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# Origin of the specificities of acetohydroxyacid synthases and glyoxylate carboligase

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ABSTRACT

Acetohydroxyacid synthases (AHAS) and glyoxylate carboligase (GCL) catalyze decarboxylation of 2-ketoacids and condensation of the resulting hydroxyalkylThDP anions/enamines with a second ketoacid to form 2-acyl-2-hydroxyacids. AHASs prefer pyruvate by >10-fold over any other ketoacid as first substrate. Steric hindrance seems to be the major determinant of this specificity; *Escherichia coli* AHAS isozyme II mutant Val375Ala allows 2-ketobutyrate ($C_2H_5COCO_2^-$) to be a good first substrate and the mutant enzyme can thus synthesize 2-propio-2-hydroxybutyrate. An Ile residue in the equivalent position in GCL (Ile393) may play the analogous role in restricting GCL to glyoxylate ($HCOCO_2^-$) as first substrate. The specificity of AHAS for 2-ketoacids as acceptor substrates is due to an arginine residue which probably interacts with the carboxylate of the second substrate (*e.g.*, Arg276 in AHASII). Mutants altered at this arginine can utilize aromatic aldehydes as second substrate and form chiral arylacyl carbinols, of interest as precursors for pharmaceutical syntheses. Analysis of AHAS II supports a mechanism in which carboligation occurs after rate-determining formation of hydroxyethylThDP. NMR measurements of the distribution of ThDP-bound intermediates showed that a faster rate constant for product release when the alkyl group derived from the acceptor substrate is ethyl compared to methyl plays a major role in product specificity. The crucial role of a Trp residue (Trp 464 in AHASII) in determining specificity may be due to control of a conformational change involved in product release rather than to affinity for 2-ketobutyrate. It is significant that in AHAS I, without the required Trp and with a low specificity for 2-ketobutyrate as acceptor substrate, the product release step is rapidly reversible.

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## 1. Introduction

Acetohydroxyacid synthases (AHASs) and glyoxylate carboligase (GCL) are members of a homologous family of thiamin diphosphate (ThDP)-dependent enzymes which decarboxylate 2-oxoacids [1–3]. In contrast to the so called catabolic acetolactate synthases (sometimes referred to as "low pH" ALSs), AHASs and GCL also require FAD as a cofactor and are very similar in structure to pyruvate oxidase (POX) from *Lactobacillus plantarum*. Unlike the case of POX, the FAD seems to play a purely structural role and internal electron transfer to the flavin is a slow, off-pathway reaction. Instead, in AHASs and GCL the decarboxylation of the oxoacid to form a bound hydroxyalkyl-ThDP$^-$ anion/enamine (HA-ThDP$^-$) is followed by the specific condensation of the intermediate with a second oxoacid to form an acylhydroxyacid (Scheme 1). While the role of the enzyme in the first stages of catalysis, *i.e.*, activation of ThDP, addition of the ThDP ylide to the carbonyl group, and decarboxylation of the substrate-ThDP adduct to form the HA-ThDP$^-$ [4], is comparable to the function of other ThDP-dependent decarboxlases [5–8], the factors controlling substrate specificity and the fate of the HA-ThDP$^-$ have not been well understood until recently. The work that will be described here has been carried out over the last five years in a collaboration between laboratories in Beer-Sheva and in Halle, and many of the experimental results have been published. This paper will attempt to integrate these results into a coherent mechanistic and structural picture.

### 1.1. Physiological function and specificity

AHAS has a crucial role in the biosynthesis of the 3 branched-chain aminoacids valine, leucine and isoleucine, so that the specificity of an AHAS, or of each of the multiple AHAS isozymes in, *e.g.*, *Eschericha coli* has been subjected to selective pressures

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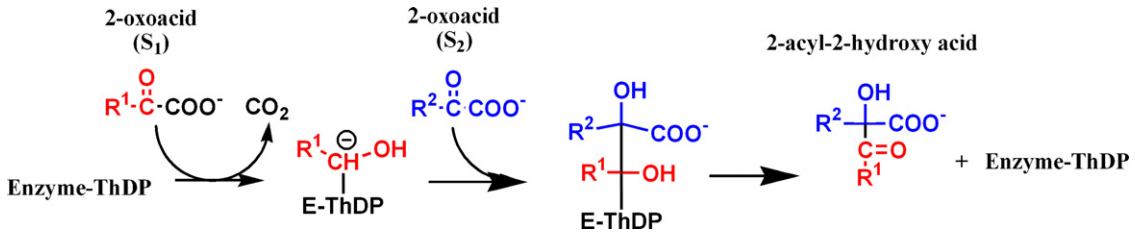
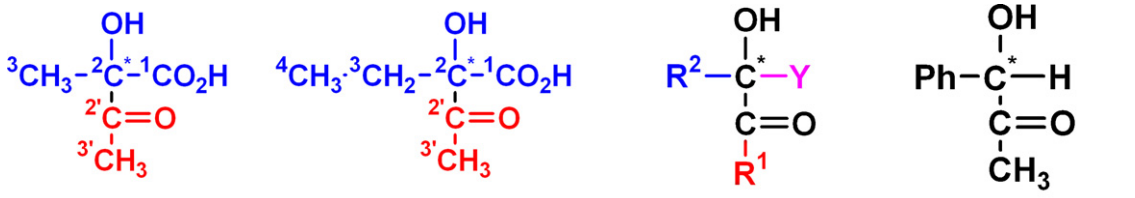



**Scheme 1.** The reaction pathway for AHASs and GCL.




**Scheme 2.** Products of decarboxylase–carboligase enzymes.

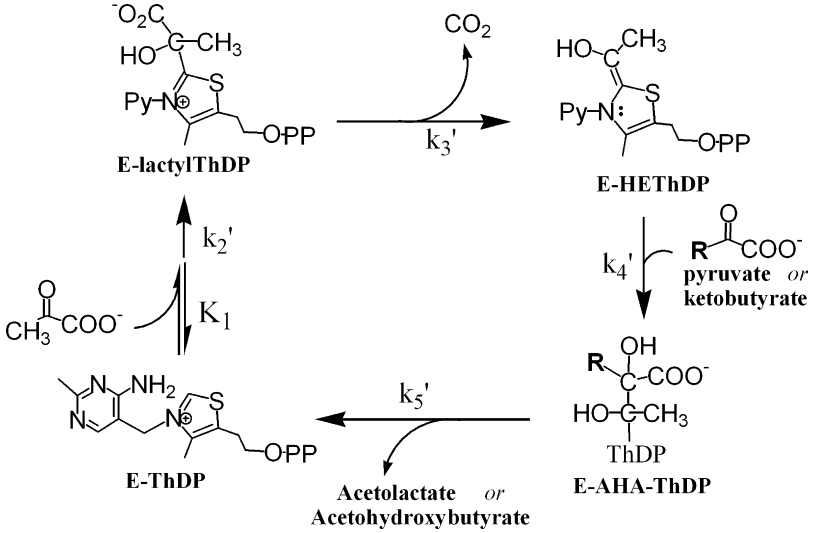
[9,10]. Under normal physiological conditions, the only products formed by AHAS are acetolactate, **1**, the precursor of valine, leucine and pantothenic acid, or acetohydroxybutyrate, **2**, the precursor of isoleucine (Scheme 2). Every AHAS ever examined can produce *both* products and the ratio of the rates of formation of **1** and **2** by a given enzyme depends on the ratio of the concentrations of 2-ketobutyrate and pyruvate, and on the selective preference of the enzyme in question [11]. Experiments carried out two decades ago [11,12] indicated that the two substrates of AHAS react in a ping-pong kinetic mechanism, with release of $CO_2$ from the first substrate preceding the association of the second (Schemes 1 and 3).

GCL has a rather different biological role, and is found in a more limited range of microorganisms. It converts glyoxylate ($O=C-COO^-$) into $CO_2$ and tartronate semialdehyde (**3** in Scheme 2, in which $R^1 = R^2 = H$, Y = COOH), which is then reduced and phosphorylated to form *R*-3-phosphoglycerate, funneling two-carbon moieties into the central glycolytic pathways [13,14]. Its substrate specificity and enantiometric preference are thus also central to its biological function.

AHAS may form alternative aryl or alkyl acylcarbinols (**3**, $R^2$ = Ar or alkyl, Y = H) [15] and GCL can catalyze the analogous reaction [16]. In order to obtain these non-physiological products *in vitro*, the reaction mixture must contain high concentrations of reactive aldehydes. Thus, these decarboxylase–carboligases must also favor 2-ketocarboxylic acids as acceptor (second) substrates. The enzymes also control the absolute configuration around C*.

## 2. Experimental approach

Three tools have been used in our laboratories to understand the origins of specificity in AHASs and GCL. The specificity of AHAS has been determined largely by competition experiments because, *e.g.*, the formation of acetohydroxybutyrate (**2**) from pyruvate and 2-ketobutyrate is always accompanied by the competing formation




**Scheme 3.** The minimal kinetic cycle for AHAS. The first step ($K_1$), in which pyruvate binds to the enzyme and the coenzyme is reversibly deprotonated, is invisible to the quench-NMR method. The addition of the ThDP ylid to the carbonyl carbon of pyruvate ($k_2'$) leads to the bound lactyl-ThDP intermediate, which decarboxylates ($k_3'$) to form bound hydroxyethylThDP anion/enamine (HEThDP$^-$). The bound HEThDP$^-$ adds to a second (acceptor) substrate ($k_4'$) to form the covalent ThDP-product compound (AHA-ThDP), which expels the product ($k_5'$) and regenerates E-ThDP.

of acetolactate (**1**). In order to ascribe specificity to enzyme structure, measured results for different enzymes (isozymes, enzymes from different organisms, or various mutants) must be compared. Measurement of the net forward rate constants for several individual steps in the reaction, and comparison of the results for related enzymes can pinpoint the specific steps.

We have previously described the method for determining the partition between acetolactate and acetohydroxybutyrate formation in AHAS isozymes and mutants, by conversion of these products to butanedione and pentanedione and simultaneous quantitative analysis of the two products by GLC using sensitive ECD detectors [17,18].

The analysis of the formation of phenylacetylcarbinol (PAC) in competition with acetolactate formation relies on a simultaneous, differential colorimetric analysis of the two products using a variation of the Westerfeld reaction [15], with the quantity of PAC produced confirmed by HPLC and its enantiomeric composition assayed by GLC on a chiral capillary column [15].

The forward (net) rate constants of elementary catalytic steps for AHAS II [19], AHAS I (Steinmetz et al., submitted) and GCL [20] in the presence of their normal substrates were determined by the quenched-flow NMR analysis method [7]. Quenched-NMR measurements of the distribution of covalent intermediates during steady-state turnover in the reactions of AHAS could not detect intermediates differing in the presence or absence of non-covalently bound substrates in the active site [19], but results were consistent with the ping-pong mechanism.

The various enzymes were expressed in *E. coli* from expression vectors as previously described [19–22], usually with a Hexahistidine peptide fused to the N-terminus of the catalytic subunit, and isolated and purified by standard methods including, when relevant, $Ni^{2+}$ affinity chromatography. Mutations were introduced using either the Overlap extension method [23] or the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA).

## 3. Results

### 3.1. Specificity for the first substrate

Abell et al. [24] provided evidence that *E. coli* AHAS II decarboxylates pyruvate 20 times faster than 2-ketobutyrate when the two substrates are present in equivalent amounts. In our laboratories in Beer-Sheva it appeared that propriohydroxybutyrate is formed as a minor product, if at all, in the reaction of the wild-type enzyme with mixtures of pyruvate and 2-ketobutyrate (M. Vyazmensky, unpublished results). As more and more genes were sequenced and the aminoacid sequences of putative ThDP-dependent enzymes accrued in the data bases, it appeared that two valine residues are located on either side of the thiazolium ring near C2 in all the enzymes whose first substrate is pyruvate. The conserved valine residue on the side of the thiazolium ring deeper in the active-site depression (farther from the enzyme surface) is V394 in *L. plantarum* POX, V497 in *Saccharomyces cerevisiae* AHAS, V391 in AHAS I and V375 in AHAS II.

The crystallographic structure of AHAS from *S. cerevisiae* in the presence of a herbicide [25]) suggests that the valine residue forms, together with a highly conserved phenylalanine and glutamine and the isoalloxazine ring of bound FAD, a tight pocket that would accommodate the methyl moiety of the first substrate (Fig. 1). In AHAS II, these residues would be Val375, Phe 109 and Gln110. Examination of the structure of several intermediates in the catalytic cycle of LpPOX by cryocrystallographic techniques show close contact between the methyl group of, *e.g.*, lactylThDP and Val394 [26]. It was striking to note that in GCL, whose donor (first) substrate

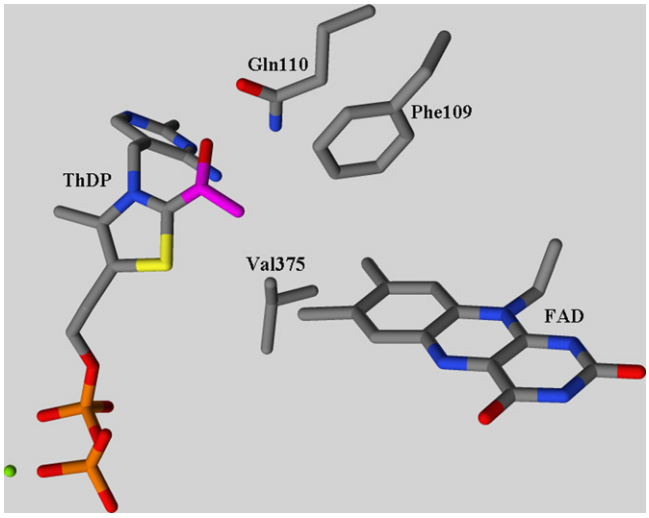



**Fig. 1.** HEThDP in the active site of AHAS II. The magenta atoms are the two carbon atoms derived from pyruvate. The groups shown here (the side-chains of Val375, Phe 109 and Gln110, as well as the isoalloxazine moiety of FAD) are in contact with the methyl group of HEThDP.

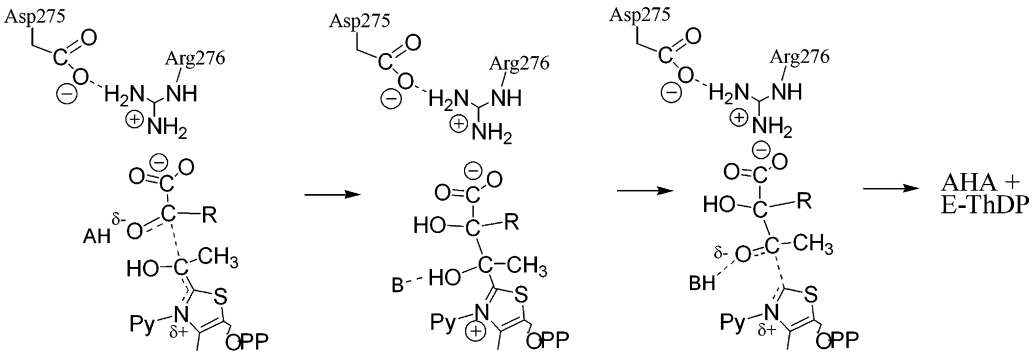
is glyoxylate rather than pyruvate, the residue equivalent to the conserved valine is isoleucine (I393) [27].

We prepared the directed AHAS II mutants Val375Ala and Val375Ile and examined their reactions. In steady-state experiments in Beer-Sheva which were analyzed by colorimetric assay and GLC with ECD detection, we found that the Val375Ala mutant is able to synthesize propiohydroxybutyrate (PHB) from 2-ketobutyrate twice as fast as it can carry out the conversion of pyruvate to acetolactate. The reactions of Val375 mutants of AHAS II were studied in more detail in Halle and showed the same inversion of specificity for the first substrate in Val375Ala. In addition, the dissociation constants of the substrate analogues methylacetylphosphonate and methylpropionylphosphonate show a similar inversion of specificity. The dissociation constants of methylacetylphosphonate are 3 and 30 μM in the wild-type enzyme and Val375Ala mutant, respectively while the dissociation constants for the 2-ketobutyrate analog methylpropionylphosphonate are 460 μM and 9 μM. Methylacetylphosphonate binds to the Val375Ile mutant with a dissociation constant of 200 μM while $K_d$ for methylpropionylphosphonate is immeasurably large. The properties of these mutants are decribed elsewhere (Steinmetz et al., submitted).

Preliminary work in Beer-Sheva also suggests that the equivalent residue in GCL, Ile393, is responsible in large part for the discrimination of this enzyme against pyruvate. Wild-type GCL produces tartronate semialdehyde (TSA) from glyoxylate with a $k_{cat}/K_m$ more 500 times its $k_{cat}/K_m$ for AL production from pyruvate [16]. The GCL mutant Ile393Ala is a crippled enzyme which is poor catalyst for the normal reaction producing TSA, but this reaction is only 2.5 time more effective than its production of AL from pyruvate.

### 3.2. The second substrate: charge is important

Although several AHASs will produce phenylacetylcarbinol (PAC; **4**) in the presence of pyruvate and benzaldehyde, none of the wild-type isozymes in bacteria are particularly efficient at this. They all preferentially produce acetolactate, even at benzaldehyde concentrations high enough to significantly inhibit the enzyme. However, replacement of the highly conserved arginine residue Arg276 in AHAS II by methionine, glutamine, or even lysine, leads to an enzyme which will catalyze the formation of PAC at least an order of magnitude faster than formation of acetolactate, in the presence of a standard reaction mixture with 30 mM each of pyruvate




**Scheme 4.** Proposed interactions in the transition states for steps 4 and 5 in the reaction of AHAS II.

and benzaldehyde [28]. We concluded that an ion-pair interaction between the carboxylate of pyruvate or 2-ketobutyrate and the positively charged side-chain of Arg27 stabilizes the transition states either for the condensation of HEThDP$^-$ with a second ketoacid, or for its release, or both (Scheme 4).

The Arg276Lys mutant is the most efficient AHAS II construct for PAC synthesis: its activity in the presence of pyruvate alone is nearly 20% that of the wild-type enzyme, while its activity in the standard PAC reaction is even higher. Analysis of the elementary steps of the carboligation process in AHAS II [19] showed that $k_4'$ is nearly two orders of magnitude lower for Arg276Lys and $k_5'$ is about 5 times lower, than for the wild-type protein. It was somewhat surprising to observe that replacement of Arg276 with Lys, which also has a long side-chain terminating in a positively charged nitrogen, is effective in slowing the normal carboligation reaction with a second ketoacid. The structure of the herbicide-inhibited *S. cerevisiae* AHAS [25] shows that the homologous arginine residue forms a close ion-pair with the aspartate residue preceding it in the protein chain, and we reasoned that a lysine residue replacing Arg276 would be held away from the reacting substrate and be effectively neutralized by this interaction.

To our surprise, AHAS I behaves differently in this regard. Replacement of the arginine in this position with glutamine (Arg289Gln) lowers $k_{cat}/K_m$ for the normal AHAS reactions by 400-fold and allows benzaldehyde to compete very effectively with pyruvate. The AHAS I Arg289Lys mutant, on the other hand, has a $k_{cat}/K_m$ for the normal reactions only 10-fold lower than wild-type, and is not significantly better than wild-type AHAS I at catalyzing the synthesis of PAC (I. Belenky, unpublished results).

The behaviour of GCL in this regard is similar [16]. Replacement of Arg284 with glutamine allows GCL to catalyze the formation of 2-hydroxyacetophenone (the spontaneously formed tautomer of phenylformylcarbinol, Scheme 5) in the presence of benzaldehyde, in competition with the formation of TSA, although both reactions are far slower than in the wild-type. The Arg284Lys mutant, on the other hand, is almost as active in the normal GCL reaction as is the wild-type, but just as inefficient at reacting with benzaldehyde.

### 3.3. The second substrate: the preference for 2-ketobutyrate

As emphasized in Section 1, the role of AHAS in the biosynthesis of Ile, Val, Leu and pantothenic acid requires that the wild-type isozymes synthesize both acetolactate (**1**) and acetohydroxybutyrate (**2**). The 3 AHAS isozymes of the enterobacteria have significant differences in substrate specificity—AHAS I will produce **1** and **2** at comparable rates in a reaction mixture with equimolar concentrations of the two ketoacid substrates, while AHAS II and III synthesize 60 and 40 times more **2** than **1**, respectively, under the same condtions [11,16,27]. The single AHASs encoded in other bacteria, plants and fungi have preferences for synthesis

**Table 1**
Analysis of kinetics of AHAS variants.

| Enzyme | R[b] | Reaction[c] | $k_{cat}$ ($s^{-1}$) | $k_2'$ ($s^{-1}$) | $k_3'$ ($s^{-1}$) | $k_4'$ ($s^{-1}$) | $k_5'$ ($s^{-1}$) |
|---|---|---|---|---|---|---|---|
| AHAS II | | | | | | | |
| Wild-type[a] | 59 | Pyr + Pyr | 20 | 24 | 530 | 1060 | 176 |
| | | Pyr + 2KB | 20 | 21 | 399 | > 2000 | > 2000 |
| Met250Ala[a] | 56 | Pyr + Pyr | 3 | 28 | 35 | 11 | 5.6 |
| | | Pyr + 2KB | 25 | 42 | 87 | 262 | > 1050 |
| Arg276Lys[a] | 28 | Pyr + Pyr | 4.6 | 9.3 | > 75 | 15 | 35 |
| | | Pyr + 2KB | 9.1 | 14.1 | > 288 | 57 | 48 |
| Trp464Leu[a] | 3 | Pyr + Pyr | 13 | 16.4 | 234 | 172 | 140 |
| | | Pyr + 2KB[d] | 13 | 16.2 | 208 | ≈ 360 | ≈ 210 |
| AHAS I[e] | | | | | | | |
| Wild-type | 1.3 | Pyr + Pyr | 69 | 83 | 490 | 3880 | 5850 |
| | | Pyr + 2KB[d] | Essentially identical to Pyr + Pyr | | | | 69 |

[a] From [19]. The individual net forward rate constants are defined by Scheme 3. See that paper for the details of the method, kinetic analysis and estimated errors for the parameters.

[b] $R$ is the characteristic 2-ketobutyrate specificity parameter for a given enzyme, defined by $R = (V_{AHB}/V_{AL})\cdot([Pyr]/[KB])$.

[c] Pyr + Pyr is the reaction at 100 mM pyruvate, forming acetolactate only. Pyr + 2KB is the reaction in the presence of 50 mM pyruvate and 50 mM 2-ketobutyrate. For the wild-type AHAS II and Met250Ala and Arg276Lys variants, the product is >98% acetohydroxybutyrate under these conditions.

[d] Because the reaction of the AHAS II Trp464Leu variant and AHAS I yield significant amounts of both products in the presence of 50 mM each of pyruvate and 2-ketobutyrate, the parameters for the reaction with 2-ketobutyrate as acceptor must be estimated from the distribution of ThDP-bound intermediates [7] and the assumption that $k_4'$ and $k_5'$ for pyruvate as the acceptor substrate is as measured with pyruvate only.

[e] Preliminary unpublished data (Steinmetz et al., submitted).

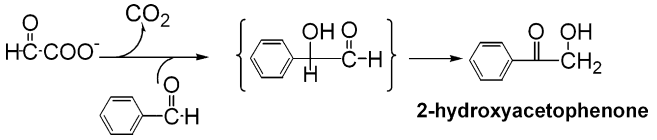
of **2** in the range of 20–60 [9]. This observation allowed us to look for differences in sequence correlated with substrate preference and to identify candidates for site-directed mutagenesis. One of these sequence differences stood out when tested by site-directed mutagenesis: a tryptophan residue which is found in all the biosynthetic AHASs with a preference for formation of **2**, but replaced by leucine in the homologous position in *E. coli* AHAS I. Replacement of Trp464 in AHAS II by *any* other aminoacid led to a sharp drop in the preference for 2-ketobutyrate as acceptor substrate, by 15–50-fold [18].

As long as one could only determine specificity, or steady-state rates of product formation as a function of substrate structure, the only conclusion one could reach was that Trp464 interacts in some way with the C4 of 2-ketobutyrate. However, the notion that a single added methyl group could lead to amplification of the energy of interaction of some species with the active site ($\Delta\Delta G$) by some $-2.5$ kcal mol$^{-1}$ was not easily explained by the binding energy of a single "extra" methyl group with a protein site, even with the indole moiety of Trp as a highly polarizable contributor to the van der Waals interactions.

Application of the quenched-NMR method to determination of the individual rate constants for the enzymatic reaction provided a new framework for considering the specificity of AHASs [19]. The results (Table 1) show that *both* the carboligation step ($k_4'$) and the product release step ($k_5'$) are significantly faster for the reaction with 2-ketobutyrate than with pyruvate, in AHAS II and in its variants which retain a strong preference for reaction with 2-ketobutyrate (R). Further, the specificities of steps 4 and 5 might be cumulative: *If* the formation of acetolactate-ThDP in step 4 has a low forward commitment factor (a smaller partition between product release in step 5 and the reversal of step 4 to release pyruvate and regenerate HEThDP) this would allow 2-ketobutyrate another chance to react. In effect, high specificity would in part be due to "kinetic proofreading" [29].

This interpretation still leaves open a critical question about the structural origin of the specificity. Why should the release of acetohydroxybutyrate (**2**) from the enzyme-bound AHA-ThDP be *faster* than release of **1**, and why should this rate differential require a Trp residue? The model of the acetohydroxybutyrate-ThDP adduct bound to wild-type AHAS II (Fig. 2) shows that the opening for departure of the product is quite narrow, and suggests that step 5 would require a conformational change, perhaps involving movement of the two subunits relative to one another. Duggleby's group showed that the region of the active site in yeast AHAS is quite disordered in crystals of the active enzyme [30], and that the binding of a sulfonylurea herbicide orders many of the disordered loops [25,31]; it is conceivable that the disordered structure of the free enzyme is relevant to product release. Does the "extra methyl" group of **2** accelerate this conformational change via contacts it makes as the conformation changes? Can we conclude that the presence of the critical Trp residue, with its large indole ring, somehow accelerate such a change (compare the rates for the Trp464Leu variant with those for wild-type, in Table 1).

A further challenge for understanding specificity is raised by AHAS isozyme I, with little or no preference for formation of **2**. In this isozyme, the intermediate HEThDP accummulates to only a small extent in the steady-state, and the calculated rates of steps 4 and 5 are fast and similar with pyruvate and 2-ketobutyrate as acceptor substrates (Steinmetz et al., submitted). The facile reversibility of the second phase of the reaction allows AHAS I to convert acetolactate to PAC with almost 100% yield, in the presence of benzaldehyde [15,32].




**Scheme 5.** Mechanism for formation of 2-hydroxyacetophenone from glyoxylate and benzaldehyde. GCL catalyzes the first step, and the enzyme product phenylformylcarbinol tautomerizes spontaneously.

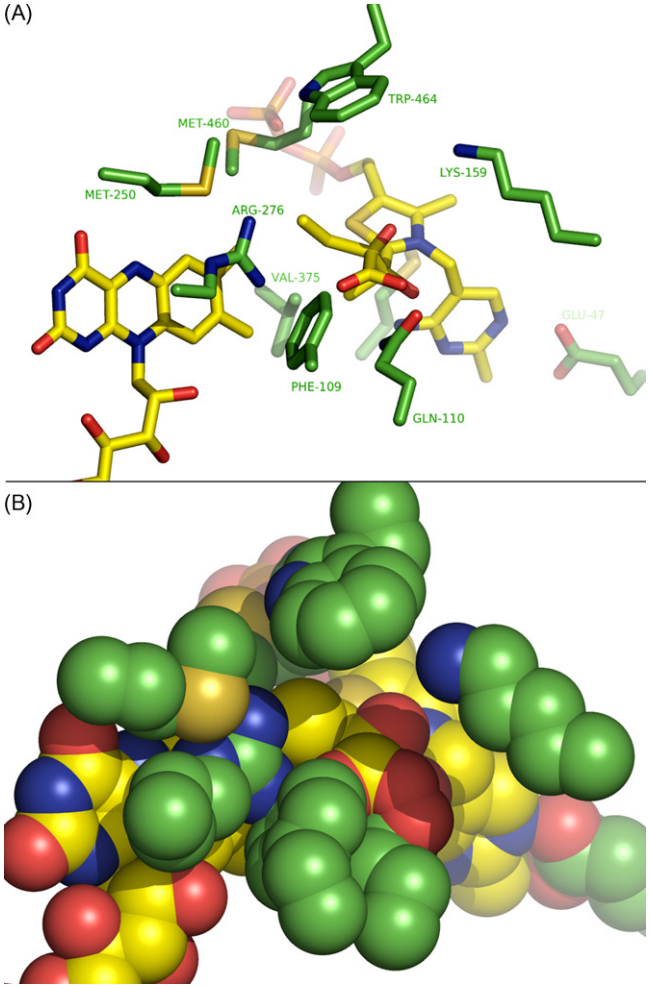



**Fig. 2.** Model of the C2 ThDP adduct of the product acetohydroxybutyrate (**2** in Scheme 2) in AHAS II. (A) Stick representation coloured by element. Side-chains of the protein have carbons in green and FAD and the ThDP adduct have carbons in light yellow. Several side-chains whose influences have been studied by us are labelled. (B) The same structure from the same viewpoint, with atoms shown as space-filling spheres.

## 4. Conclusions

A variety of experiments in our laboratories have made it possible to describe the steps involved in the determination of the substrate and product specificities of the related ThDP-dependent decarboxylase–carboligases AHAS and GCL. We can construct plausible models for several of the covalent ThDP-bound intermediates along the reaction pathway in some cases, but these models need more experimental support (perhaps from crystal structures of bound analogs). We can be fairly certain that three rather different kinds of effects are involved in the specificity of these enzymes: Steric exclusion (restricting the first substrate of AHAS to pyruvate and of GCL to glyoxylate), coulombic interactions (leading to preference for an oxoacid as acceptor substrate, dependent on a conserved arginine) and effects of product structure on a conformational change involved in product release, whose details remain

to be clarified. This group of enzymes reminds us once again that the specificity of an enzymatic process cannot be separated from the enzyme's reaction mechanism.

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