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Significant Catalytic Roles for Glu47 and Gln 110 in All Four of the C−C Bond-Making and -Breaking Steps of the Reactions of Acetohydroxyacid Synthase II

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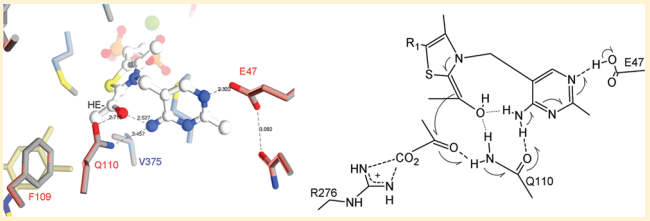
Supporting Information

ABSTRACT: Acetohydroxy acid synthase (AHAS) is a thiamin diphosphate (ThDP)-dependent enzyme that catalyzes the first common step in the biosynthesis of branched-chain amino acids, condensation of pyruvate with a second 2-ketoacid to form either acetolactate or acetohydroxybutyrate. AHAS isozyme II from *Escherichia coli* is specific for pyruvate as the first donor substrate but exhibits a 60-fold higher specificity for 2-ketobutyrate (2-KB) over pyruvate as an acceptor substrate.



In previous studies relying on steady state and transient kinetics, substrate competition and detailed analysis of the distribution of intermediates in the steady-state, we have identified several residues which confer specificity for the donor and acceptor substrates, respectively. Here, we examine the roles of active site polar residues Glu47, Gln110, Lys159, and His251 for elementary steps of catalysis using similar approaches. While Glu47, the conserved essential glutamate conserved in all ThDP-dependent enzymes whose carboxylate is in H-bonding distance of the ThDP iminopyrimidine N1′, is involved as expected in cofactor activation, substrate binding, and product elimination, our studies further suggest a crucial catalytic role for it in the carboligation of the acceptor and the hydroxyethyl-ThDP enamine intermediate. The Glu47-cofactor proton shuttle acts in concert with Gln110 in the carboligation. We suggest that either the transient oxyanion on the acceptor carbonyl is stabilized by H-bonding to the glutamine side chain, or carboligation involves glutamine tautomerization and the elementary reactions of addition and protonation occur in a concerted manner. This is in contrast to the situation in other ThDP enzymes that catalyze a carboligation, such as, e.g., transketolase or benzaldehyde lyase, where histidines act as general acid/base catalysts. Our studies further suggest global catalytic roles for Gln110 and Glu47, which are engaged in all major bond-breaking and bond-making steps. In contrast to earlier suggestions, Lys159 has a minor effect on the kinetics and specificity of AHAS II, far less than does Arg276, previously shown to influence the specificity for a 2-ketoacid as a second substrate. His251 has a large effect on donor substrate binding, but this effect masks any other effects of replacement of His251.

Acetohydroxyacid synthase (AHAS, EC 2.2.1.6.) is a thiamin diphosphate (ThDP)-dependent decarboxylase-carboligase with sequence and tertiary homology to the pyruvate oxidase family. It catalyzes the decarboxylation of pyruvate followed by the condensation of the resulting hydroxyethyl-ThDP (HEThDP) carbanion/enamine with another 2-ketoacid (pyruvate or 2-ketobutyrate) to form an acetohydroxyacid. In addition to ThDP, it requires a divalent metal ion and FAD, although the flavin has no catalytic function and serves a structural purpose.[1,2] AHAS is found in bacteria, algae, fungi, and higher plants and is a key enzyme in the biosynthesis of the branched-chain amino acids (BCAA) valine, leucine, isoleucine as well as in biosynthesis of pantothenate and compounds derived from it.[3−5]

The catalytic cycle of AHAS (Scheme 1) involves at least six discrete steps, beginning with formation of the reactive cofactor ylide (k_0'), binding of pyruvate to the active center (K_1) and subsequent carbonyl addition (k_2') to the ThDP C2 carbanion to produce the tetrahedral intermediate 2-lactyl-ThDP (LThDP). Decarboxylation of LThDP (k_3') leads to the 2-hydroxyethyl-ThDP carbanion/enamine ($HEThDP^-$) intermediate. In the second phase of the reaction, $HEThDP^-$ undergoes carboligation with either pyruvate or 2-KB (k_4') as alternative acceptors.

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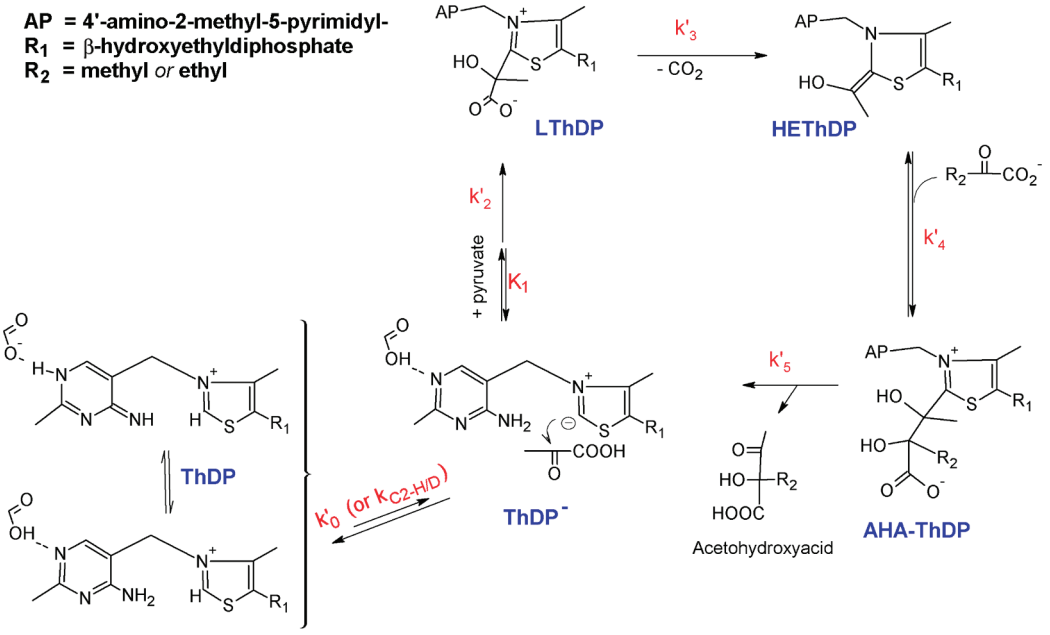
Scheme 1. Minimal Catalytic Scheme for the Mechanism of AHAS, with the Net Forward Pseudo-First Order Rate Constants As Used Here Labeled in Red



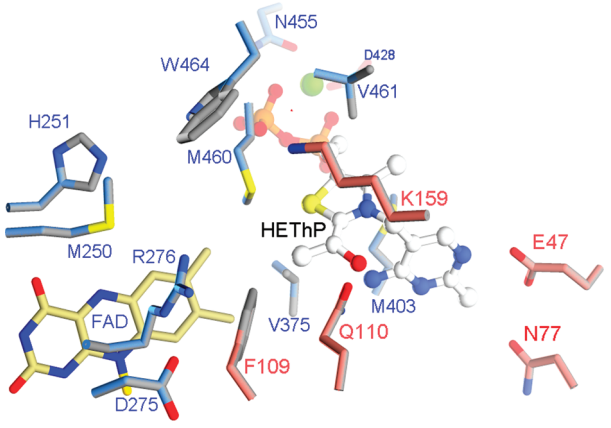


Figure 1. Active site of AHAS II. HEThDP is depicted in ball-and-stick form, with white C atoms and the remainder of the atoms colored in the CPK convention. The magnesium ion is shown as a green sphere. Amino acid sidechains are depicted in stick form for relevant residues, with the carbon atoms in the two different catalytic subunits in pale blue and pale red. FAD (isoalloxazine moiety only) is depicted with pale beige carbon atoms. The structure was drawn with UCSF Chimera[49] using a model based on the crystal structure of yeast AHAS.[7]

Liberation of the product from the covalent acetohydroxyacid-ThDP (AHA-ThDP) intermediate thus formed (k_5') completes the cycle. The several steps of the catalytic cycle involve interactions of different regions of the active site with the two substrates and with the product and have different influences on specificity.

Although the only available experimental structures for AHAS catalytic subunits are crystallographic structures of the enzymes from yeast[6−8] and *Arabidopsis thaliana*,[8−10] the most extensive studies directed at understanding the catalytic mechanism have involved AHAS isozyme II (AHAS II) from *Escherichia coli*. Homology models based on the crystal structures directed the design of experiments aimed at understanding the specificity[11,12] and catalytic mechanism of AHASs.[4,12−14] The roles of the conserved residues Glu47, Thr70, Met250, Arg276, Asp428, Met460, and Trp464 (Figure 1) have each been studied by site-directed mutagenesis and examination of the kinetics and/or specificity of the variants.[12,13,15−17] In a recent paper,[18] we studied residues Val375 and Phe109 of AHAS II, which influence chiefly the first phase (steps 1−3, Scheme 1) of the catalytic cycle and were able to define their roles more precisely. In the present paper, we consider the roles of the polar residues whose positions in the active site suggest they might interact with the cofactor or reacting substrates; Glu47, Gln110, Lys159, and His251 (Figure 1). In this context, we aimed at identifying the active site general acid/base catalysts required for the individual catalytic steps, in particular for carboligation of the carbanion/enamine intermediate and the acceptor. Previous studies on related ThDP enzymes that similarly catalyze a carboligation of two substrates such as transketolase or benzaldehyde lyase indicated that histidine residues are likely to protonate the acceptor carbonyl in these cases.[19−22]

Glu47 is the presumptive conserved "essential glutamate" in AHAS II (Supplemental Figure S1 in the Supporting Information), which plays a critical role in the ionization and tautomerization of the coenzyme (Scheme 2).[23−25] Our earlier study of variants at this position was limited to steady-state kinetics and the rate of H/D exchange at the thiazolium C2−H of ThDP,[26] but consideration of the proposed mechanism (Scheme 1) and the presumed active site architecture predict that it might be an important player in every step of the reaction.

The other polar residues we consider here might also be involved in both phases of the catalytic process, decarboxylation and carboligation. Gln110 is in H-bonding distance to the active center ThDP (Figure 1) and highly conserved in AHASs and

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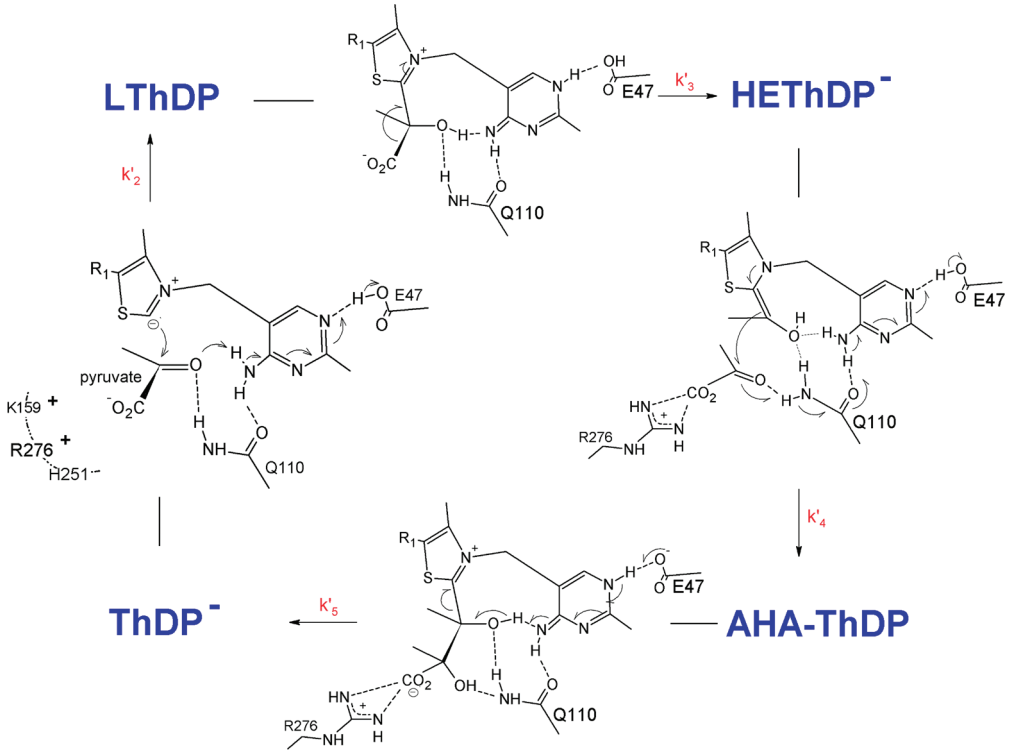
Scheme 2. Four Steps of the Catalytic Cycle Expanded to Show the Roles of Residues in the Active Site in Each Step, As Proposed on the Basis of the Results of This Study and Some Previous Data



in the related glyoxylate carboligase[27] and catabolic acetolactate synthases[1] (Supplemental Figure S1 in the Supporting Information). All of these enzymes are decarboxylase-carboligases. Mutation of Lys251 in yeast AHAS (analogous to Lys159 in AHAS II, see Supplemental Figure S1 in the Supporting Information) to Thr leads to resistance to sulfonylurea herbicides,[28,29] and this lysine is in close contact with the bound sulfonylurea in the inhibited wild-type enzyme.[7] It was suggested that the charge on this lysine, or on the other positively charged group in the "active site channel" (Arg276 in AHAS II), might interact with an acceptor carboxylate.[29] His251 is another polar residue close to the putative active site which is conserved in AHASs.

We have now applied the kinetic and thermodynamic tools at our disposal to these four polar residues and describe the conclusions one can reach concerning their involvement in the catalytic process.

MATERIALS AND METHODS

The reagents as used in this work were as previously described.[18] Plasmids which express variant AHAS II were prepared from plasmid pQEV-GM.[15] Mutations at positions corresponding to residues Gln110, Lys159, and His251 were introduced using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA), while the Overlap Extension method[30] was used in mutagenesis at Glu47. Each plasmid used for protein expression was sequenced.

Expression and Purification. Bacterial growth for protein preparation was carried out under two slightly different conditions. For variants at Gln110 and His251, growth was carried out as described previously.[26] In studies of AHAS II variants at Lys159, expression was induced with 0.5 mM IPTG at 18 °C overnight. This led to an isolated wild type enzyme with a 2-fold higher specific activity than the preparation prepared under the previous growth conditions, presumably because a smaller fraction of the protein was improperly folded. Wild type AHAS II and its variants described here were obtained as N-terminal hexahistidine-tagged proteins overexpressed in XL-1-Blue MRF′ (Stratagene, La Jolla, CA) and were purified on Ni^{2+}-nitrilotriacetic acid-agarose (Qiagen, Hilden, Germany) as described elsewhere.[15] The proteins were stored at −20 °C in 50 mM potassium phosphate buffer (pH 7.6) containing 20 μM FAD, 1 mM DTT, and 50% glycerol. Protein concentration was measured by the method of Bradford[31] using bovine serum albumin as a standard.

Catalytic Properties. Enzyme assays were carried out using the methods previously described[26,32] in 0.1 M potassium phosphate buffer (pH 7.6) containing 10 mM $MgCl_2$, 0.1 mM ThDP, and 75 μM FAD, with 100 mM pyruvate as the substrate, except where otherwise indicated. K_M for pyruvate and $K_{0.5}$ for ThDP in acetolactate (AL) formation were determined in the above buffer and reaction medium at 37 °C.

The variants with Ala, Asn, His, or Glu in place of the conserved Gln110 were studied earlier and independently of the variants at Glu47, Phe109, Lys159, and Val375, which are described here and in a recent report.[18] The control wild type protein in this case had half the activity observed for the wild type sample used in parallel with variants at the other positions. Details of the standard protein isolation in this case were slightly different, in particular the conditions for induction of expression (see Expression and Purification, above). The results here refer

to the Glu110 variants, and these are compared with the wild type prepared in parallel with them.

The characteristic substrate specificity of a given enzyme for 2-KB as second substrate, R, is defined by $R = (V_{AHB}/V_{AL}) \times ([Pyr]/[2\text{-KB}])$,[33] and was determined in a competition experiment with 2-KB and pyruvate under the standard conditions, by measuring AHB and AL formation simultaneously.[34] The results were analyzed by fitting the data with the program SigmaPlot 2000 (SPSS, Inc.). At a given pyruvate concentration, the total rate of production of the acetohydroxy acids by wild type and many variants of AHAS II is constant,[15,17,34] and the 2-KB dependence of the individual rates (V_{AL} and V_{AHB}) can be fit simultaneously to eqs 1 and 2,

$$V_{AL} = V_{max}\left(\frac{[Pyr]}{[Pyr] + R[2\text{-KB}]}\right) \quad (1)$$

$$V_{AHB} = V_{max}\left(\frac{R[2\text{-KB}]}{[Pyr] + R[2\text{-KB}]}\right) \quad (2)$$

where [2-KB] is the concentration of 2-KB, and V_{AHB} and V_{AL} are the rates of formation of AHB and AL. For variants where the total rate ($V_{AL} + V_{AHB}$) is not constant, one can conclude that the second phase of the reaction cycle (Scheme 2), including steps 4 and 5, is partially rate-determining.[15]

Reactions with pyruvate and benzaldehyde were carried out as described previously[15] at 30 °C in the presence of 0.1 M Hepes/KOH (pH 7.0), 5 mM $MgCl_2$, 0.1 mM ThDP, 50 μM FAD, 1 mM DTT, 10% DMSO, 30 mM pyruvate, and 30 mM benzaldehyde, with a colorimetric method for the simultaneous assay of AL and phenylacetylcarbinol (PAC).[35] The enantiometric purity of the PAC formed was determined using GLC analysis.[35]

For GLC−MS analyses, a RTX-1 30 m capillary column (Supelco, Bellefonte, PA) was used with helium as the carrier gas, on a GLC instrument equipped with a Hewlett-Packard 5971 mass selective detector. The temperature regime was 5 min at 50 °C, followed by a linear increase to 250 °C at 15 °C/min.

Net Forward Unimolecular Rate Constants. The analysis of covalent reaction intermediates by ^{1}H NMR spectroscopy after acid quench isolation and calculation of the net forward unimolecular rate constants of Scheme 1 was carried out as recently described by us.[36]

Analysis of Methyl Acetylphosphonate Binding by CD. Methyl acetylphosphonate (MAP), an analogue for the substrate pyruvate, adds to C2 of ThDP yielding the tetrahedral compound analogous to the predecarboxylation intermediate LThDP. It has been shown that formation of such carbonyl adducts of ThDP is associated with formation of the aminopyrimidine 1′,4′-imino tautomer of ThDP with a CD signal whose maximum is around 300−310 nm.[25,37] CD titration experiments were carried out as previously described.[18]

Kinetic Analysis of MAP Binding by Stopped-Flow Absorbance. The binding kinetics of the donor substrate analogue MAP were analyzed as previously described[18] by transient stopped-flow kinetics relying on the UV absorbance signal at 310 nm of the 1′,4′-iminotautomeric form of the resultant covalent adduct. Apo-FAD AHAS II was reconstituted with 0.2 mM ThDP and 1 mM Mg for 10 min at 37 °C. The enzyme was then reacted with defined concentrations of MAP (0−40 mM) dissolved in the same buffer as the enzyme, in a 1 to 1 mixing ratio at 37 °C using a thermostatted SX20 or SX18 stopped-flow spectrophotometer from Applied Photophysics (Leatherhead, U.K.) and an optical path length of 1 cm. A total of 2000 data points at 310 nm were collected over 1 s. Experiments were carried out in triplicate, and the averaged transients were fitted to a single exponential equation unless otherwise stated, using programs SigmaPlot 11 and KaleidaGraph 3.08. The derived rate constants k_{obs} showed hyperbolic dependence on the MAP concentration, as expected for a reversible two-step mechanism with rapid equilibrium formation of a Michaelis complex:

$$\text{E-ThDP} + \text{MAP} \overset{K_1}{\rightleftharpoons} \text{E-ThDP*MAP} \quad \text{(Michaelis complex)} \quad (3)$$

$$\text{E-ThDP*MAP} \overset{k_{+2}}{\underset{k_{-2}}{\rightleftharpoons}} \text{E-MAP} - \text{ThDP} \quad \text{(covalent adduct)} \quad (4)$$

The dependence of k_{obs} on the MAP concentration was fit to eq 5.

$$k_{obs} = k_{-2} + \frac{k_{+2}[MAP]}{K_1 + [MAP]} \quad (5)$$

RESULTS

Glutamate 47. In AHAS II, the "conserved essential glutamate" that plays a critical role in the ionization and tautomerization of the coenzyme is Glu47.[24,25] Our original study of variants at this position was limited to steady-state kinetics and the rate of H/D exchange at the thiazolium C2−H.[26] In the present work we prepared two variants of AHAS II, with Glu47 replaced by Ala and Gln and purified and analyzed them in parallel in greater detail. The properties of these variants are summarized in Table 1. The unimolecular rate constants of H/D exchange at C2 (k_0') are decreased about 50- and 40-fold, respectively, in the Ala and Gln variants but are larger than k_{cat} in both cases demonstrating that cofactor activation is not rate-limiting. The decrease in k_0' is expected, but it is important to note that this decrease is by a considerably smaller factor than is observed in several other ThDP-dependent enzymes.[24,38] The catalytic efficiency (k_{cat}/K_M) of these two variants is reduced to a lesser degree, by 16- and 30-fold, as the ionization of C2−H is only one of a number of steps in the catalytic process. More significantly, every one of the net forward rate constants in the catalytic cycle is reduced by at least 10-fold in variant E47Q, as estimated by NMR-based analysis of the intermediate distribution at steady-state (Table 1, Figure 2). This finding indicates that the Glu47-aminopyrimidine proton shuttle is critically involved in all major catalytic steps: carbonyl addition of donor, decarboxylation, carboligation, and product release.

Methyl acetylphosphonate (MAP) was used as an analogue for the substrate pyruvate to kinetically and thermodynamically characterize binding of the donor substrate. The MAP carbonyl adds to C2 of ThDP yielding a tetrahedral compound analogous to the predecarboxylation intermediate LThDP. Because of the stable C2α−P bond, this tetrahedral adduct can only revert to ThDP and MAP.[39] The formation of such carbonyl adducts of ThDP is associated with formation of the aminopyrimidine 1′,4′-imino tautomer which gives rise to a CD signal with a maximum around 300−310 nm.[25,37] CD titration experiments were carried out as previously described.[18] Although the

Table 1. Properties of AHAS II Glu47 Variants

reaction measured	parameters	enzyme: wild type	enzyme: E47A	enzyme: E47Q
2 pyruvate → AL[a] (steady state)	K_M, mM	6.6 ± 0.4	7.1 ± 0.8	7.7 ± 0.5
	k_{cat}, s^{-1}	40.3 ± 0.6	2.7 ± 0.7	1.6
	k_{cat}/K_M, M^{-1} s^{-1}	6100	375	208
	$k_0' \equiv k_{C2\text{-}H/D}$, s^{-1}	332	5.7	8
2 pyruvate → AL[b] (microscopic rate constants by quench/NMR)	k_2', s^{-1}	~48	n.d.[e]	3.9
	k_3', s^{-1}	~1000	n.d.[e]	~22
	k_4', s^{-1}	~2100	n.d.[e]	~87
	k_5', s^{-1}	~350	n.d.[e]	~18
MAP binding (by CD)[c]	K_D^{app}, μM	2.7 ± 1.4	n.d.[e]	206 ± 57
MAP binding (stopped-flow kinetics)[d]	K_1, mM	5.0 ± 1.3	n.d.[e]	15.7 ± 7.9
	k_{+2}, s^{-1}	180 ± 17	n.d.[e]	0.31 ± 0.07
	k_{-2}, s^{-1}	1.8 ± 1.2	n.d.[e]	0.04 ± 0.01

[a] The kinetic parameters of the enzymatic reaction with pyruvate as the sole substrate were determined by the colorimetric method as described in the Materials and Methods, at 37 °C, with pyruvate as the sole substrate in 0.1 M potassium phosphate buffer (pH 7.6) containing 10 mM $MgCl_2$, 0.1 mM ThDP, and 75 μM FAD. [b] Reactions were carried out with 50 mM pyruvate and quenched and analyzed by NMR analysis.[18,36] [c] Dissociation equilibrium of the enzyme-bound covalent analogue to form free holoenzyme and MAP in solution, determined at 20 °C by CD spectroscopic titration as described in the Materials and Methods. [d] Dissociation equilibrium of noncovalently bound MAP in the active site at 37 °C determined by stopped-flow absorbance spectroscopy as described in the Materials and Methods. [e] n.d., not determined.

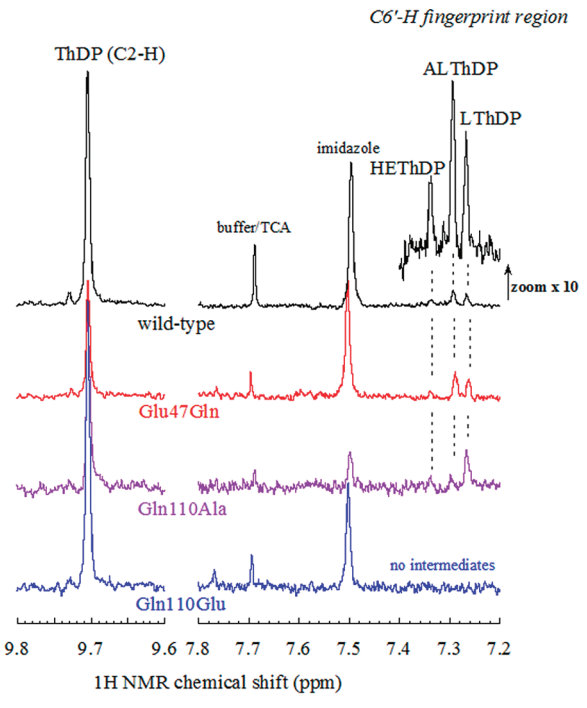


Figure 2. Distribution of reaction intermediates in AHAS II wild type (black) and variants E47Q (red), Q110A (magenta), and Q110E (blue) at steady-state as analyzed by 1D ^{1}H NMR spectroscopy after acid quench isolation. Sections of ^{1}H NMR spectra are shown, where the C6′−H and C2−H resonances of intermediates and of C2-unsubstituted ThDP are detected.

equilibrium constant K_1 for dissociation of noncovalently bound MAP from the active site of the E47Q variant is only 3-fold larger than for the wild type enzyme, the overall dissociation constant K_D^{app} $[(K_1K_2)/(1 + K_2)]$ of MAP from the enzyme-bound LThDP is nearly 75-fold larger (Table 1). It is significant to note that in the E47Q variant, the formation of the covalent adduct between MAP and ThDP is not accompanied by buildup of the prominent ThDP iminopyrimidine band at ~310 nm as observed for wild type and the other variants (in E47Q a band at $\lambda < 300$ nm is formed). This emphasizes the crucial role of Glu47 for control of the protonic and tautomeric equilibria of the bound cofactor aminopyrimidine (Figure 3). Carbonyl addition of MAP to ThDP at saturating concentrations of the analogue (k_{+2}) is ~600-fold compromised in E47Q compared to the wild type (Table 1, Figure 4).

Glutamine 110. Four variants of AHAS II with Ala, Asn, His, or Glu in place of the conserved Gln110 were prepared and studied in detail (Tables 2 and 3). Note that these proteins were studied earlier than the other variants reported here, with a different control wild type (see details in the Materials and Methods). The data in Tables 2 and 3 refer to the Glu110 variants and the wild type prepared in parallel with them.

The most active of the variants, Q110N, has a catalytic efficiency (k_{cat}/K_M) lower than that of the wild type by more than an order of magnitude. Part of this difference is due to a lower k_{cat}, but the larger part is due to a higher apparent K_M. The least active, Q110E, is some 700-fold less active. This variant also has $K_{0.5}$ for ThDP 20-fold higher than that for the wild type. The three variants Q110A, Q110N, and Q110E are characterized by increasing total activity as the concentration of 2-KB increases (see V_b/V_a in Table 2); this implies that the second phase of these reactions, which involves the acceptor substrate, is at least partially rate-determining.[15] Single step analysis of Q110A and Q110E variants by NMR-based intermediate analysis highlights the central role of Q110 for substrate binding and catalysis (Table 3, Figure 3). While each microscopic step is compromised by at least 1 order of magnitude in Q110A, paralleling the results on Glu47 variants (see above), carbonyl addition of bound pyruvate to ThDP is overwhelmingly rate-determining in the case of Q110E. There was no acetaldehyde produced in the reaction of Q110E with pyruvate, as is regularly observed with some PDC variants (data not shown). Affinity for substrate

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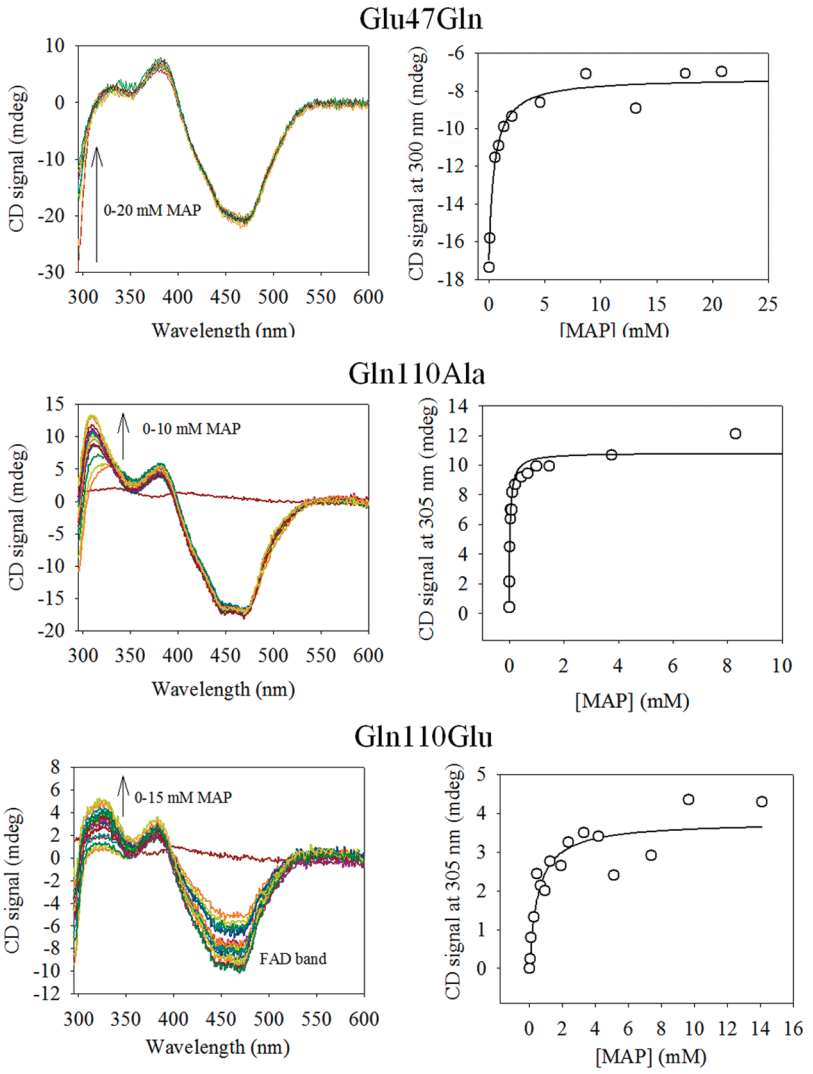

Figure 3. Thermodynamic analysis of MAP binding to AHAS variants by CD spectroscopy. (Left panel) CD spectra obtained after addition of increasing concentrations of MAP to AHAS. Experimental details are given in Materials and Methods. (Right panel) Dependence of the CD signal intensity at either 300 or 305 nm on MAP concentration and corresponding fit of the data according to eq 5.

analogue MAP is decreased by one (Q110A) or even two (Q110E) orders of magnitude in the variants, almost entirely due to the dramatically reduced rates of carbonyl addition of bound analogue to ThDP (Table 3). It is noteworthy that formation of the intermediate analogue in Q110E is associated with changes of the CD band of the flavin at ~400−470 nm, indicating changes of the local cofactor binding site.

Lysine 159. Duggleby and his co-workers showed that Lys159 is in close contact with the bound inhibitor in the wild type enzyme−sulfonylurea herbicide complex[7] and suggested that in the normal catalytic reaction the positively charged ε-ammonium group might interact with the carboxylate anion of a substrate or product of the enzyme.[40] Each of three variants of AHAS II at Lys159 (K159A, K159M, K159Q) is indeed more than 2 orders of magnitude less sensitive to the inhibitor SMM than is the wild type enzyme; mutations at the other residues studied do not show such effects on SMM sensitivity (not shown). The affinities of the variants at Lys159 for ThDP are also decreased, by 30−90-fold. On the other hand, most of their other catalytic properties are quite similar to those of the wild type (Table 4). Their catalytic competence in the formation of AL is only slightly compromised, and the poorest variant (K159M) has a value of k_{cat}/K_M only about 6-fold lower than the wild type enzyme.

We have shown previously[15] that in a competition between uncharged benzaldehyde and negatively charged pyruvate as the second acceptor substrate (to form the nonphysiological product phenyl acetyl carbinol, PAC), an AHAS II variant at the conserved arginine, R276Q, is 35-fold better than the wild type enzyme in this nonphysiological reaction (i.e., less specific in its preference for formation of AL). We could conclude that Arg276 specifically stabilizes an intermediate on the pathway to formation of an acetohydroxy acid rather than an acetyl carbinol,[15] probably by Coulombic interaction of its positive charge with the substrate carboxyl anion. In contrast, the variants at Lys159 (K159A, K159 M, and K159Q) are each only about 5- to 7-fold less selective for pyruvate than the wild type (Table 4), a significantly smaller effect than that of mutation at Arg276.[15] The product PAC formed by variant K159Q is ≥98% the *R* enantiomer, similar to the product of variant R276Q.

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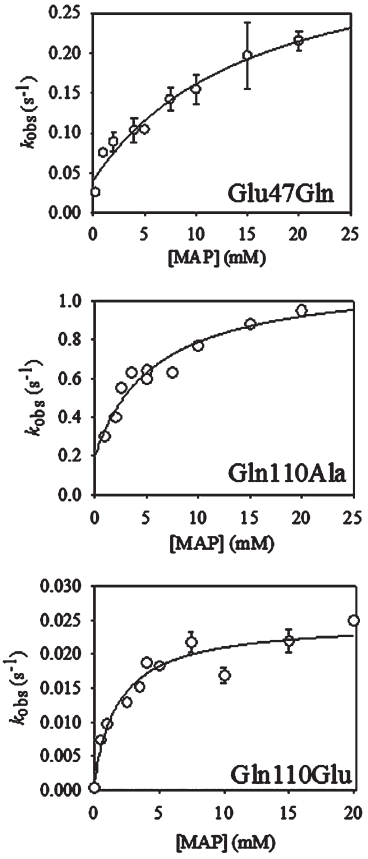

Figure 4. Kinetic analysis of MAP binding to AHAS variants by stopped-flow kinetics, following absorbance at 310 nm after addition of MAP. Details are given in Materials and Methods. Dependence of the observed rate constants on the MAP concentration is shown. Data were fitted to eq 5.

Histidine 251. Three variants were prepared in which His251 was replaced by Ala, Asn, or Gln. All three variants were about 20-fold less effective catalysts than the wild type, with about 15−25-fold higher apparent $K_{0.5}$ for ThDP (data not shown). The specificity of these variants for reaction with 2-KB in the presence of pyruvate is about half that of the wild type protein, and this difference, while moderate, is statistically significant.

The covalent intermediates in the reaction of the H251A variant were analyzed after acid quench,[18,36] but only ThDP itself (C2-unsubstituted) could be detected (data not shown). This indicates that the addition of the donor substrate (k_2') is slow and rate determining for this variant. The vital role of His 251 for donor binding is apparently also reflected in the equilibrium binding of MAP to the H251A variant, for which K_D^{app} is 1.08 mM (compared to 2.7 μM for the wild type). The observed pseudofirst order rate constant of association of MAP determined by stopped-flow absorbance spectroscopy shows a linear rather than a hyperbolic dependence on [MAP], with $k_{+2}/K_1 \sim 7.7$ mM^{-1} s^{-1} and $k_{-2} = 2$ s^{-1}, indicating that His251 is critically required for formation of the Michaelis complex in the course of substrate binding.

DISCUSSION

The paradigmatic role of the highly conserved glutamate in ThDP-dependent enzymes, in a position to form a hydrogen bond to N_1' of the cofactor, is participation in the aminopyrimidine−iminopyrimidine tautomerization of the cofactor and promotion of the abstraction of the thiazolium C2−H proton by the C4′−N (Scheme 1, k_1').[24,38,41,42] The data for the two variants studied here, E47A and E47Q, fit this paradigm and further demonstrate the significant effects of Glu47 on all of the individual net forward rate constants for AL formation. This implies that the amino-iminopyrimidine tautomer couple plays an enabling role in a proton shuttle that first abstracts the proton from C2, returns it to the carbonyl of the substrate in the course

Table 2. Steady-State Kinetic Properties of AHAS II Q110 Variants

	enzyme				
parameters [a]	wild type	Q110A	Q110N	Q110H	Q110E
specific activity, U mg^{-1}	16.6 ± 0.9	2.3 ± 0.1	4.9 ± 0.4	1.4 ± 0.1	0.35 ± 0.02
K_M, mM	6.9 ± 1.4	30.2 ± 2.8	29.5 ± 3.7	40.4 ± 4.7	110.6 ± 12.8
k_{cat}, s^{-1}	19.5 ± 1.1	2.7 ± 0.1	5.8 ± 0.5	1.6 ± 0.1	0.41 ± 0.02
k_{cat}/K_M, M^{-1} s^{-1}	2830	90	200	40	4
$K_{0.5}$ for ThDP,[b] μM	1.2 ± 0.1	0.80 ± 0.04	5.7 ± 0.3	16.1 ± 0.9	24.3 ± 1.9
K_i for SMM, μM	0.92 ± 0.08	8.1 ± 1.0	0.65 ± 0.11	2.4 ± 0.2	3.7 ± 0.3[e]
R[c]	59 ± 3	33 ± 5	19 ± 3	32 ± 6	50 ± 6
V_b/V_a[d]	~1	1.8	2.2	~1	4.1

[a] The kinetic parameters of the enzymatic reactions were determined as described under Material and Methods and in footnote a of Table 1. [b] The enzymatic reactions of AHAS II wild type and variants were carried out in 0.1 M potassium phosphate buffer (pH 7.6) containing 10 mM $MgCl_2$, 75 μM FAD, and varying concentrations of ThDP. The pyruvate concentration was 40 mM in the reaction with wild type, 100 mM in the reactions with the variants Q110A, Q110N, and Q110H, and 400 mM pyruvate in the reaction with the variant Q110E. $K_{0.5}$ is defined as the concentration of ThDP that is required for half-maximum of AHAS II activity under the conditions used with each variant. [c] The specificity for 2-KB as the second substrate, R, is defined by $R = (V_{AHB}/V_{AL})([Pyr]/[2\text{-KB}])$, where [2-KB] is the concentration of 2-KB and V_{AHB}/V_{AL} is the relative rate of formation of AHB and AL, respectively. R was determined with 50 mM pyruvate and the varying concentrations of 2-KB under standard conditions by measuring AHB and AL formation in a competition experiment.[34] The results were analyzed by fitting the data to eqs 1 and 2 with the program SigmaPlot 2000 (SPSS, Inc.). [d] At a given constant pyruvate concentration, the total rates of the acetohydroxy acid production for variants Q110A, Q110N, and Q110E are not constant. V_a is the rate in the absence of 2-KB, and V_b is the rate of AHB formation at saturation with 2-KB.[15] [e] In this case, the enzymatic reaction was carried out with 400 mM pyruvate in 0.1 M potassium phosphate buffer (pH 7.6) containing 10 mM $MgCl_2$, 0.1 mM ThDP, 75 μM FAD, and varying concentrations of SMM.

Table 3. Properties of AHAS II Variants at Gln110[a]

reaction measured	parameters	enzyme: wild type	enzyme: Q110A	enzyme: Q110E
2 pyruvate → AL[b] (steady state)	K_M, mM	6.6 ± 0.4	29.5 ± 1.7	128 ± 29
	k_{cat}, s^{-1}	40.3 ± 0.6	4.1 ± 0.1	2.6 ± 0.5 n_H = 1.4 ± 0.1
	k_{cat}/K_M, M^{-1} s^{-1}	6100	138	21
	R[c]	55.1 ± 3.2	28 ± 8	50 ± 9
2 pyruvate → AL[d] (microscopic rate constants by quench/NMR)	k_2', s^{-1}	~48	~5.4	~2.6
	k_3', s^{-1}	~1000	~28	n.d.[g]
	k_4', s^{-1}	~2100	~90	n.d.[g]
	k_5', s^{-1}	~350	~90	n.d.[g]
MAP binding (by CD)	K_D^{app}, μM[e]	2.7 ± 1.4	35 ± 5	468 ± 140
MAP binding (stopped-flow kinetics)	k_{+2}, s^{-1}	180 ± 17	0.89 ± 0.11	0.0024 ± 0.0002
	k_{-2}, s^{-1}	1.8 ± 1.2	0.29 ± 0.04	~0
	K_1, mM[f]	5.0 ± 1.3	8.4 ± 3.2	1.95 ± 0.01

[a] The parameters in Table 3 were determined in the German laboratories with protein purified by a slightly different protocol from that used in Beer-Sheva (results in Table 2) with an additional purification step by column chromatography. [b] The kinetic parameters of the enzymatic reactions were determined as described under the Material and Methods and in footnote a of Table 1. [c] The substrate specificity for 2-KB as the second substrate, R, was determined as described under the Materials and Methods and in footnote c of Table 2. [d] Reactions were carried out with 50 mM pyruvate and quenched and analyzed by NMR analysis.[18,36] [e] Dissociation equilibrium of the enzyme-bound covalent analogue to free enzyme and MAP in solution, determined at 20 °C by CD spectroscopic titration as described in the Materials and Methods. [f] Dissociation equilibrium of noncovalently bound MAP in the active site at 37 °C determined by stopped-flow absorbance spectroscopy as described in the Materials and Methods. Since variant Q110E is prone to aggregation at 37 °C, all kinetic stopped-flow measurements were carried out at 20 °C in this case. [g] n.d., not determined.

Table 4. Steady-State Kinetic Properties of AHAS II Lys159 Variants

reaction measured	parameters	enzyme: wild type	enzyme: K159A	enzyme: K159M	enzyme: K159Q
2 pyruvate → AL[a]	specific activity, U mg^{-1}	34.3 ± 0.5	12.4 ± 0.5	12.3 ± 0.4	8.0 ± 0.2
	K_M, mM	6.6 ± 0.4	25.6 ± 2.3	27.2 ± 2.0	8.2 ± 0.2
	k_{cat}, s^{-1}	40.3 ± 0.6	14.5 ± 0.6	14.4 ± 0.5	9.4 ± 0.2
	k_{cat}/K_M, M^{-1} s^{-1}	6100	570	530	1150
	$K_{0.5}$ for ThDP, μM[d]	0.61 ± 0.18	52.0 ± 3.4	25 ± 2	21.2 ± 1.3
	K_i for SMM, μM[e]	1.1 ± 0.1	>350	174 ± 51	162 ± 7
pyruvate + 2-KB → AL + AHB[b]	R[f]	55.1 ± 3.2	60.1 ± 7.2	66.5 ± 6.7	47.2 ± 5.3
benzaldehyde + pyruvate → AL + PAC[c]	R-PAC formation, U mg^{-1}	3.3 ± 0.7	2.7 ± 0.3	2.3 ± 0.3	3.2 ± 0.5
	AL formation, U mg^{-1}	2.9 ± 0.4	0.37 ± 0.03	0.44 ± 0.05	0.52 ± 0.07
	$R_{(PAC/AL)}'$[g]	1.1	7.3	5.2	6.2

[a] The kinetic parameters of the enzymatic reaction with pyruvate as the sole substrate were determined as described in the Material and Methods and in footnote a of Table 1. [b] The substrate specificity for 2-KB as a second substrate, R, was determined with 50 mM pyruvate and the varying concentrations of 2-KB in the standard conditions as described in the Materials and Methods. [c] Enzymatic reaction with benzaldehyde was determined by measuring PAC and AL formation simultaneously in a competition experiment with 30 mM pyruvate and 30 mM benzaldehyde as described in the Materials and Methods. [d] $K_{0.5}$ defined as concentration of ThDP that is required for half-maximum of AHAS II activity. [e] K_i was determined from the rates of reactions at varied SMM concentrations carried out in the presence of 100 mM pyruvate. [f] The specificity for 2-KB as the second substrate, R, is defined by $R = (V_{AHB}/V_{AL})([Pyr]/[2\text{-KB}])$, where [2-KB] is the concentration of 2-KB and V_{AHB}/V_{AL} is the relative rate of formation of AHB and AL, respectively. R was determined with 50 mM pyruvate and the varying concentrations of 2-KB under standard conditions by measuring AHB and AL formation in a competition experiment.[34] The results were analyzed by fitting the data to eqs 1 and 2 with the program SigmaPlot 2000 (SPSS, Inc.). [g] $R' = V_{PAC}/V_{AL}$ under given conditions.[35]

of the addition (k_2'), and abstracts it from the C2α−OH of the ThDP-product in the course of release of the product (k_5') (Scheme 2). Furthermore, Glu47 apparently has a role (via the iminopyrimidine) in the addition of the HEThDP anion/enamine to the second substrate (k_4'), either by direct protonation of the carbonyl oxygen which will become the C2β−OH or more likely as part of a proton transfer system involving the conserved Gln110 (Scheme 2).

In addition to their expected low catalytic efficiencies in the formation of acetolactate (Table 2), variants Q110A, -N, -H, and -E show the changes in substrate specificity R and/or in total reaction rate (as seen from values of V_b/V_a) indicative of a carboligation step which has become partially rate-determining.[15] Gln110 is in a position to play a major role in stabilization of intermediates on the reaction pathway and in proton transfers in the course of the reaction (Figure 1 and Scheme 2). In each of the structures of the

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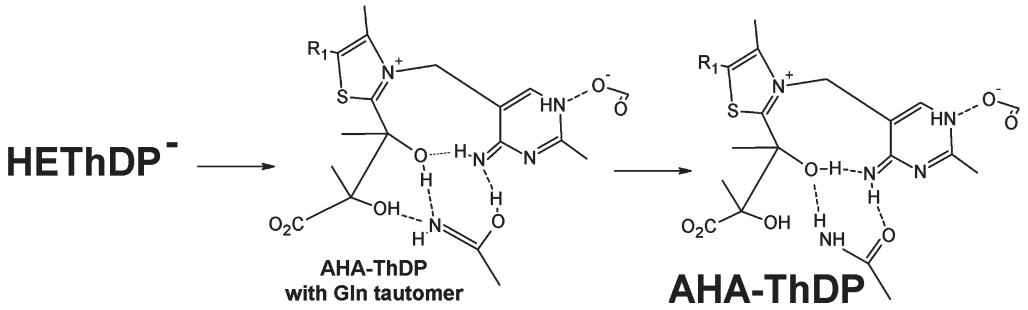
Scheme 3. Possible Mechanism for the Carboligation Step (k_4'), Assisted by Gln110 and the Glu47-Aminopyrimidine Dyad [a]



[a] According to this suggestion, a transient tautomer of the glutamine sidechain is formed, which reverts to the stable carboxamide by a net charge-neutral cyclic proton transfer.

intermediates (or their analogues) in the reaction catalyzed by pyruvate oxidase,[21,43] the carboxamide of the Gln side chain and the carbonyl group of the bound reacting substrate are in good H-bonding distance. The detailed kinetics (Table 3) imply that Gln110 has important roles in every step in catalysis by AHAS II, including both donor substrate binding (K_1, see Table 3) and product release (Scheme 1).

Jaña et al. recently reported a computational study of the mechanism of the carboligation reaction (k'_4 in Scheme 1 here) of AHAS,[44] using the experimental structure of yeast AHAS inhibited by chlorimuron ethyl as the basis of their calculations. They conclude that the yeast homologue of Gln110 (Gln202) has too high a pK_a to be a proton donor to the carbonyl of the acceptor substrate in the carboligation step and that there is no other protein group which can play this role. They propose instead an unassisted addition of HEThDP$^-$ to the acceptor carbonyl, to form a transient anion at the incipient C2-β hydroxyl group of AHA-ThDP, followed by proton transfer from the C2α hydroxyl group. However, given the experimental evidence (Tables 3 and 4) that Gln110 (Gln202 in *S. cerevisiae* AHAS) makes a significant contribution to the rate of carboligation, k_4', it must have some role in this step. This formal step might proceed via transient ions which are stabilized by hydrogen bonding to Gln110. We suggest as an alternative, speculative possibility that the Gln110 (Gln202 in *S. cerevisiae* AHAS) residue could simultaneously donate a proton to the acceptor substrate and accept a proton from the aminopyrimidine N_4' (Scheme 2) in the carboligation step. This would effectively lead to charge-neutral tautomerization of the Gln side chain carboxamide to a transient imino-carbinol (imidic acid) form (Scheme 3).

AHASs (and by structure and sequence similarity also related decarboxylase-carboligases such as, e.g., GCL) appear to have evolved a unique and effective mechanism for stablization of the incipient adduct. Either the transient oxyanion is stabilized by H-bonding to a glutamine side chain or carboligation and product release are under tautomeric control (proton transfer from C2α−OH to C2β−O$^-$) so that the two elementary reactions occur in a concerted manner. Intriguingly, the suggested mechanism of carboligation in decarboxylase-carboligases involving the conserved Gln and the Glu-aminopyrimidine dyad is different from those proposed for ThDP enzymes catalyzing carboligation equilibrium reactions such as transketolase or benzaldehyde lyase. In those enzymes, structural and kinetic data support a mechanism in which His residues function as general acid/base catalysts for reversible protonation of an acceptor carbonyl.[19−22,45,46] Since these enzyme catalyze true equilibrium reactions, His residues are well suited to function both as general acids and bases, a situation different to decarboxylase-carboligase where a large forward commitment is required.

Glu47 and Gln110 both have very significant effects on K_D^{app} for the pyruvate analogue MAP, largely through their influence on the formation of the covalent bond between MAP and enzyme-bound ThDP (Tables 1 and 3). Glu47, Gln110, and the iminopyrimidine moiety thus apparently have important roles in the stabilization of LThDP, in addition to their involvement in the rates of proton transfers to and from the substrate oxygens.

Gln110 of AHAS II is in a position homologous to one of the two adjacent invariant His residues of pyruvate decarboxylases. AHASs and other enzymes of the pyruvate oxidase subfamily have reaction pathways which do not allow for protonation of the HEThDP enamine (HEThDP$^-$) and thus cannot have residues which are good reversible proton donors near C2α.

In contrast to Glu47 and Gln110, Lys159 is not critical to the catalytic efficacy of AHAS II (Table 4). Despite earlier suggestions (e.g., ref 40), Lys159 does not seem to play a very important role in the preference of AHAS II for a charged ketoacid (pyruvate or 2-ketobutyrate) rather than an uncharged aldehyde (e.g., benzaldehyde) as the second (acceptor) substrate. The variants at Lys59 were all only about 5-fold less selective for pyruvate than the wild type (Table 4), and variant K159Q retained all of its enantioselectivity. Lys159 contributes to the overall positive charge of the active site and may promote the formation of a physiological acetohydroxyacid product, but it is not the major determinant of the specificity of AHAS II. As we have shown in previous work,[15] the positive charge of Arg276 probably plays a major role in the interaction of the second 2-ketoacid substrate with the enzyme during the carboligation step.

His251 is conserved in AHASs but not in other ThDP-dependent enzymes. The loop LGMLGMHG (residues 245−252 in AHAS II) is conserved in AHASs, but the histidine is replaced by Gln in glyoxylate carboligase (a homologous decarboxylase-ligase which carries out a reaction like that of AHAS, with glyoxylate as the substrate) and by Ile in *Lactobacillus plantarum* pyruvate oxidase (Supplemental Figure S1 in the Supporting Information). The side chain of His251 appears to be quite far from the cofactor in the structures of herbicide-inhibited eukaryotic AHASs,[7,9] although the two variants at this residue do have significantly reduced apparent affinity for ThDP. The role of His251 is thus far less clearly defined than that of the other polar residues of AHAS that have been studied, particularly because its large effect on donor substrate binding (k_2') masks any effect that

replacement of His251 has on other steps. We can only guess from its position in the solvent channel between the ThDP and the surface of the enzyme that it plays a vital role in an extended hydrogen-bonding network.

CONCLUSIONS

The active sites of AHASs have many highly conserved residues, more than a dozen of which have now been studied by analysis of the properties of variants. Some of these appear to function to hold the catalytic cofactor ThDP in position, and others are critical to the specific nonpolar interactions with the donor substrate pyruvate (V375, F109) [18] or with 2-ketobutyrate as a preferred second substrate (M460, W464).[13] However, despite the fact that the catalytic cycle of AHASs involves four discrete different bond-making and -breaking polar reaction steps, only three different conserved polar residues appear to be critical participants in the reaction cycle. These are (1) the conserved glutamate (E47 in AHAS II) which is essential to the role of ThDP in [almost] every enzyme which uses this coenzyme; (2) the conserved glutamine (Q110 in AHAS II) which is found in all members of the pyruvate oxidase subfamily and is involved in all steps of the reaction; and (3) the conserved arginine (R276 in AHAS II) found in decarboxylase-carboligases, which confers preference for 2-ketoacids as a second substrate. This parsimony is due, of course, to the use of ThDP and the enormous catalytic power of *Umpolung* (inversion of polarity) caused by covalent attachment of the substrate to the thiazolium ring in tandem with the proton transfer functions of the aminopyridine moiety.

ASSOCIATED CONTENT

Supporting Information. Alignment of part of the amino acid sequences of several AHASs, glyoxylate carboligase, and pyruvate oxidase, determined with the program ClustalW[47] and drawn with EsripT.[48] The first row is the subject of this paper, the catalytic subunit of *E. coli* isozyme II, followed by the large subunits of *E. coli* isozymes I and III, *Mycobacterium tuberculosis*, *Bacillus anthracis*, the yeast *Saccharomyces cerevisiae*, and the plant *Arabidopsis thaliana*. The last two rows are *E. coli* glyoxylate carboligase and pyruvate oxidase from *Lactobacillus plantarum*. The residues which have been studied in this paper are marked with red triangles under the alignment. Other residues shown in the depiction of the active site in Figure 1 are marked with green triangles. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

AHAS, acetohydroxyacid synthase; BCAA, branched-chain amino acids; AHA, acetohydroxyacid; AL, acetolactate; AHB, acetohydroxybutyrate; 2-KB, 2-ketobutyrate; MAP, methyl acetylphosphonate; ThDP, thiamin diphosphate; LThDP, 2-lactyl-ThDP; HEThDP, 2-hydroxyethyl-ThDP; PAC, phenylacetylcarbinol.

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