ELSEVIER

Mechanisms of acetohydroxyacid synthases

David M Chipman[1], Ronald G Duggleby[2] and Kai Tittmann[3]

Acetohydroxyacid synthases are thiamin diphosphate- (ThDP-) dependent biosynthetic enzymes found in all autotrophic organisms. Over the past 4–5 years, their mechanisms have been clarified and illuminated by protein crystallography, engineered mutagenesis and detailed single-step kinetic analysis. Pairs of catalytic subunits form an intimate dimer containing two active sites, each of which lies across a dimer interface and involves both monomers. The ThDP adducts of pyruvate, acetaldehyde and the product acetohydroxyacids can be detected quantitatively after rapid quenching. Determination of the distribution of intermediates by NMR then makes it possible to calculate individual forward unimolecular rate constants. The enzyme is the target of several herbicides and structures of inhibitor–enzyme complexes explain the herbicide–enzyme interaction.

Addresses
[1] Department of Life Sciences, Ben-Gurion University POB 653, Beer-Sheva 84105, Israel
[2] School of Molecular and Microbial Sciences, University of Queensland, Brisbane QLD 4072, Australia
[3] Department of Biochemistry/Biotechnology, Martin-Luther-University Halle-Wittenberg, Kurt-Mothes-Str. 3, Halle/Saale, D-06120, Germany

Corresponding author: Chipman, David M (chipman@bgumail.bgu.ac.il)

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Introduction

The acetohydroxyacid synthases (AHASs) are a group of biosynthetic enzymes apparently found in all plants, fungi and bacteria that are capable of *de novo* synthesis of the branched-chain amino acids. The enzymes known by this name are all capable of synthesizing the alternative products (*R*)-acetolactate (AL) from pyruvate and of (*R*)-acetohydroxybutyrate (AHB) from pyruvate and 2-ketobutyrate, in proportions dependent only on the concentrations of the two substrates in the medium [1,2] (Figure 1). They require as essential cofactors thiamin diphosphate (ThDP), FAD and a divalent metal ion. Enzymes with a certain functional similarity to AHASs, which do not utilize FAD, synthesize acetolactate almost exclusively and have an optimum pH for activity below 6.5, are now generally known as acetolactate synthases [3,4], although this name is sometimes also applied to AHASs.

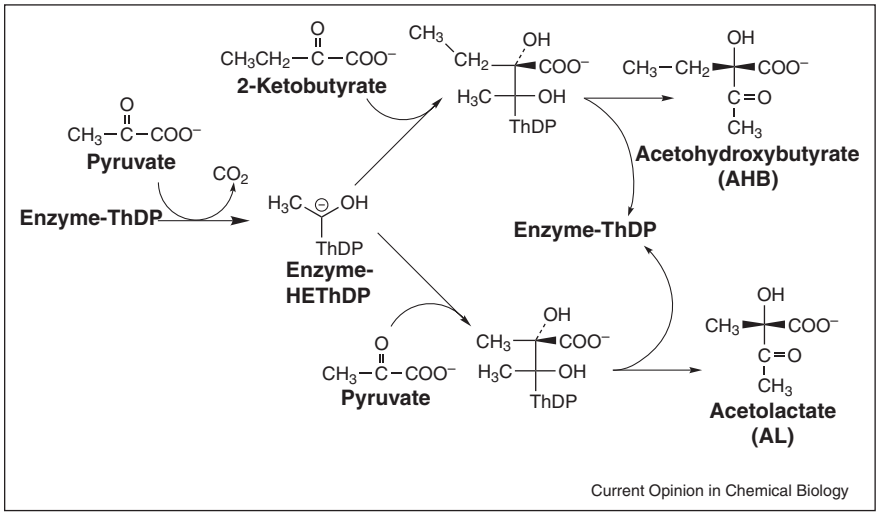
The AHASs are composed of two kinds of subunits, catalytic subunits of molecular mass around 60–70 kDa and the much more diverse regulatory subunits of mass between 9.5 and 54 kDa. The bacterial AHAS is composed of two copies of each subunit, or possibly an integral multiple of that [5,6]. The molecular size of the plant and fungal enzymes is less certain and the enzymes as isolated frequently contain the catalytic subunits only.

The binding sites for the natural modulators (valine and, in some cases, leucine or isoleucine) are located in the regulatory subunit [7•,8–10]. There is evidence that in plant AHAS there are two regulatory sites with different specificities, leading to a complex pathway for activity regulation [9]. Even in the simpler bacterial enzymes, separate domains of the regulatory subunit interact in complex ways [7•].

Most of the direct structural and mechanistic data on AHAS have come from a single isozyme, AHAS II from *Escherichia coli* [11–13]. It is important to point out that this isozyme has several atypical properties. Its regulatory subunits have important sequence differences from those of other AHASs and AHAS II does not show any feedback regulation; unlike most other AHASs, the catalytic subunit of AHAS II is not active alone. The side reaction of AHAS II with molecular oxygen (see below), which leads to formation of peracetate (and perhaps other side reactions), is at least an order of magnitude faster than occurs in AHAS III [14•]. AHAS I also has unique kinetic properties.

AHAS is a target for sulfonylureas, imidazolinones and other herbicides that are in wide use and of great practical importance. This has provided an important practical push for structural studies of AHASs, and there are now high-resolution crystal structures (resolution $\leq$ 2.5 Å) available for the catalytic subunit from yeast [15•,16••,17•], with and without bound herbicides. Despite the fact that holoenzymes have not yet been crystallized, these partial structures are extremely informative. Pairs of catalytic subunits form an intimate dimer containing two active sites, each of which lies across a dimer interface and involves both monomers [15•]. This review attempts to summarize the advances made in the past five years in our understanding of the mechanism of AHASs, including the intermediates on the reaction pathway and the origins of the specificity of different isozymes.

Figure 1

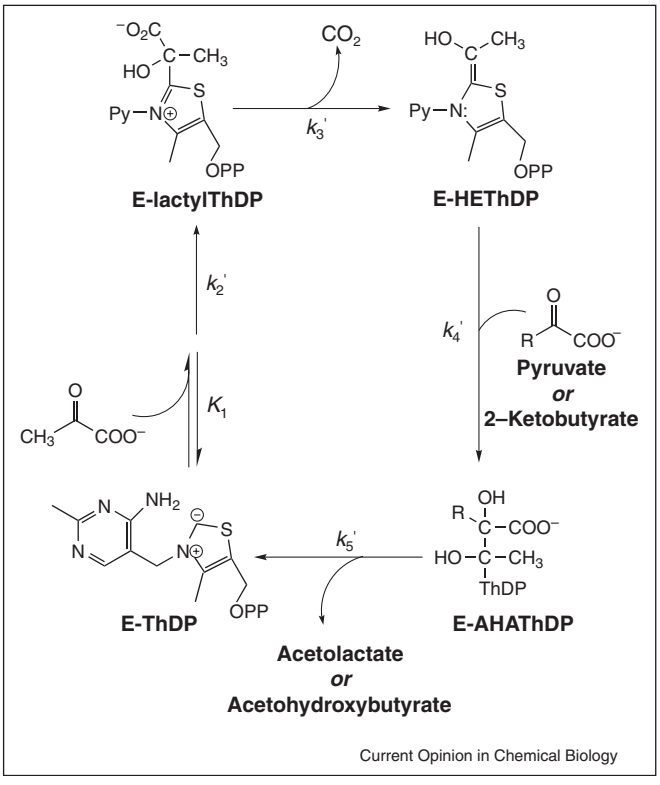


The two competing reactions catalyzed by acetohydroxyacid synthases.

Specificity

The enzyme-sequestered ThDP plays a central role in the mechanism (Figure 2). A molecule of pyruvate is attacked by the ThDP anion and undergoes decarboxylation [18]. In subsequent steps, the hydroxyethylthiamin diphosphate (HEThDP) enamine/carbanion thus formed attacks the second substrate (the acceptor) carbonyl to form the product–ThDP adduct, which in a further step dissociates to form ThDP and the free product [19••]. Direct competition between alternative

Figure 2



The mechanism of the AHAS reactions, with a minimum of five discrete steps.

acceptor substrates for the bound HEThDP determines the ratio of products formed. In the wild-type enzymes, the product ratio depends only on the relative amounts of the acceptors present and k_{cat} is nearly independent of the amount or identity of the acceptors [19••,20••]. This implies that k_{cat} is determined by steps preceding the product-determining, carboligation step so that the steps contributing to discrimination among 'second substrates' have little effect on the overall rate of reaction.

The kinetic behaviors of several mutant forms of AHAS II are completely different [20••]. For example, modification of Met250, Phe109 or Arg276 leads to mutants for which reaction with the *second* substrate is at least partly rate-determining [20••]. In reactions of mutants, the addition of the HEThDP enamine to the carbonyl of the second substrate, the release of the acetohydroxyacid product, or both are significantly slower than for the wild-type enzyme, and significant amounts of the covalent intermediates can be detected in the steady-state [20••].

In all of the ThDP-dependent enzymes, the bound cofactor adopts a *V*-conformation in the active site, fixing the 4′-NH_2 group very close to the C2-H of the thiazolium group [21]. In addition, the N1′ is held close to a conserved glutamate side chain. Studies of H/D exchange in ThDP-dependent enzymes (AHAS among them) revealed that the typical 10^5-fold rate acceleration of proton exchange at the ThDP C2 is accomplished by a shuttle consisting of the conserved glutamate, the aminopyrimidine moiety and the C2-H [22,23]. However, mutation of the conserved Glu to Gln or Ala results in a reduction of the rate of deprotonation at C2 of only 10^2. Reconstitution of AHAS II with N3′-pyridyl-ThDP leads to a totally inactive enzyme. The N1′-Glu *interaction* is thus insufficient to account by itself for activation of ThDP in AHAS. However, replacement of the N1′ atom by carbon or displacement of the 4′-amino group also totally abolishes the proton shuttle. Jordan and co-workers [24•] have accumulated spectroscopic evidence for the occurrence of the elusive 1′-4′-imino tautomer of ThDP as a potent base in the active site.

Choi and co-workers have carried out a long series of kinetic studies of mutant variants of tobacco AHAS [25•,26,27]. The kinetic studies revealed two acid/base catalysts (with pK_a values of 6.5 and 8.2) in the mechanism of the tobacco enzyme. They have also begun tests of the mechanism based on a homology model derived from the yeast enzyme.

Intermediate steps in the reaction

The ThDP adducts of the lactyl group (LThDP), HEThDP, and the product acetohydroxyacids (ALThDP and/or AHBThDP) can be detected quantitatively in the mixture after rapid quenching [19••]. Determination of the distribution of the key reaction intermediates by ^{1}H NMR analysis then make it possible to calculate the forward unimolecular rate constants for individual microscopic steps. The forward (net) rate constant for formation of LThDP, the first detectable step, is overwhelmingly rate-determining for catalysis, whereas the subsequent steps occur with similar, but much larger rates (e.g. the rates of decarboxylation, carboligation and product release are comparable) [18,20••]. Such relative rates explain why k_{cat} for a given enzyme is essentially the same for AHB and AL formation, despite the 60-fold preference for 2-ketobutyrate as acceptor. The occurrence of a step that is overwhelmingly rate determining (LThDP formation) is unique for AHAS; in other ThDP-dependent enzymes, the energies of all the transition states are similar. The molecular origin for the slow, rate-determining formation of LThDP is an important puzzle in the AHAS mechanism.

The rate-determining formation of LThDP has an interesting consequence for 'unnatural' reactions of AHAS. AHAS I and II are both able to catalyze formation of phenylacetyl carbinols from pyruvate and benzaldehyde (or many of its derivatives) in competition with AL and/or AHB [28]. Many mutant AHASs that are compromised in their ability to synthesize the natural acetohydroxy acid products but not in phenylacetyl carbinol (PAC) formation are both excellent potential synthetic tools and provide insights into enzyme–substrate interactions in these enzymes. A group of enzyme residues in the active site, particularly a conserved arginine [29•], are required for rapid and specific reaction of the intermediate with pyruvate or 2-ketobutyrate, but not aromatic aldehydes (e.g. mutagenesis of Arg276 in AHAS II leads to an enzyme that can synthesize PAC as rapidly as the wild-type forms AL or AHB, but maintains the stereospecificity of the wildtype [28]).

A tricyclic thiamin cofactor-intermediate has been detected in the crystal structure of *Klebsiella pneumoniae* acetolactate synthase [30•]. Until this observation, model studies had revealed only a non-enzymatic tricyclic carbinolamine derived from 2-acetylThDP. It remains to be seen whether such intermediates could be formed by other enzymes; this would require a re-assessment of mechanistic assumptions.

Oxygen-consuming side reactions

Some, but not all, of the AHASs demonstrate a significant oxygenase side reaction [31] in which molecular oxygen electrophilically attacks a highly reactive carbanion/enamine to form a peroxy-adduct that decomposes to ThDP and peracetic acid. The peracetic acid can further react with pyruvate to form two moles of acetate. This oxygenase reaction is partly inhibited by high concentrations of the acceptor ketoacids, but even at very high acceptor levels the inhibition of the oxygenase side

reaction saturates, leaving some 10–15% residual oxygenase activity. A classical Theorell–Chance mechanism involving expulsion of carbon dioxide simultaneous with reaction of the acceptor can thus be ruled out. One alternative is that oxygen might use paths to access the active site that are different from those used by the acceptor substrates. Recent results with AHAS II [14•] have shown that there is an additional path for oxygen consumption via a futile redox cycle in which FAD is reduced by the enamine and reoxidized by molecular oxygen. Stopped-flow kinetics show that the apparent rate of FAD reduction at substrate saturation is only slightly dependent on whether pyruvate or 2-ketobutyrate is the acceptor used. The lack of a large rate differential is unexpected, because AHAS II prefers 2-ketobutyrate over pyruvate as acceptor by 60-fold.

Reactivity of the flavin in AHAS

Although the flavin plays a crucial role in the structural integrity of those AHASs that have been examined, no catalytic function in the normal reaction has ever been demonstrated. Flavin is reduced in the course of catalysis as a result of an internal redox side reaction (1–2 s^{-1} at 37 °C for AHAS II) at a rate that is at least two orders of magnitude slower than for the *Lactobacillus plantarum* pyruvate oxidase (*Lp*POX), where FAD reduction is necessary for catalysis. From a kinetic point of view, the slow apparent rate of reduction is at least in some part the result of the direct competition between electron transfer and the extremely fast carboligation ($k' > 1000\ s^{-1}$) and may thus be significantly slower than the true microscopic rate constant. The distance between the thiamin and FAD is almost the same as in *Lp*POX and the flavin is distorted in *Lp*POX in the same way as in AHAS II [17•]. The bent conformation in the AHAS flavin suggests that its redox potential should be higher than that of the free flavin, but there may be other factors contributing to the high potential of the cofactor in this enzyme. Generally, the redox potential of a flavin may be modulated by the protein environment through three major mechanisms: first, donating or accepting hydrogen bonds with the isoalloxazine moiety, in particular with the pyrazine and pyrimidine part; second, placement of a charge proximal to positions of the flavin where charges may develop on reduction; or third, insertion of the flavin into a microenvironment with a high or low effective dielectric constant. Studies that address these issues are currently underway in the authors' laboratories.

Herbicides

A large number of papers on AHAS published in recent years are dedicated to the interaction of the enzyme with sulfonylurea or imidazolinone herbicides and the identification of herbicide-resistant AHAS mutants. Despite this intensive effort, there are still some aspects of the inhibition that remain puzzling. The inhibition of AHAS by herbicides is a time-dependent process with an initial weak inhibition followed by a slow transition into a final steady-state where the inhibition is more potent [32–35]. The initial and final inhibition, and the speed of the transition, are all dependent on the concentration of the inhibitor.

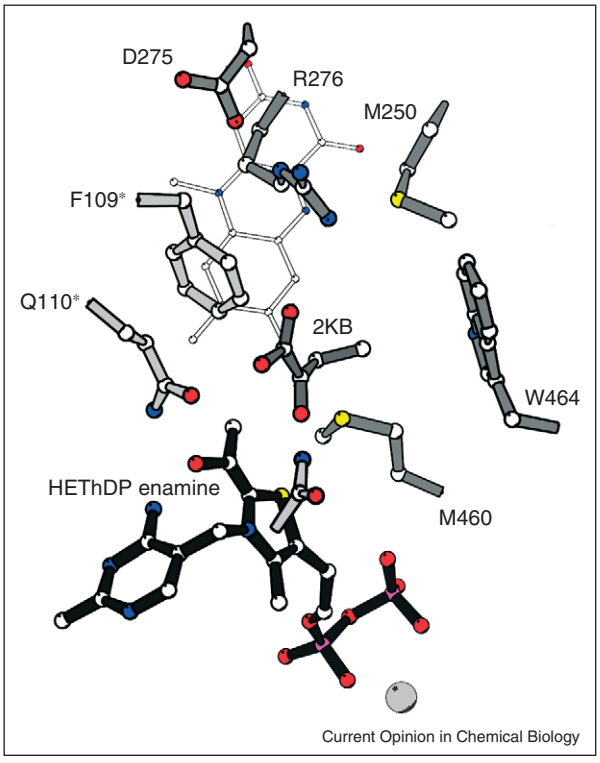
The inhibition by sulfonylureas has been reported to be non-competitive [36,37] and nearly competitive [38,39] with respect to pyruvate. By contrast, the imidazolinones behave as non-competitive [39,40] or uncompetitive inhibitors [35,36,41]. The differences in the responses, coupled with the structural diversity between the two types of herbicide, suggest that these inhibitors bind to different sites on the enzyme. However, other studies have shown mutually exclusive binding of sulfonylureas and imidazolinones to AHAS [36,40,42,43].

Whether the inhibition by the herbicides is reversible remains controversial. Using gel filtration and dialysis, it has been reported that the bound inhibitors can be completely, albeit slowly, separated from the enzyme [33,34,36,40]. However, the recovery of enzymatic activity after inhibitor removal is variable. In other studies, irreversible inactivation of the enzyme by both sulfonylureas and imidazolinones has been reported, despite complete removal of the herbicide [33,36,44].

One explanation for time-dependent irreversible inactivation of AHAS by herbicides is the oxygenase side-reaction of the enzyme mentioned earlier [45]. If these inhibitors bind preferentially to the enamine form of the HEThDP enzyme intermediate, it would promote accumulation of the oxygen-sensitive complex as well as interfering with the release of a peroxide product. Thus, the trapped peroxide would cause progressive oxidative inactivation of the enzyme. There are, unfortunately, observations that conflict with this explanation. For example, Ortéga *et al.* [44] showed that barley AHAS and *E. coli* AHAS II are inhibited by the sulfonylurea thifensulfuron methyl, and do not regain activity when the inhibitor is removed by gel filtration or by dilution. However, if precipitation of AHAS using ammonium sulfate is used for inhibitor removal, full recovery of activity for the bacterial enzyme, but not barley AHAS, is observed. The inactivation is observed only if the enzyme is incubated with ThDP and Mg^{2+}, irrespective of whether pyruvate is added.

The location of the herbicide binding site started to become clear when the crystal structure of yeast AHAS was published [15] (Figure 3). Although the protein was crystallized in the absence of any herbicide, the location of herbicide-resistance mutations clearly mapped out where the herbicide would bind. This experimental structure strongly validated earlier homology models [46,47] that were based on the structure of *Lp*POX

Figure 3



Cartoon illustrating the likely binding mode in which 2-ketobutyrate (2KB) binds to the HEThDP form of the AHAS active site. Not labelled and shown in the background with thin bonds is the isoalloxazine portion of bound FAD.

[48]. The subsequent determination of several structures of yeast AHAS with bound sulfonylureas [16••,17•] has firmly established that these herbicides bind in a channel that leads to the active site. This channel is at a subunit interface, and is lined with amino acids that, when mutated, give rise to sulfonylurea resistance in yeast AHAS [49,50]. It should be noted that these structures contain no bound substrate, which may seem to contradict the earlier suggestion that these inhibitors bind to the enzyme-bound covalent adduct, HEThDP. However, the sulfonylurea concentrations used for crystallization (1 mM) are around four orders of magnitude greater than their inhibition constants (3–130 nM). The concordance between the structural and mutational data leaves little doubt that the observed sulfonylurea binding location and conformation is responsible for the inhibition, and is not an artefact of the very high concentrations used.

One curious observation in all of the yeast AHAS structures is that the ThDP cofactor is damaged, in some cases so substantially that the fragments are not readily recognizable. This invites the speculation that the time-dependent inhibition of AHAS results from progressive ThDP destruction. There is evidence both for and against this hypothesis, but it is an idea that may be worth pursuing.

Future perspectives

There has been enormous progress in the knowledge and understanding of AHASs over the past few years, but this has emphasized a number of questions that remained in the background. Why is the first chemical step in the mechanism, formation of LThDP so slow? How does feedback inhibition by valine affect the rate of the reaction? How do other inhibitors impede catalysis? Why is the flavin bound in the enzyme rather unreactive in its resting state? Is the redox side reaction kinetically or thermodynamically controlled? To answer these questions, it will be necessary to bring all the dynamic and structural tools available, including structures of the intermediates of the major pathway trapped in the active site, to bear on this system.

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