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Suicide inhibition of acetohydroxyacid synthase by hydroxypyruvate

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Abstract
Acetohydroxyacid synthase (Ec 2.2.1.6) catalyses the thiamine diphosphate-dependent reaction between two molecules of pyruvate yielding 2-acetolactacte and CO_2. The enzyme will also utilise hydroxypyruvate with a k_{cat} value that is 12% of that observed with pyruvate. When hydroxypyruvate is the substrate, the enzyme undergoes progressive inactivation with kinetics that are characteristic of suicide inhibition. It is proposed that the dihydroxyethyl-thiamine diphosphate intermediate can expel a hydroxide ion forming an enol that rearranges to a bound acetyl group.

Keywords: *Acetohydroxyacid synthase, hydroxypyruvate, suicide inhibition, thiamine diphosphate*

Abbreviations: AHAS, acetohydroxyacid synthase, ThDP, thiamine diphosphate

Introduction

Acetohydroxyacid synthase [1,2] (AHAS; EC 2.2.1.6) catalyses the first committed step in the biosynthesis of valine, leucine and pantothenate in plants and micro-organisms. The reaction involves the decarboxylation of a molecule of pyruvate followed by reaction with a second molecule of pyruvate, forming 2-acetolactate (Figure 1). The same enzyme participates in the biosynthesis of isoleucine by virtue of its ability to accept 2-ketobutyrate instead of the second pyruvate, yielding 2-aceto-2-hydroxybutyrate. The enzyme from most species, and isoforms within a species, have a substantial preference in the second step for 2-ketobutyrate over pyruvate, thereby compensating for the low intracellular concentration of 2-ketobutyrate [3,4]. The enzyme can also use 2-ketobutyrate in place of the first pyruvate [5] although this activity is unlikely to be of any biological significance.

Elucidation of the three-dimensional structure of AHAS [6,7] and mutagenesis studies [8,9] indicate that the alkyl side-chain of the 2-ketoacid substrates interact with hydrophobic amino acid residues in the active site that control substrate specificity. Therefore it would be expected that the polarity of hydroxypyruvate would make it a poor substrate but, as far as I am aware, it has never been tested. Here I confirm that it is a poor substrate and demonstrate that it also acts as a suicide inhibitor. The data supporting these conclusions and their mechanistic interpretation are reported.

Materials and methods

Recombinant *Escherichia coli* AHAS isoenzyme II was expressed and purified as described previously [10]. Activity measurements were conducted at 37° in 0.1 M potassium phosphate buffer (pH 7.5) containing the three cofactors (10 mM $MgCl_2$, 1 mM thiamine diphosphate (ThDP), and 10 μM FAD), and pyruvate or hydroxypyruvate as required for any particular experiment. Substrate utilization was measured at 333 nm [11].

Results

When acting on pyruvate as substrate, *E. coli* AHAS II has a k_{cat} of 47 sec^{-1} and a K_m of 5 mM [10].

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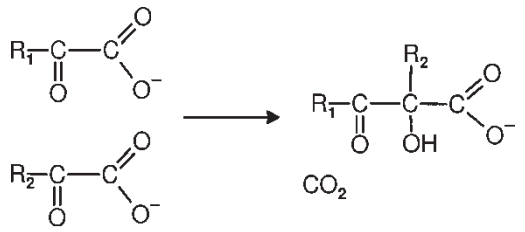

Figure 1. *The reaction catalyzed by AHAS.* Two 2-ketoacids react to give an acetohydroxyacid and carbon dioxide. The normal physiological reactions are those where pyruvate ($R_1 = CH_3$) reacts with pyruvate ($R_2 = CH_3$) or 2-ketobutyrate ($R_2 = C_2H_5$).

The enzyme will also act on hydroxypyruvate with a k_{cat} of $5.47 \pm 0.14\,sec^{-1}$ and a K_m of 8.31 ± 0.60 mM (data not shown). During the course of making these measurements, I observed that substrate utilization ceases before hydroxypyruvate is exhausted. Moreover, the extent of substrate consumption depends upon the amount of AHAS added as illustrated in Figure 2A. When the reaction ceases, addition of more AHAS (arrows) causes substrate utilization to recommence. These data clearly suggested that AHAS undergoes inactivation during the reaction. The relationship between the extent of hydroxypyruvate utilization and the AHAS concentration was characterized more fully (Figure 2B). If enough AHAS is added, hydroxypyruvate is fully converted to product (open circles). At lower AHAS concentrations there is a strictly linear relationship (filled circles).

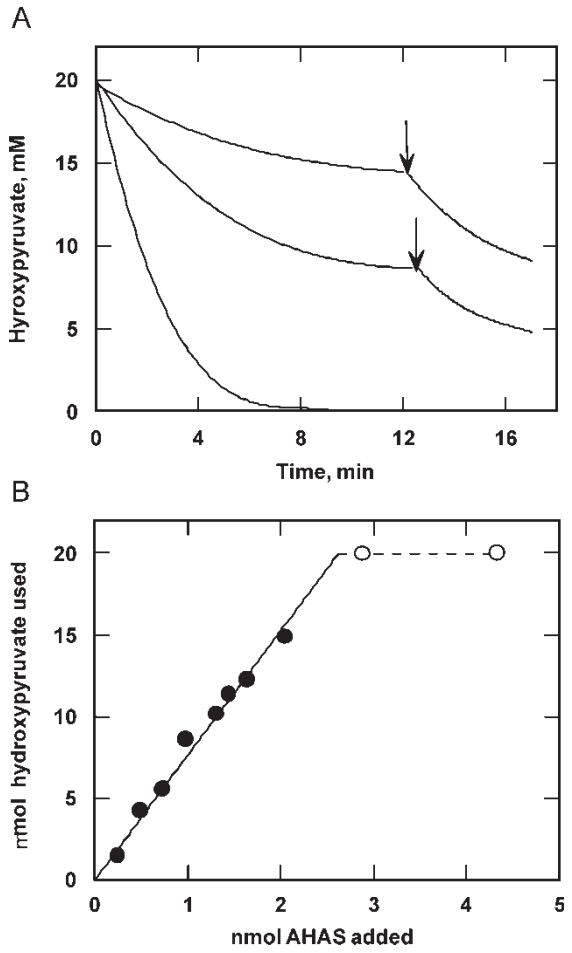

Figure 2. *Partial hydroxypyruvate utilization by AHAS II.* Reactions were as described in Materials and Methods using 20 mM hydroxypyruvate as substrate, in a total volume of 1 ml. In **Panel A**, the amounts of AHAS added were (top to bottom) 0.72, 1.45 and 4.34 nmol. At the points indicated by arrows, a further 1.45 nmol AHAS was added. In **Panel B**, experiments were as in Panel A and the amount of hydroxypyruvate utilized is plotted against the quantity of AHAS added. Open circles indicate reactions in which there was complete consumption of hydroxypyruvate. The solid line is the best fit to the data shown as filled circles of a straight line passing through the origin.

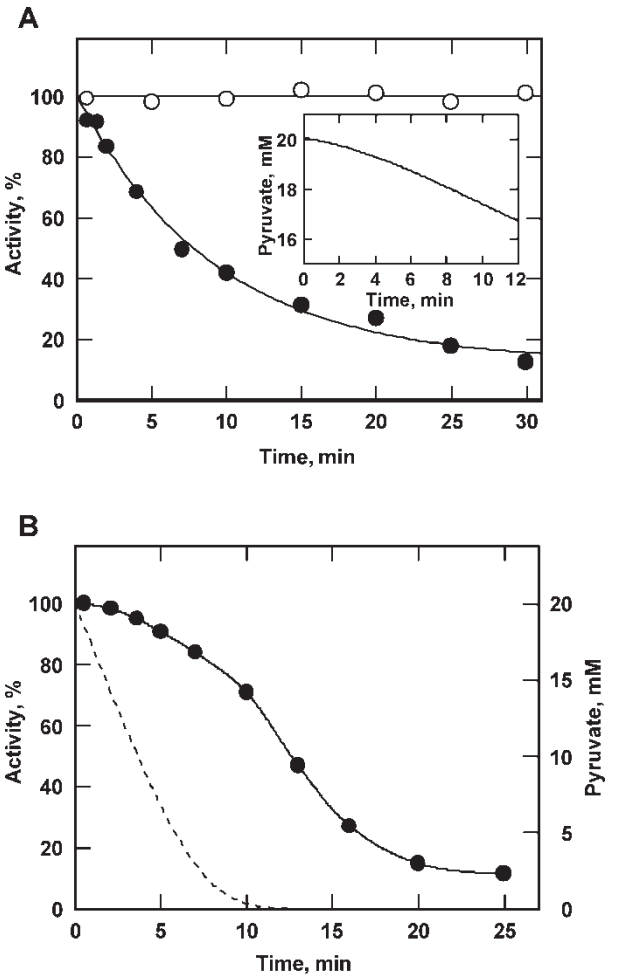

Figure 3. *Progressive inactivation of AHAS II.* Reactions were as described in Materials and Methods using 1.45 nmol of enzyme per ml. In **Panel A**, 20 mM hydroxypyruvate was provided as substrate (filled circles) and samples of 0.96 ml were removed at intervals and mixed with 40 µl of 0.5 M pyruvate (final concentration of 20 mM) to measure residual activity. The data indicated by open circles were similar except that the initial incubations contained no hydroxypyruvate. The inset shows an identical reaction to the 20 mM hydroxypyruvate/30 minute sample that was desalted by gel filtration before mixing with pyruvate. **Panel B** shows a similar experiment to that shown in Panel A (filled circles) except that 20 mM pyruvate was used in the initial incubations. The broken line shows the consumption of 20 mM pyruvate under these reaction conditions, scaled on the right ordinate.

The inactivation of AHAS was investigated further by withdrawing samples while AHAS was acting on hydroxypyruvate and assaying the residual activity using the natural substrate, pyruvate (Figure 3A, filled circles). There is a substantial loss of activity over a 30 minute period with a half-life of approximately 7 minutes. AHAS is a notoriously unstable enzyme but the observed inactivation is entirely dependent upon the presence of hydroxypyruvate, as shown in a parallel experiment in which this substrate was omitted (Figure 3A, open circles). Enzyme that had been incubated with hydroxypyruvate for 30 minutes was separated by gel filtration from product formed and unused hydroxypyruvate, and then assayed with pyruvate. There is little activity initially but this increases with time (Figure 3A, inset), showing that the inactivation of AHAS can be reversed.

Schloss and colleagues [12] have described an oxygenase activity of AHAS, which can lead to enzyme inactivation when pyruvate is the substrate. However, the kinetics of this process are quite different from hydroxypyruvate-induced inactivation, as illustrated in Figure 3B. Initially inactivation is slow and it is only as substrate exhaustion approaches (broken line) that pyruvate-induced inactivation becomes rapid. This is because the steady-state fraction of the oxygen-sensitive carbanion intermediate is low in the earlier stages of the reaction [13]. It is only when the pyruvate concentration approaches zero that this intermediate becomes the dominant enzyme species [14].

Discussion

E. coli AHAS II is capable of using hydroxypyruvate as a substrate, at about 12% of the maximum rate observed with pyruvate. Simultaneously it undergoes progressive inactiveation (Figure 3A). Although pyruvate also induces a loss of activity, the kinetics are quite different (Figure 3B), suggesting that the two processes occur by different mechanisms. Hydroxypyruvate utilization ceases before it is exhausted (Figure 2A) and the amount remaining is proportional to the enzyme concentration (Figure 2B). This linear relationship is expected when the free enzyme is stable but a catalytic intermediate is not [15]. From the slope of this line, and allowing for the fact that each catalytic cycle consumes two molecules of hydroxypyruvate, one can calculate a partition ratio of 3807 $\pm$ 75. This means that there is an average of nearly 4000 successful catalytic cycles for each event in which the intermediate is diverted into the inactivation pathway.

In common with many other enzymes that catalyse the decarboxylation of 2-ketoacids, AHAS requires ThDP as an essential cofactor (Figure 4). The thiazolium carbanion (**I**) reacts with pyruvate to give lactyl-ThDP (**II**), which then undergoes decarboxylation to the resonating enamine (**III**) and α-carbanion (**IV**) of hydroxyethyl-ThDP. Formation of this α-carbanion is an obligatory step for reaction with the second substrate. When hydroxypyruvate is the substrate, the corresponding enamine (**IIIa**) can have an alternative fate. Rather than forming the required

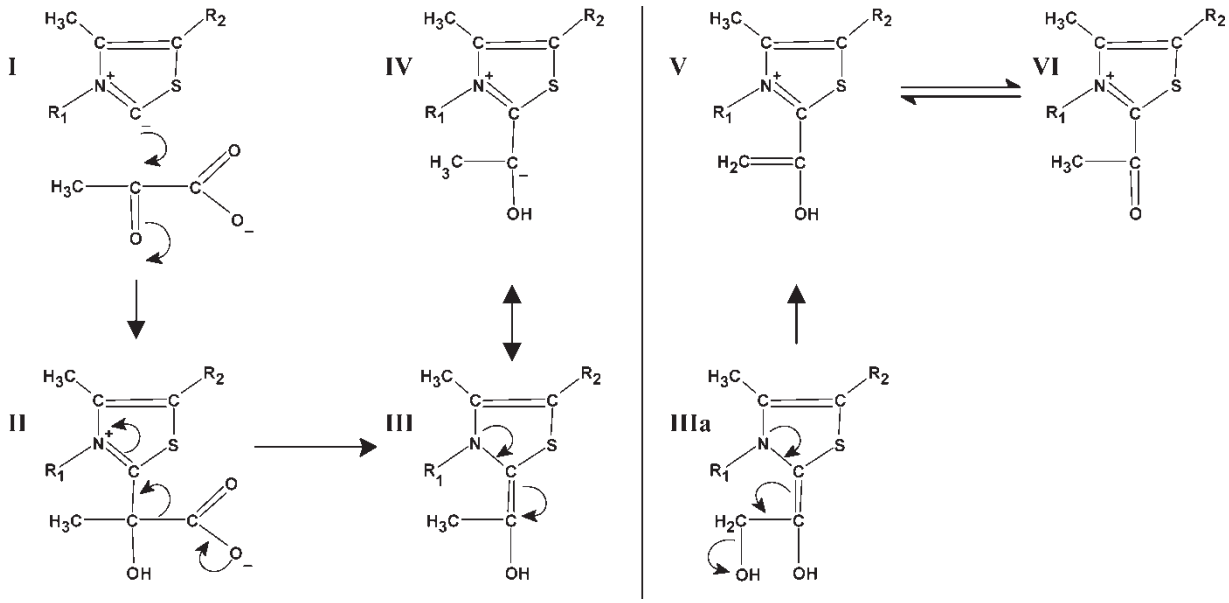

Figure 4. *Proposed mechanism of AHAS and its inactivation by hydroxypyruvate.* R_1 represents the dimethylaminopyrimidine group of ThDP while R_2 is the ethyl diphosphate side-chain of the cofactor. The left half of the scheme shows the normal reaction sequence when pyruvate is the substrate. Reaction of pyruvate with the thiazolium carbanion (**I**) gives lactyl-ThDP (**II**). This undergoes decarboxylation to the resonating enamine (**III**) and α-carbanion (**IV**) of hydroxyethyl-ThDP. The α-carbanion would then attack C2 of the second 2-ketoacid substrate. The right half of the scheme shows the postulated events when hydroxypyruvate is the substrate. The enamine (**IIIa**) can form the α-carbanion (not shown), or it can eliminate a hydroxide ion giving an enol (**V**) that will tautomerise to acetyl-ThDP (**VI**).

α-carbanion (not shown), it can eliminate a hydroxide ion giving an enol (**V**) that will tautomerise to acetyl-ThDP (**VI**). I suggest that it is this acetylated form that is the inactive enzyme. This hypothesis is based on a similar proposal for the inactivation by fluoropyruvate of the closely-related enzyme pyruvate decarboxylase, where a fluoride ion is ejected [16].

Inactivation of some ThDP-dependent enzymes by hydroxypyruvate has been reported previously [17,18]. In contrast, pyruvate dehydrogenase is not affected [17]. For another ThDP-dependent enzyme, transketolase, **IIIa** is an obligatory intermediate that is formed by reaction of the enzyme with its ketose sugar donor substrate, or with hydroxypyruvate [19]. Thus, the active site of transketolase must of necessity suppress the abortive formation of acetyl-ThDP. For pyruvate dehydrogenase, either expulsion of a hydroxide ion is suppressed or the enzyme cannot accept hydroxypyruvate as an alternative substrate.

In solution, acetyl-ThDP is hydrolysed rapidly but when bound to the active site of AHAS it is significantly more stable [20]. Hydrolysis would result in the regeneration of ThDP and recovery of AHAS activity. This process may underlie the observed progressive increase in activity illustrated in Figure 3a (inset) although exchange of bound acetyl-ThDP with unmodified ThDP in solution cannot be ruled out.

Acknowledgements

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