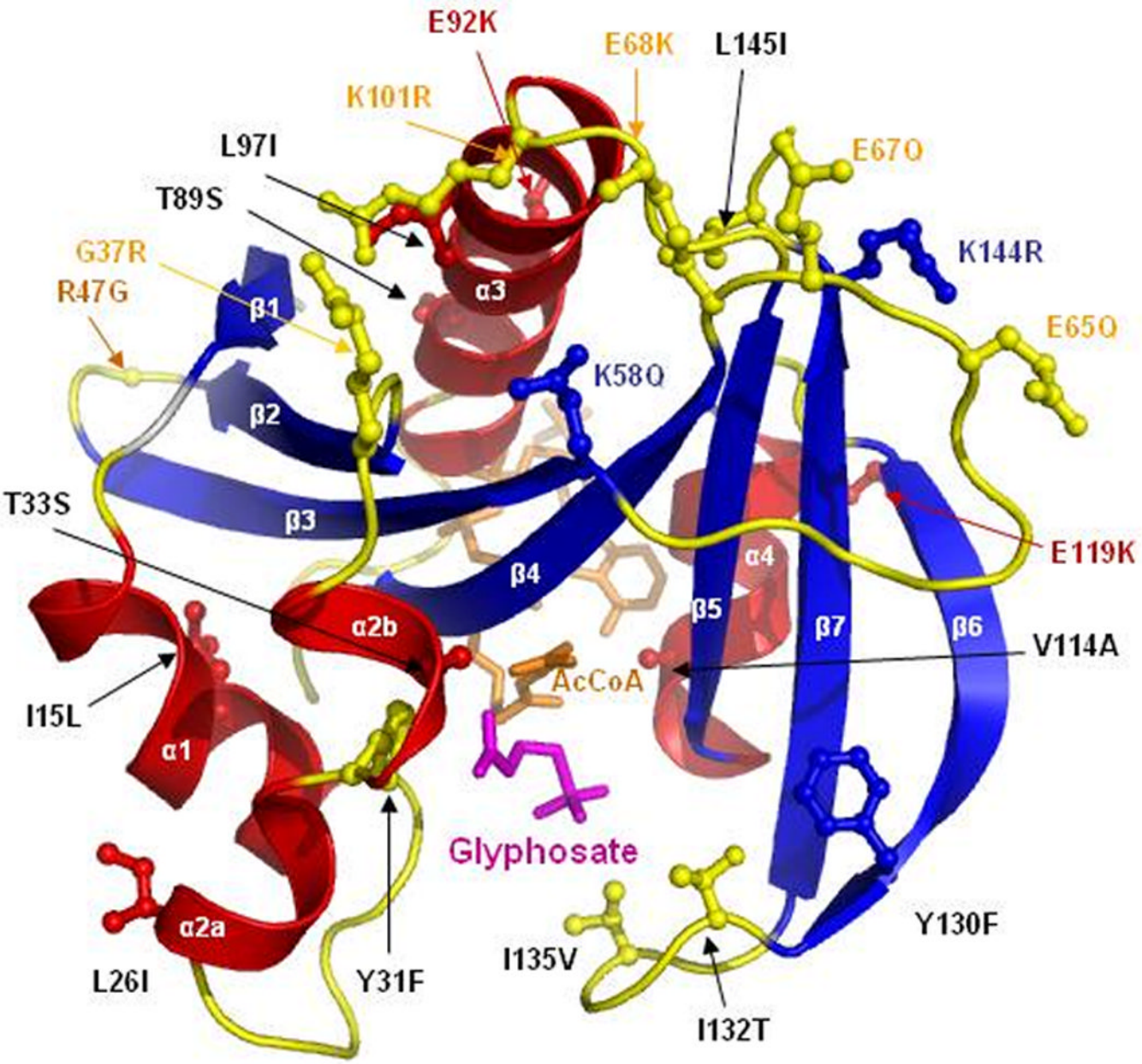

Fig. 6.

R7 GLYAT ligated with glyphosate and acetyl coenzyme A (Z. Hou, Pioneer Hi-Bred, unpublished, based on **PDB: 2jdd**). The altered residues (R7 vs. native) and ligands are shown with ball-and-sticks.

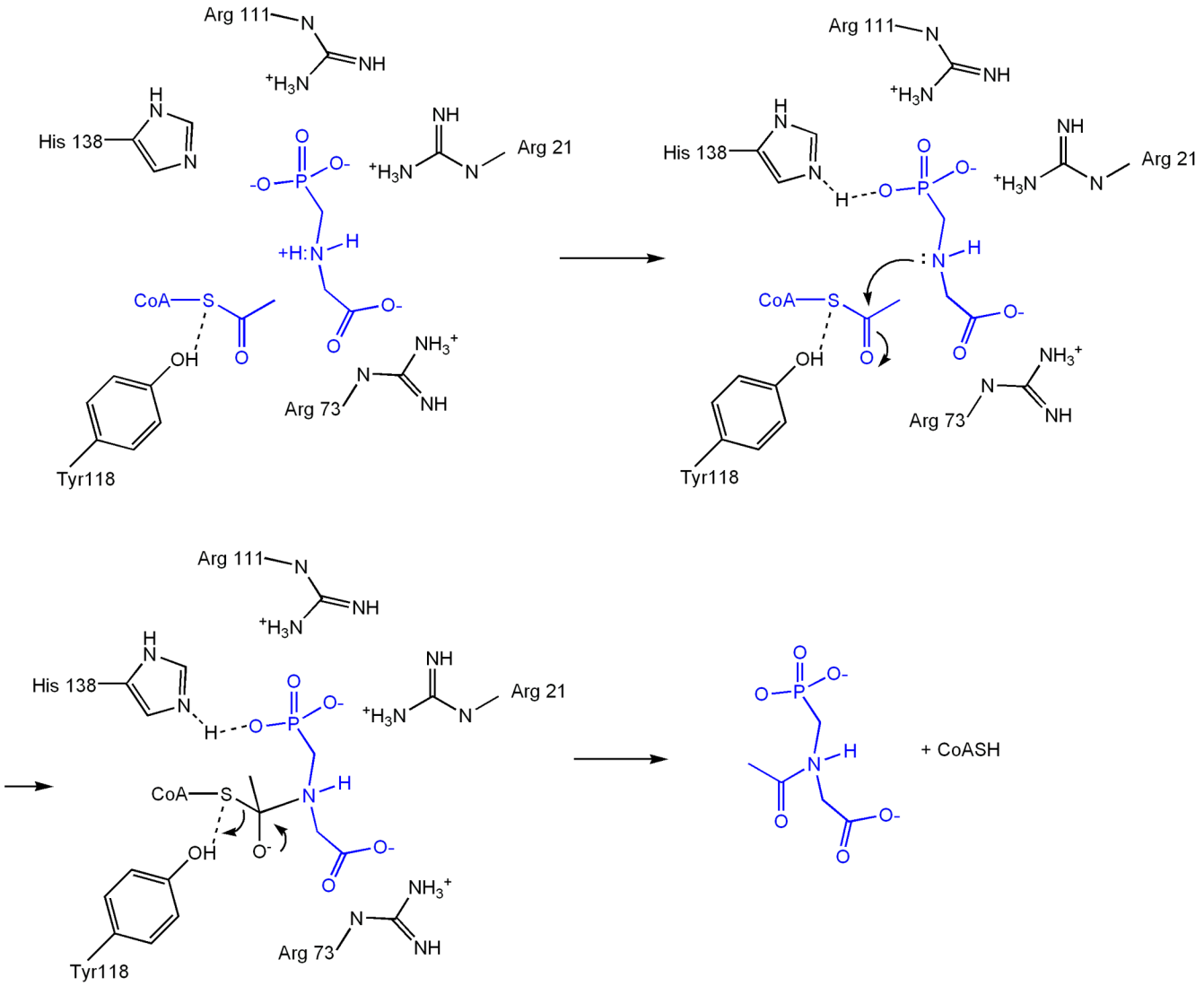

Fig. 7.

GLYAT reaction mechanism [55]. Glyphosate, whose nitrogen pK is 10.3, enters the active site as the protonated form and binds with its phosphonate group ligated by charge interactions with Arg21 and Arg111, and its carboxyl group in contact with Arg73. The shortness of the hydrogen bond between the N-ε of His138 and a phosphonate oxygen of glyphosate suggests a specific mechanism in which a proton from the secondary amino group of glyphosate is stabilized on a phosphonate oxygen atom, resulting in formation of the strong hydrogen bond between His-138 and glyphosate and activation of the substrate amine. This substrate-assisted proton transfer mechanism is consistent with the observed pH dependence of k_{cat} and explains the dual role of His138 in substrate binding and as a catalytic base. To complete the reaction, attack by the lone pair of the glyphosate nitrogen on the carbonyl carbon of AcCoA results in a tetrahedral intermediate. Tyr118 is perfectly positioned to protonate the sulfur atom of coenzyme A as the tetrahedral intermediate breaks down to yield the products. This research was originally published in [55].

Table 1
Evolution of a GOX variant active on glyphosate

Comparison of the apparent kinetic parameters with glyphosate determined for wild-type GOX and variants obtained by random mutagenesis [42].

	$V_{max,app}$ [a]	$K_{m,app}$	$V_{max,app} / K_{m,app}$
	(U/mg protein)	(mM)	
Wild–type	0.8	27.0	0.03
S84G/K153R/H334R	0.6	2.6	0.23
H334R	0.6	2.6	0.23
H334K	0.7	9.9	0.07
H334N	0.6	19.6	0.03

[a] One unit corresponds to the conversion of 1 εmol of glyphosate per minute, at 30 °C.

Table 2

Evolution of a GO variant active on glyphosate

Comparison of the apparent kinetics parameters on glycine and glyphosate, thermostability and protein expression in *E. coli* determined for wild-type and variants of GO obtained by site-saturation mutagenesis of the positions identified by docking analysis or by introducing multiple mutations [49].

	Glycine		Glyphosate		SSC[a]	Melting temperature[b]	Expression yield
	$k_{cat,app}$ (s^{-1})	$K_{m,app}$ (mM)	$k_{cat,app}$ (s^{-1})	$K_{m,app}$ (mM)		(°C)	(mg /L culture)
Wild–type	0.60 ± 0.03	0.7 ± 0.1	0.91 ± 0.04	87 ± 5	0.01	57.8	13.7
Single-point variants:							
H244A	0.63 ± 0.06	1.5 ± 0.3	0.77 ± 0.03	78 ± 4	0.02	55.0	21.0
A54R	1.2 ± 0.1	28 ± 3	1.50 ± 0.02	4.4 ± 0.3	8.5	45.7	7.0
G51R	0.35 ± 0.02	53 ± 8	1.8 ± 0.1	6.5 ± 0.7	40	42.1	7.2
Multiple-point variants:							
G51R/A54R	0.70 ± 0.03	59 ± 4	0.70 ± 0.03	1.0 ± 0.1	58	34.9	7.7
G51S/A54R	0.91 ± 0.02	35 ± 1	1.05 ± 0.05	1.3 ± 0.1	31	46.1	8.5
G51S/A54R/H244A	1.5 ± 0.1	105 ± 11	1.05 ± 0.05	0.5 ± 0.03	150	45.8	14.0

[a] Substrate specificity constant (SSC) is calculated as the ratio of the apparent kinetic efficiency ($k_{cat,app}/K_{m,app}$) for glyphosate to that for glycine.

[b] Melting temperatures were determined by following protein and fluorescence changes during temperature ramp experiments.

Table 3
Kinetic parameters of site-directed mutants of R7 GLYAT

	k_{cat}	K_m	k_{cat} / K_m
	(min^{-1})	(mM)	($min^{-1}mM^{-1}$)
Wild–type	5.3 ± 0.1	1.3 ± 0.1	4.1
R7	1040 ± 40	0.24 ± 0.01	4330
Site-directed mutations in R7:			
H138A	9.4 ± 0.3	10.6 ± 0.6	0.9
R111A	40 ± 1	61 ± 3	0.7
R21A	240 ± 10	41 ± 4	5.9
R73A	820 ± 20	41 ± 4	20
Y118F	60 ± 3	5.2 ± 0.1	11.5
Reversions in R7 to native amino acids:			
T132I	1470 ± 30	0.74 ± 0.04	1990
V135I	2100 ± 90	1.5 ± 0.1	1400
F31Y	1080 ± 40	0.38 ± 0.01	2840
A114V	2100 ± 80	3.2 ± 0.2	660
All four	34.1 ± 2.0	1.8 ± 0.1	18.8

Modified from research originally published in [55].