HERBICIDES THAT INHIBIT ACETOLACTATE SYNTHASE

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1 INTRODUCTION

Herbicides are an essential pillar in modern agricultural practice. They are used to selectively reduce the growth of weeds in crops and natural environments. Early weed mitigating chemicals date back to Roman times and include substances as simple as table salt, sulfuric acid and carbon bisulfide. However, these were non-selective, required high application rates and could be toxic not only to the target weeds but to other plants and to animals. The first major advance in the development of tailored herbicides began in the mid 1940s with the development of the first organic growth regulators such as 2,4 dichlorophenoxyacetic acid (2,4-D)[1].

The next major advance came in the 1970s with the discovery of glyphosate[2], marketed as Roundup, a herbicide that is routinely used to eradicate broad leaf weeds and grasses. Due to the low cost to produce, the small quantities that need to be applied to the field, and the development of glyphosate-resistant crops, it has been the dominant herbicide for the past 30 years. As a result of its success, recent efforts in herbicide discovery have been subdued. Glyphosate functions by acting as a transition state inhibitor of 5-enolpyruvylshikimate-3-phosphate (EPSP) synthase, an enzyme in the aromatic amino acid biosynthesis pathway. Even though glyphosate remains as a world leading commercial herbicide, an increase in the number of resistant weeds is a concern[3]. In the 1980s, another revolutionary class of herbicide, the sulfonylureas (SUs), was discovered by Dr. George Levitt of DuPont. Several years later their mode of action was shown to be inhibition of acetolactate synthase (ALS), also known as acetohydroxyacid synthase (AHAS; EC 2.2.1.6)[4]. ALS is the first enzyme in the biosynthetic pathway of the branched chain amino acids (BCAAs) (i.e., leucine, isoleucine and valine), fundamental components of most proteins. Thus, there are striking similarities in the glyphosate and SU classes of herbicide (i.e., both discovered in the 1970s−1980s, both target amino acid biosynthesis pathways, though aromatic versus branched chain, both target pathways that are found in plants and bacteria but not in animals, and in both cases weed resistance is increasing in frequency and distribution). Nonetheless, these two herbicide classes continue to be at the forefront of crop and land protection strategies employed by farmers, graziers and government agencies across the world. Since there are many reviews that track the success and history of glyphosate, it will not be discussed further here. However, recent advances to our knowledge of ALS and its herbicidal inhibitors and updates to the current status of weed resistance due to application of ALS-inhibiting herbicides warrants review at this time.

2 ACETOLACTATE SYNTHASE

ALS is the first enzyme in the BCAA pathway, where it catalyzes the decarboxylation of pyruvate and its condensation with either 2-ketobutyrate or a second molecule of pyruvate to give 2-aceto-2-hydroxybutyrate or 2-acetolactate, respectively (Fig. 1(a)). The reaction products are precursors of the three BCAAs. The complete enzyme has two subunits, a catalytic subunit (CSU) and a regulatory subunit (RSU) and has three cofactors: thiamine diphosphate (ThDP)-Mg$^{2+}$ and flavin adenine dinucleotide (FAD). ThDP participates directly in catalysis by covalently binding to the reaction intermediates. The role of Mg$^{2+}$ is to anchor ThDP to the surface of the
Fig. 1 Chemical reactions for ALS and structures of ALS-inhibiting herbicides. (a) The chemical reactions of ALS. (b) The five different chemical classes of ALS-inhibiting herbicide, imidazolinones (IMIs) in pink, pyrimidinyl benzoates (PBs) in brown, a selection of sulfonylureas (SUs) in black, triazolopyrimidines (TPs) in blue, and the sulfonylamino-carbonyl-triazolinones (SCTs) in purple.
enzyme. The function of FAD is more controversial. Historically, FAD has been suggested to not be directly involved in the mechanism of catalysis. This view is based on the fact that there is no net oxidation or reduction and the fact that there is an equivalent enzyme to ALS in the fermentation pathway which can perform the same reaction as ALS but has no FAD present. However, it has recently been proposed that an alternative mechanism directly involving FAD and $O_2$ could occur[5]. Irrespective of mechanism, FAD needs to be in the reduced form for catalysis to proceed[6]. The structure of the holoenzyme of ALS, consisting of the CSUs and RSUs, was recently solved using cryo-EM and revealed that it has an arrangement of eight catalytic subunits (four dimer pairs) with four RSUs forming a central core[7]. The shape of the complex is similar to a Maltese cross with CSU dimers at the extremities of the cross (Fig. 2(a)). Comparison of this structure and the structure of the CSU in the absence of the RSU shows the enhanced activity of the complex is due to (1) stabilization of the dimer and (2) an interaction between a key CSU catalytic loop (referred to as the Q-loop because of the presence of glutamine at the tip) and the RSU that guides movement of the loop and thus assists catalysis (Fig. 2(b)). A comparison of the structure of the holo-enzyme in the presence and absence of valine shows that this feedback inhibitor decouples the link between the Q-loop and the RSU, thereby returning catalytic activity to a baseline level[7]. This new structural data will be useful in informing future herbicide discovery and design efforts, whether that be to use the same binding site as existing herbicides (see below) or to target alternative functionally important sites (e.g., Q-loop, subunit interfaces and BCAA binding pockets).

3 AHAS-INHIBITING HERBICIDES

Fifty-eight inhibitors of AHAS have been developed into

![Fig. 2 Cryo-EM and crystal structures of Arabidopsis thaliana ALS. (a) The complex between the CSUs (blue) and the RSUs (white) as determined by cryo-EM. (b) Close up of the interaction between the CSUs and RSUs. FAD, ThDP, Q-loop and valine are shown in yellow, green, red and blue, respectively. (c, d) Crystal structure of the complex between the CSU of A. thaliana ALS and the herbicide, penoxsulam. Penoxsulam is shown as a ball and stick model in with magenta carbon atoms. (c) The interface between the two CSU subunits (green and blue) is shown as a surface. Herbsicide resistance sites are in red. (d) Details of the herbicide binding site. FAD, ThDP and peracetate are shown as stick models. The herbicide resistance sites are labeled and highlighted in red.}
commercial herbicides\cite{1}. They are categorized into five chemical classes (i.e., sulfonylureas, SUs; imidazolinones, IMIs; triazolopyrimidines, TPs; pyrimidinylbenzoates, PBs; and sulfonylaminocarbonyl-triazolinones, SCTs) (Fig. 1(b)). A common scaffold of these compounds is the presence of two aromatic ring moieties (one being a heterocycle) joined by linkers of variable length. In the SUs and SCTs the linker is typically 3–4 atoms, in the TPs and PBs the linker is 1–2 atoms and in the IMIs the linker is a covalent bond. The initial success of the SUs and IMIs in the 1980s led agrochemical companies to invest heavily in the development of this class of herbicide. As a result, six IMIs were developed by BASF, five PBs by Dupont, Kumiai, LG chemicals, and Syngenta, 36 SUs by BASF, Bayer, DuPont, Isagro Ricera, Monsanto, Nihon Nohyaku Syngenta, Takeda, and, four SCTs by Arysta and Syngenta, and seven TPs by Dow. These herbicides are applied to protect a broad range of crops including rice, wheat, barley, oats, tomato, cotton, sugarcane, soybeans and peanuts from broadleaf weeds and annual grasses and more specifically to guard against *Averrhoa carambola*, *Avena fatua*, *Cyperus rotundus*, *Cyperus difformis*, *Phalaris paradoxa*, *Sagittaria calycina* as well as many other weeds. This early herbicide development was conducted in the absence of any published experimental three-dimensional structures, knowledge that is considered vitally important in modern inhibitor design efforts. However, in 2006, the first crystal structures of some of these herbicides in complex with a plant ALS (i.e., from *Arabidopsis thaliana*) were determined\cite{2}. This study showed that the herbicides bind in a location above the active site, making contact with ~15–18 amino acids (depending on the herbicide), but with minimal contact to FAD and no contact with ThDP. These herbicides function by blocking a channel that leads to the active site, preventing the substrate from gaining access to the reactive ThDP\cite{2}. As a result of these interactions the herbicides become almost completely enclosed within the enzyme structure making a major contribution to the very low $K_i$ values (nmol·L$^{-1}$ to pmol·L$^{-1}$) for many of these herbicides\cite{2}. The IMIs are an exception, with $K_i$ values in the μmol·L$^{-1}$ range but still largely enclosed by the enzyme surface\cite{2}. The structure of only one IMI, imazaquin, has been determined in complex with *A. thaliana* ALS\cite{8}. Although it does bind in the same region as the other four herbicide classes, its contact points with the enzyme do not completely overlap with those of the other four classes of herbicides.

In addition to direct binding to ALS by the herbicides, there are other factors that also contribute to their herbicidal and inhibitory potency. Kinetic studies have shown that a single molecule of herbicide is able to inactivate many enzyme molecules\cite{2}. There are several likely reasons that lead to this unusual phenomenon. Among them is the degradation and or modification of ThDP by the herbicides leading to the formation of aminoethenethiol diphosphate, thiamine thiazolone diphosphate or other ThDP derived products that inactivate ALS\cite{2}. Evidence for these changes is provided by the electron density maps from the crystal structures of the CSUs of ALS in complex with different herbicides and by mass spectrometry analysis of solution samples of the complexes\cite{2}. Inhibition can range from reversible to virtually irreversible, depending on the inhibitor. It is likely that inhibitors that impose a low rate of recovery on enzyme activity should be highly effective herbicides, even though their $K_i$ value for ALS may be relatively high (i.e., in the μmol·L$^{-1}$ range). Consistently, imazaquin is a highly effective herbicide in this category having a modest $K_i$ value of 18.5 μmol·L$^{-1}$ for the enzyme, but with virtually irreversible inhibition\cite{2}.

An exceptional example of ThDP modification occurs when penoxsulam inhibits ALS. In the crystal structure of penoxsulam in complex with Saccharomyces cerevisiae a ThDP-peracetate adduct is observed whereas in the *A. thaliana* ALS complex a molecule of peracetate is trapped between ThDP and the inhibitor\cite{2}. The origin of the peracetate, a powerful oxidant, is a side reaction of ALS where an O$_2$ molecule located in a pocket near the active site can bind to the resonating hydroxyethyl-ThDP/enamine intermediate instead of pyruvate or 2-ketobutyrate\cite{2}. The emergence of peracetate from the active site likely contributes to oxidative inactivation of ALS. Within the plant cell, inactivation of other enzymes may also occur.

## 4 RESISTANCE TO ALS-INHIBITING HERBICIDES

ALS-inhibiting herbicides have been in use for crop protection for more than 30 years. Inevitably, however, resistance is expected to develop over time for any deployed herbicide or drug. For the ALS-inhibiting herbicides three major mechanisms of resistance have been identified: (1) metabolism of their chemical structures, thus preventing them from binding to ALS, (2) mutation of the active site within ALS, and (3) restriction of cell permeability. Although these herbicides do not directly compete for binding to the substrate they do bind in a site of significance, which is within the active site pocket and also the location where soluble quinone derivatives can bind and regulate ALS activity by oxidation of the FAD cofactor\cite{2}. Thus, adaptive mutations at this site need to reduce or prevent the binding of herbicide but at the same time, for metabolic maintenance, the enzyme must remain
functional. To document site-of-action herbicide resistance, Ian Heap maintains a website that identifies weed resistant biotypes\textsuperscript{[3]}\textsuperscript{,}. For ALS inhibitors, mutations to eight site-of-action residues have been observed in weeds (Fig. 2(c,d)), these are A122 (mutation to T, V, Y, S and N in 11 different weeds), P197 (mutation to T, H, R, L, Q, S, A, I and N in 49 weeds), A205 (mutation to V or F in six weeds), D376 (mutation to E in 14 different weeds), R377 (mutation to H in one weed), W574 (mutations to L, M, G and R across 42 weeds), S653 (mutations to N, T and I across ten weeds) and G654 (mutation to E or D in two weeds)[1]\textsuperscript{,}. Thus P197 and W574 represent the vast majority and most susceptible sites for mutations to occur. In summary, although a significant number of resistant weeds have emerged, there is hope that new inhibitors of ALS can now be developed based on informed structural data that specifically avoid contact points that mutate more frequently in target weeds.

Based on this expectation, Yang and coworkers have proposed a strategy to overcome site-of-action conferred herbicide resistance. Their proposal is to design “smart” ALS inhibitors with a self-adaptive conformation in the inhibitor binding site suited to both wild-type and mutant ALS\textsuperscript{[11]}\textsuperscript{,}. Molecular simulations suggested novel inhibitors that could bind into the herbicide site by forming new interactions with the mutant enzyme, thereby maintaining potency\textsuperscript{[13]}\textsuperscript{,}. These putative inhibitors were successfully synthesized and subsequently shown to possess strong inhibition (low µmol·L\textsuperscript{−}1) of both mutant and wild-type ALS\textsuperscript{[11–13]}\textsuperscript{,}. These findings show that ALS inhibitors with conformational flexibility are less likely to drive the development resistance in weeds due to site-of-action mutations, especially at the crucial P197 and W574 sites. Thus, as mutations in other sites emerge it is feasible that these can also be successfully overcome by intelligent design strategies.

5 CONCLUSIONS

Herbicides will continue to be an essential component of crop and environmental weed management. Their use is expected to increase with the upward trend in human population growth and the consequent need to continue to implement sustainable and modern farming technologies. Indeed, the current situation of food supply is already imposing near to impossible demands. This issue stems from the fact that the current range of herbicides is limited in number, is not totally effective, can induce non-target toxicity, and can be neutralized by resistance mechanisms. A major concern is the current and growing number of herbicide resistant weed species with herbicide resistant populations, a fact which is now prompting agrochemical chemical companies and government research agencies to again invest in herbicide development. Therefore, a better understanding of the molecular basis for herbicide action and how resistance develops is required if we are to continue to protect crops from weed competition. Recent advances in understanding the catalytic mechanism of ALS and its regulation show that it is an extremely complex enzyme, and each of its activities represent a potential target that could be used to design new, safe, sustainable and environmentally-friendly herbicides which are less likely to lose efficacy through development of target weed resistance.

REFERENCES

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