Research Focus

How an enzyme answers multiple-choice questions

Jennifer A. McCourt and Ronald G. Duggleby

School of Molecular and Microbial Sciences, University of Queensland, St Lucia, Brisbane, QLD 4072, Australia

Acetohydroxyacid synthase (AHAS) is the first common enzyme in the pathway for the biosynthesis of branched-chain amino acids. Interest in the enzyme has escalated over the past 20 years since it was discovered that AHAS is the target of the sulfonylurea and imidazolinone herbicides. However, several questions regarding the reaction mechanism have remained unanswered, particularly the way in which AHAS 'chooses' its second substrate. A new method for the detection of reaction intermediates enables calculation of the microscopic rate constants required to explain this phenomenon.

Acetohydroxyacid synthase

Acetohydroxyacid synthase (AHAS; EC 2.2.1.6) [1] catalyses the thiamin diphosphate (ThDP)-dependent synthesis of both 2-*S*-acetolactate (AL) and 2-*S*-aceto-2-hydroxybutyrate (AHB) by reacting a molecule of pyruvate with either a second molecule of pyruvate or 2-ketobutyrate (2-KB), to form the two respective products (Figure 1). Despite the structural similarity between the two alternative substrates, the enzyme exerts a preference for 2-KB over pyruvate, the magnitude of which can be described by the specificity constant (R) defined in Eqn 1.

$$R = \frac{\text{AHB formed/[2-KB]}}{\text{AL formed/[pyruvate]}} \qquad \text{(Eqn 1)}$$

However, until early this year, the mechanism by which the enzyme 'chooses' a larger substrate over its smaller competitor in the second half of the reaction was not clear. In their recent report, Tittmann *et al.* [2] investigate how AHAS accomplishes this task by measuring the individual rate constants of the elementary steps in the catalytic cycle. The significance of this research lies not only in the enhanced understanding of the reactions catalysed by AHAS, but with the prospect for extending this method in the future to better understand other ThDP-dependent enzymes.

ThDP-dependent enzymes

Enzymes using the vitamin B1-derived cofactor, ThDP, have interested scientists over the past 70 years for a variety of reasons. Unlike many other cofactors, ThDP is capable of catalysing reactions without the benefit of a protein environment [3]. For this reason alone, it is of great interest to comprehend how such enzymes can enhance a reaction rate by a factor of up to 10^{12}. A detailed understanding of the elementary steps of these reactions is essential for the rational development of inhibitory compounds and, in some cases, to clarify why some existing inhibitors are so effective. In addition, because these enzymes are involved in numerous biological reactions that produce chiral compounds with great efficiency and high enantiomeric selectivity, they are of great appeal to the pharmaceutical industry for the chemoenzymatic synthesis of drugs.

At least 27 different ThDP-dependent enzymes have been isolated and characterized to some degree, and there are now 62 crystal structures of 11 different ThDP-dependent enzymes deposited in the RCSB (Research Collaboratory for Structural Bioinformatics) protein data bank. Information from these structures, together with detailed kinetic studies, has assisted enzymologists in formulating reaction mechanisms for most ThDP-dependent enzymes. However, because a full description of enzyme mechanism should include the identification of all catalytic groups, elucidation of all the chemical species involved in the reaction(s) and the values of all rate constants (both forward and reverse), there are still several unanswered questions pertaining to the reaction mechanism of individual enzymes.

Enzymes convert substrates to products via a series of intermediates and their intervening transition states. In most cases, these intermediates are too ephemeral to be isolated, let alone quantified. ThDP-dependent enzymes are unusual in this respect, in that the reaction pathway passes through a series of covalent intermediates that are relatively stable when separated from the enzyme. Typically, two or three intermediates (plus free ThDP) can be identified and these can be quantified simultaneously by the NMR-based technique that was pioneered by Tittmann *et al.* [4]. At steady state, the relative amount of each of these ThDP derivatives depends on the net rate constant for its forward conversion. Consequently, the mechanism can be dissected and characterized in terms of these rate constants that define individual steps around the catalytic cycle.

Provided that large quantities of enzyme are available, the method devised by Tittmann and coworkers [4] for the detection and quantification of covalent intermediates is straightforward. The enzyme is mixed rapidly with substrate before halting the reaction with a deuterated acid solution. Denatured protein is subsequently removed by centrifugation, and the supernatant is analysed by ^{1}H NMR spectroscopy over a spectral width of 12 ppm. Although the NMR spectrum of an acid-quenched enzyme reaction is composed of numerous overlapping peaks, Tittmann *et al.* [4] were able to demonstrate that the

Corresponding author: Duggleby, R.G. (ronald.duggleby@uq.edu.au).
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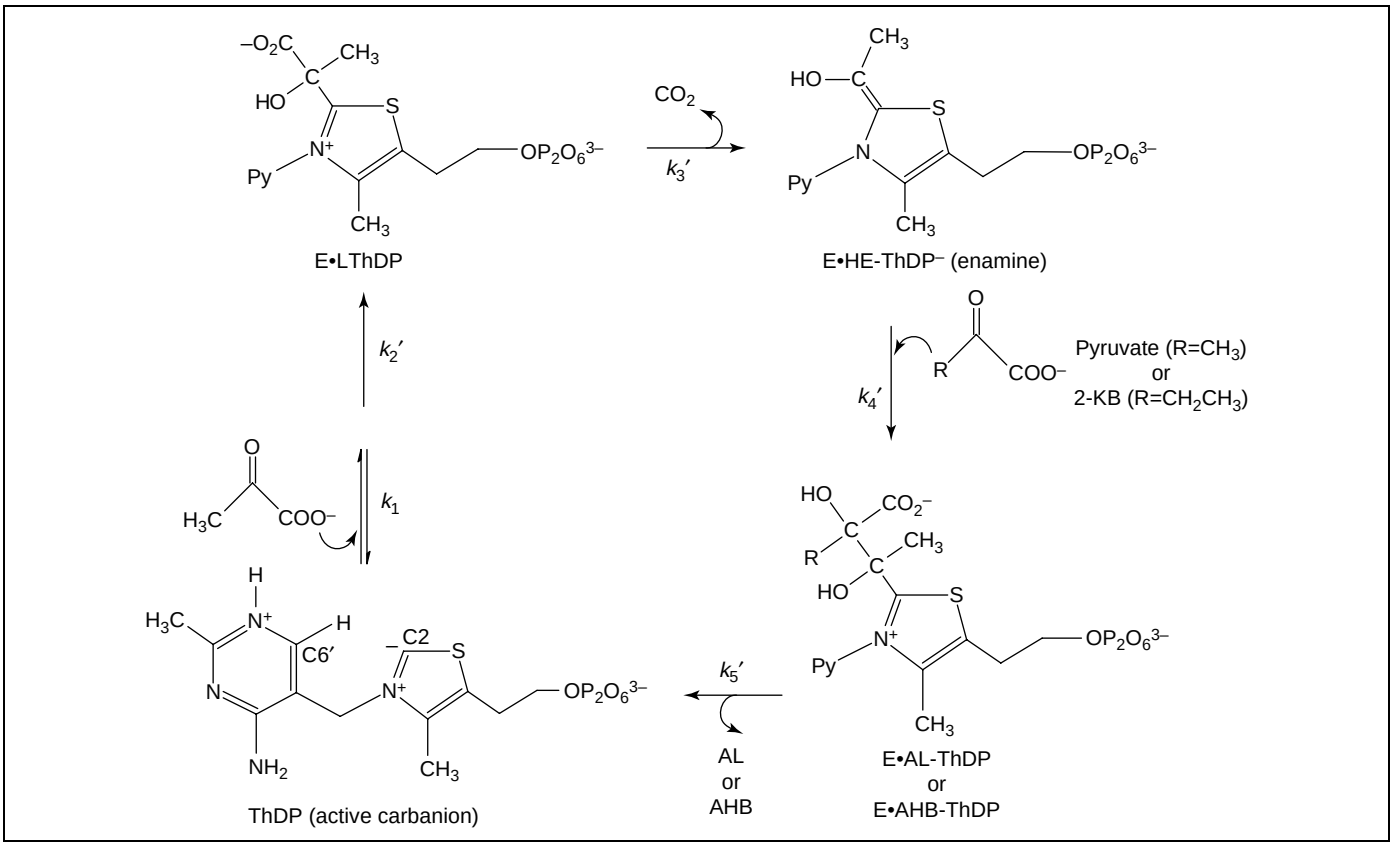

Figure 1. The catalytic cycle of AHAS. ThDP binds pyruvate reversibly before reacting with it (step 2) to form LThDP. The product, E•LThDP, then undergoes decarboxylation (step 3), addition of the second substrate (step 4) and release of the product (step 5). By quantifying each of the intermediates shown, the rate constants k'_2–k'_5 can be calculated. Because the initial binding of pyruvate is readily reversible, k'_2 represents the combined steps from ThDP to LThDP. Adapted, with permission, from Ref. [2].

chemical shift for the C6′ proton lies within a region of ~7.2–8.6 ppm, and that this chemical shift is distinct for each of seven covalently bound ThDP reaction intermediates. By integrating the peaks, the amount of each intermediate can be estimated and used to calculate net forward microscopic rate constants [2,4].

Characteristics of the specificity ratio for AHAS

The specificity ratio, R, has been measured for AHAS enzymes from several different species and, with the exception of a single isoform within the Enterobacteria, 2-KB is the preferred second substrate [5,6]. The Enterobacteria have evolved to harbour multiple isoforms of AHAS (AHAS I, II and III), each with a different R value, to adapt under conditions in which the substrate availability might change. In this way, if an organism is forced to survive on a poor carbon source such as acetate, it will rely predominantly on AHAS I, which has little or no preference for 2-KB ($R=1$ [7]) as the second substrate, to meet its metabolic requirements [8]. Conversely, AHAS II ($R=60$) and AHAS III ($R=40$), which both have a strong preference for 2-KB as the second substrate, are of more importance when the intracellular concentration of pyruvate is high [9].

The specificity ratio is not altered by the addition of branched-chain amino acids (feedback inhibitors) or changes in pH [7]. Furthermore, the R value and the overall rate remain unchanged if, at a single concentration of pyruvate, the concentration of the competing substrate 2-KB is varied. This implies that the rate-determining step for the overall reaction occurs before binding of the second substrate [7]. Indeed, Tittmann and coworkers [4] demonstrated that that the formation of lactyl ThDP (LThDP) is the rate-limiting step in the AHAS reaction. In 1996, it was discovered that an *Escherichia coli* AHAS isoenzyme II (*Ec*AHAS II) mutant (Trp464Leu) had lost its preference for 2-KB as the second substrate ($R=1.3$ [10]) suggesting that Trp464 is directly involved in recognition of the second substrate. However, as indicated by Gollop *et al.* [5], the difference between the interaction energies (calculated from the R values) for 2-KB and that of pyruvate is too large to be attributed solely to the additional hydrophobic contacts for the extra methylene group of 2-KB.

In an attempt to learn more about the mechanism of AHAS, based on the active site from the crystal structure of yeast AHAS (PDB code: 1N0H [11]) and that of the *Ec*AHAS II homology model [10] derived from the structure of pyruvate oxidase, Engel *et al.* [12] designed various mutants of *Ec*AHAS II (Phe109, Met250, Arg276 and Trp464). The kinetic properties of the wild-type and mutant enzymes were all characterized in the presence of pyruvate, pyruvate with 2-KB and pyruvate in combination with the newly discovered unnatural substrate benzaldehyde, which can be ligated to the hydroxyethyl-ThDP anion (HE-ThDP$^-$) to form R-phenylacetylcarbinol [13]. It was observed that not only are the R values for Arg276 and Trp464 mutants reduced, but that the acetohydroxyacid production of Arg276, Met250 and Phe109 mutants is so severely impaired that the

formation of products becomes rate-determining for the overall reaction [12]. However, because the rate of formation of *R*-phenylacetylcarbinol remains unchanged for Arg276Lys, the authors suggest that it is likely that this residue is involved in carboxylate recognition of the 2-ketoacid substrates [12].

How does an enzyme choose between two substrates?

In their follow-up study, Tittmann *et al.* [2] identify and quantify the key covalently bound intermediates of the AHAS reaction and, using this information, determine the rate constants for the minimal reaction steps depicted in Figure 1 for both wild-type and mutant (Arg276Lys, Met250Ala and Trp464Leu) *Ec*AHAS II. Figure 2 illustrates the distribution of intermediates formed by the Trp464Leu mutant from pyruvate alone. Like the ^{1}H NMR spectrum for the wild-type enzyme [4], the amounts of LThDP, HE-ThDP^{-} and 2-*S*-acetolactyl ThDP adduct (AL-ThDP) are similar but also much less than ThDP itself. To calculate rate constants from the distribution of intermediates, Tittmann *et al.* [2] give somewhat complicated expressions but these simplify to $k_x = k_{cat}\Sigma/[X]$, where k_x is the rate constant for forward conversion of intermediate X and Σ is the total concentration of all intermediates. Thus, the dominance of free ThDP indicates that k'_2 is rate limiting.

Trp464Leu has an activity similar to wild type, but a drastically reduced *R* value of 3.0 (compared with $R = 59$ for wild type). The difference is well explained by the rate constants with substantial increases in k'_4 and k'_5 for wild type when 2-KB is present but little change for Trp464Leu. Curiously, Arg276Lys shows a similar insensitivity of k'_4 and k'_5 to 2-KB, which is superficially inconsistent with the *R* value of 28. Tittmann *et al.* [2] explain this discrepancy by noting that k'_4 and k'_5 are overall rate constants for two-step processes. For example, conversion of AHAS•AL-ThDP to AHAS•ThDP would involve the reversible formation of a non-covalent AHAS•ThDP•AL complex followed by release of the product. It is proposed that AHAS•ThDP•AL or AHAS•AL-ThDP can be converted back to AHAS•HE-ThDP^{-} (releasing pyruvate), which provides a second opportunity for competition by 2-KB. Thus, it seems that AHAS has evolved so that formation of AHB is more highly committed than that of AL. In this way, release of pyruvate from the AL-ThDP complex gives 2-KB another chance to react, thereby, increasing the likelihood of AHB formation [2]. Although this explanation is plausible, it highlights a weakness in this NMR technique; at least for this mutant, steps that are essentially hidden to the method determine second-substrate specificity. Although the kinetic scheme indicated in Figure 1 is simplistic and steps 4 and 5 of the reaction mechanism are, in reality, undoubtedly more complicated, a detailed examination of these steps would not invalidate the evidence which indicates that Arg276 is a crucial residue for recognition of the second ketoacid substrate and that it is probably involved in recognition of the carboxylate moiety [2,13].

Another curiosity in the data is that Met250Ala seems to show statistically significant changes in k'_2 and k'_3 in the presence of 2-KB. Because these two rate constants govern steps prior to second substrate binding, it is not obvious how they can be affected by 2-KB. One explanation is that the second substrate can influence these rate constants because it is bound in the active site early in the catalytic cycle (K. Tittmann, personal communication). A similar proposal has been made previously [14] based on entirely different observations and reasoning.

Concluding remarks

Tittmann *et al.* [2] have extended their technique for quantifying ThDP intermediates to several mutants of AHAS. They have demonstrated that the catalytic cycle can be dissected into four individual steps, for which the net forward rate constants have been determined. These rate constants provide a reasonable explanation of how this enzyme chooses between two similar substrates and favours 2-KB over pyruvate to compensate for the difference in their intracellular concentrations.

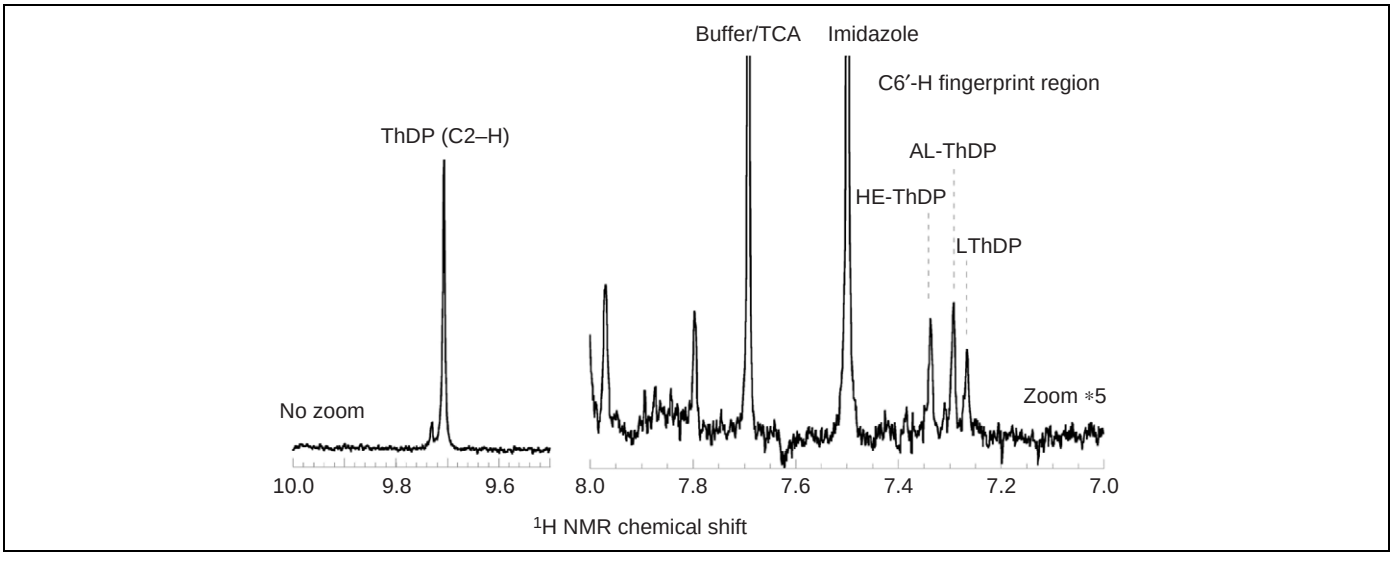

Figure 2. Identification of ThDP intermediates by NMR. ThDP itself is identified from the C2 proton resonance, whereas intermediates have characteristic peaks for the C6′ proton in the 'fingerprint' region (shown here magnified fivefold). Reproduced, with permission, from Ref. [2]. © (2005) National Academy of Sciences, U.S.A.

There are several other features of AHAS activity that would warrant investigation by this technique. For example, the enzyme possesses a regulatory subunit that stimulates activity and confers upon it sensitivity to inhibition by valine. What step(s) are accelerated by binding of the regulatory subunit? Does valine act by slowing the same step(s), or does in interfere with other parts of the catalytic cycle? AHAS is the target of several commercial herbicides, such as the sulfonylurea and imidazolinone families, which act as potent inhibitors. At what step(s) in the catalytic cycle do these compounds exert their effect? Do both the sulfonylureas and imidazolinones inhibit by a common mechanism?

In addition to answering these questions, we expect that the technique described by Tittmann *et al*. [4] can be extended to other ThDP-dependent enzymes, provided that their intermediates have a uniquely identifiable NMR fingerprint.

References

1 Duggleby, R.G. and Pang, S.S. (2000) Acetohydroxyacid synthase. *J. Biochem. Mol. Biol.* 33, 1–36
2 Tittmann, K. *et al*. (2005) The carboligation reaction of acetohydroxyacid synthase II: steady-state intermediate distributions in wild type and mutants by NMR. *Proc. Natl. Acad. Sci. U. S. A.* 102, 553–558
3 Kern, D. *et al*. (1997) How thiamine diphosphate is activated in enzymes. *Science* 275, 67–69
4 Tittmann, K. *et al*. (2003) NMR analysis of covalent intermediates in thiamin diphosphate enzymes. *Biochemistry* 42, 7885–7891
5 Gollop, N. *et al*. (1989) Kinetics and mechanism of acetohydroxy acid synthase isozyme III from *Escherichia coli*. *Biochemistry* 28, 6310–6317
6 Gollop, N. *et al*. (1990) Physiological implications of the substrate specificities of acetohydroxy acid synthases from varied organisms. *J. Bacteriol.* 172, 3444–3449
7 Barak, Z. *et al*. (1987) Physiological implications of the specificity of acetohydroxy acid synthase isozymes of enteric bacteria. *J. Bacteriol.* 169, 3750–3756
8 Dailey, F.E. and Cronan, J.E., Jr. (1986) Acetohydroxy acid synthase I, a required enzyme for isoleucine and valine biosynthesis in *Escherichia coli* K-12 during growth on acetate as the sole carbon source. *J. Bacteriol.* 165, 453–460
9 Epelbaum, S. *et al*. (1998) Branched-chain amino acid biosynthesis in *Salmonella typhmurium*: a quantitative analysis. *J. Bacteriol.* 180, 4056–4067
10 Ibdah, M. *et al*. (1996) Homology modeling of the structure of bacterial acetohydroxy acid synthase and examination of the active site by site-directed mutagenesis. *Biochemistry* 35, 16282–16291
11 Pang, S.S. *et al*. (2003) Molecular basis of sulfonylurea herbicide inhibition of acetohydroxyacid synthase. *J. Biol. Chem.* 278, 7639–7644
12 Engel, S. *et al*. (2004) Role of a conserved arginine in the mechanism of acetohydroxyacid synthase. *J. Biol. Chem.* 279, 24803–24812
13 Engel, S. *et al*. (2003) Acetohydroxyacid synthase: a new enzyme for chiral synthesis of *R*-phenylacetylcarbinol. *Biotechnol. Bioeng.* 83, 833–840
14 Duggleby, R.G. *et al*. (2004) Structure and properties of acetohydroxyacid synthase. In *Thiamine: Catalytic Mechanisms in Normal and Disease States* (Jordan, F. and Patel, M.S., eds), pp. 251–274, Marcel Dekker

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Deoxyribonucleoside kinases: two enzyme families catalyze the same reaction

Michael P.B. Sandrini[1,2] and Jure Piškur[1]

[1]Cell and Organism Biology, Lund University, Sölvegatan 35, SE-22732 Lund, Sweden
[2]BioCentrum-DTU, Technical University of Denmark, DK-2800 Kgs. Lyngby, Denmark

Mammals have four deoxyribonucleoside kinases, the cytoplasmic (TK1) and mitochondrial (TK2) thymidine kinases, and the deoxycytidine (dCK) and deoxyguanosine (dGK) kinases, which salvage the precursors for nucleic acids synthesis. In addition to the native deoxyribonucleoside substrates, the kinases can phosphorylate and thereby activate a variety of anti-cancer and antiviral prodrugs. Recently, the crystal structure of human TK1 has been solved and has revealed that enzymes with fundamentally different origins and folds catalyze similar, crucial cellular reactions.

Salvage of nucleic acid precursors

In the cell, deoxyribonucleotides (the building blocks of DNA synthesis) are synthesized by the *de novo* [1] and salvage pathways [2]. In the salvage pathway, deoxyribonucleosides are converted into monophosphates, diphosphates and, finally, triphosphates. The salvage pathway is also used for activation of several antiviral prodrugs (i.e. pharmacologically inactive derivatives of active drugs), such as Zovir (acyclovir) and Zidovudine [azidothymidine (AZT)], and established anti-cancer prodrugs, such as cladribine, gemcitabine and fludarabine. The first reaction of the salvage pathway – between the phosphate donor (usually ATP) and a deoxyribonucleoside recipient – represents the 'bottle neck' of the pathway and is catalyzed by deoxyribonucleoside kinases.

In mammals, four deoxyribonucleoside kinases with overlapping substrate specificities for the four native DNA precursors can be found: (i) cytoplasmic thymidine kinase 1 (TK1) phosphorylates only thymidine (dT); (ii) mitochondrial thymidine kinase 2 (TK2) phosphorylates dT and deoxycytidine (dC); (iii) cytoplasmic deoxycytidine

Corresponding author: Piškur, J. (Jure.Piskur@cob.lu.se).
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