

## The Cofactors Role on Chemical Mechanism of Recombinant Acetohydroxy Acid Synthase from Tobacco

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Acetohydroxy acid synthase (AHAS) is one of several enzymes that require thiamine diphosphate and a divalent cation as essential cofactors. The active site contains several conserved ionizable groups and all of these appear to be important as judged by the fact that mutation diminishes or abolishes catalytic activity. Recently, we have shown [Yoon, M.-Y., Hwang, J.-H., Choi, M.-K., Baek, D.-K., Kim, J., Kim, Y.-T., Choi, J.-D. FEBS Letters 555 (2003), 185-191] that the activity is pH-dependent due to changes in  $V_{\max}$  and  $V/K_m$ . Data were consistent with a mechanism in which substrate was selectively catalyzed by the enzyme with an unprotonated base having a pK 6.48, and a protonated group having a pK of 8.25 for catalysis. Here, we have in detail studied the pH dependence of the kinetic parameters of the cofactors (ThDP, FAD,  $Mg^{2+}$ ) in order to obtain information about the chemical mechanism in the active site. The  $V_{\max}$  of kinetic parameters for all cofactors was pH-dependent on the basic side. The pK of ThDP, FAD and  $Mg^{2+}$  was 9.5, 9.3 and 10.1, respectively. The  $V/K_m$  of kinetic parameters for all cofactors was pH-dependent on the acidic and on the basic side. The pK of ThDP, FAD and  $Mg^{2+}$  was 6.2-6.4 on the acidic side and 9.0-9.1 on the basic side. The well-conserved histidine mutant (H392) did not affect the pH-dependence of the kinetic parameters. The data are discussed in terms of the acid-base chemical mechanism.

**Key Words :** Acetohydroxy acid synthase, Chemical mechanism, pH study, Cofactors

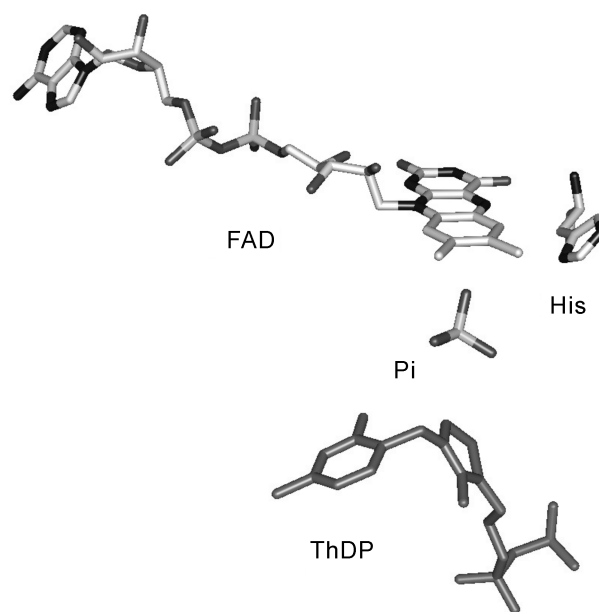
### Introduction

Acetohydroxy acid synthase (AHAS, EC 4.1.3.18 also referred to as acetolactate synthase) catalyzes the initial common step in the biosynthesis of the branched-chain amino acids, valine, leucine, and isoleucine in plants and microorganisms.<sup>1,2</sup> AHAS catalyzes the condensation of two molecules of pyruvate to form acetolactate in the biosynthesis of valine and leucine, or the condensation of pyruvate and 2-ketobutyrate to form 2-aceto-2-hydroxybutyrate in the biosynthesis of isoleucine.

AHAS requires thiamine diphosphate (ThDP), divalent metal ion and FAD as cofactors for its catalytic function. The requirement for FAD is not unprecedented and has also been described for glyoxylate carboligase,<sup>3</sup> which is structurally related to AHAS, as well as the unrelated enzyme chorismate synthase.<sup>4</sup> Much attention was stimulated by the discovery that it is the target site of at least four structurally diverse families of herbicides, namely sulfonylureas, imidazolinones, triazolopyrimidines, and pyrimidinyl oxybenzoates.<sup>2</sup> These compounds bear no resemblance to the substrate and are not competitive inhibitors, suggesting that they do not bind at the active site. The structure and natural role of this herbicide-binding site is unknown.

The crystallization of the catalytic subunit<sup>5</sup> and AHAS

enzyme<sup>6</sup> from yeast was recently reported at 2.6 Å resolution. This structure revealed the location of several active site features, including the position and conformation of the cofactors ThDP,  $Mg^{2+}$  and FAD (Figure 1, showing ThDP



**Figure 1.** Structure of the active site of *Saccharomyces cerevisiae* AHAS. The position of Pi is that occupied by substrates. His residue is shown in side chain atoms and in the A subunit. The figure was created using the Chimera package from the Computer Graphics Laboratory, UCSF.

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and FAD). The structure, in combination with molecular modeling, also suggested the geometry and location of the binding site for the imidazolinone herbicide imazapyr (2-(4-isopropyl-4-methyl-5-oxo-2-imidazolin-2-yl) nicotinic acid). A number of chemical modification studies, including site-directed mutagenesis, have revealed that Trp490,<sup>7</sup> Cys411,<sup>8</sup> His487,<sup>9</sup> and Lys219<sup>10</sup> residues are essential for the catalytic function in tobacco AHAS. Lee *et al.* carried out steady-state kinetic studies of recombinant tobacco AHAS using pyruvate and 2-ketobutyrate as substrates.<sup>11</sup> They proposed that recombinant tobacco AHAS catalyzes the reaction in the manner of a Uni Uni Ping Pong Bi Bi mechanism. Recently, we have studied the pH dependence of kinetic parameters in order to obtain information about the chemical mechanism of the active site.<sup>12</sup> Data were consistent with a mechanism in which substrate was selectively catalyzed by the enzyme with an unprotonated base having a pK 6.48, and a protonated group having a pK 8.25 for catalysis. The temperature dependence of kinetic parameters was pH dependent, and the enthalpies of ionization,  $\Delta H_{\text{ion}}$ , calculated from the slope of pK<sub>1</sub> and pK<sub>2</sub> were both pH independent. The solvent perturbation of kinetic parameters were pH dependent, and the pK<sub>1</sub> from the acidic side and the pK<sub>2</sub> from the basic side were shifted down 0.4 pH units and shifted up 0.6 units as water was replaced by 15% ethanol, respectively. Although some information is available on the aspects of the AHAS reaction, little is known of the chemical mechanism.<sup>13-16</sup> In an attempt to determine the ionization state of the enzyme during catalysis, we have further carried out an investigation of the pH dependence by the cofactors (ThDP, FAD as well as Mg<sup>2+</sup>) and the well-conserved residues of AHAS from tobacco. Data will be discussed in terms of the reaction mechanism for AHAS, particularly acid-base chemistry.

### Materials and Methods

**Chemicals.** Pyruvic acid sodium salt, tris (hydroxymethyl) aminomethane, flavin adenine dinucleotide (FAD), thiamin diphosphate (ThDP),  $\alpha$ -naphthol, creatine, glutathione, isopropyl- $\beta$ -D-thiogalactoside (IPTG), NaCl, Triton X-100, glutathione (GSH) and MgCl<sub>2</sub> were all purchased from Sigma Chemical Co. (St. Louis, USA). Epoxy-activated Sepharose 6B was obtained from Pharmacia Biotech (Uppsala, Sweden). All other chemicals were obtained from commercial sources and were of the highest quality available.

**Enzyme Purification.** Expression and purification of the recombinant acetohydroxyacid synthase was performed with modifications as described by Chang *et al.*<sup>17</sup> Briefly, *E. coli* DH5 $\alpha$  cells containing the expression vector pGEX-AHAS were grown at 37 °C in Luria-Bertani (LB) medium containing 50  $\mu$ g/mL ampicillin to an OD<sub>600</sub> of 0.7-0.8. Cells were induced by the addition 1.0 mM isopropyl-D-thiogalactoside (IPTG) and grown for an additional 4 hours at 30 °C. Cells were harvested by centrifugation at 6000 rpm for 15 min. The cell pellet was resuspended in PBST buffer (150 mM Tris-HCl, pH 7.5, 1 mM pyruvate, 10% (v/v) ethylene glycol,

10 mM MgCl<sub>2</sub>) containing protease inhibitors (2  $\mu$ g/mL Leupeptin, 4  $\mu$ g/mL Aprotinin, 2  $\mu$ g/mL Pepstatin A). The cell suspension was lysed by sonication at 4 °C. The homogenate was centrifuged at 20,000 rpm for 20 min and the supernatant was applied to a GSH-coupled Sepharose 6B column with PBST buffer. The GST-AHAS fusion protein was recovered from the column with an elution buffer (50 mM Tris-HCl, pH 8.0, 20 mM GSH, 10% (v/v) ethylene glycol). The isolated protein was identified by SDS-PAGE analysis and the protein concentration was determined by the method of Bradford.

**Enzyme Assay.** Enzyme activities of the purified AHAS were measured according to the method of Westerfeld<sup>18</sup> with modifications as reported previously.<sup>19</sup> The standard reaction mixture contained 100 mM potassium phosphate buffer (pH 7.5), 1 mM ThDP, 10 mM MgCl<sub>2</sub>, 20  $\mu$ M FAD, 75 mM pyruvate, and the enzyme in the absence or presence of various concentrations of inhibitors. Assays were initiated by the addition of AHAS at 37 °C for 60 min and terminated by the addition of 6 N H<sub>2</sub>SO<sub>4</sub>. The reaction product, acetohydroxy acid, was allowed to decarboxylate at 60 °C for 15 min. The acetoin formed by acidification was incubated and colorized with 0.5% creatine and 5%  $\alpha$ -naphthol at 60 °C for 15 min. All data were collected using a Shimadzu spectrophotometer. All reactions were carried out in a 1-mL cuvette with a 1 mm-light path-length. The absorbance of the reaction mixture was monitored at 525 nm. The concentration of reactants was corrected for the concentration of the metal chelate complexes according to Dawson *et al.*<sup>20</sup> One unit (U) of activity was defined as the amount required to form 1  $\mu$ mol of acetohydroxy acid per minute under the assay conditions described above. Specific activities of AHAS were expressed as units (U) per mg of protein.

**pH Studies.** The pH stability of AHAS was determined by incubating enzyme at the desired pH and assaying aliquots as a function of time up to 10 min at pH 7.5 as described above. The enzyme is stable with no activity loss from pH 6 to 9. At pH 5.5, some degree of denaturation occurs. Since substrate is present in the assay, which is likely to afford some protection against denaturation, and since enzyme is added from a stock solution at pH 7.5 to the assay mixture at the desired pH, small activity losses will not affect the measurement of velocity.

In order to obtain estimates of the K<sub>m</sub> values for cofactors, steady state kinetic patterns in the absence of products were obtained at pH 6.0, 7.5, and 9.0 by measuring the rate at various concentrations of cofactor and at saturating concentrations of other cofactors and pyruvate, unless otherwise mentioned. The saturation curve for each cofactor was obtained at a fixed saturating concentration of other cofactors and pyruvate as a function of pH. Buffers used at 100 mM concentration were MES at 6.0 to 6.5, MOPS at pH 6.5 to 8.0 and TAPS at pH 8.0 to 9.5. All buffers were titrated to the appropriate pH with KOH. Assays were repeated at a given pH using different buffers to eliminate the possibility of activation by the buffers.

**Data Analysis.** Reciprocal values of the steady state rate were plotted as a function of the reciprocal of substrate concentrations. Data were analyzed according to the appropriate rate equations by using the FORTRAN programs of Cleland.<sup>21</sup> Individual saturation curves were fitted to Eq. (1). Data for pH profiles giving limiting slopes were fitted to Eq. (2). Data for pH profiles that decreased with a slope of +1 at low pH and a slope of -1 at high pH were fitted to Eq. (3).

$$v = VA/(K + A) \quad (1)$$

$$\text{Log } y = \text{log } C/(1 + K_2/[H^+]) \quad (2)$$

$$\text{Log } y = \text{log}\{C/(1 + [H^+]/K_1 + K_2/[H^+])\} \quad (3)$$

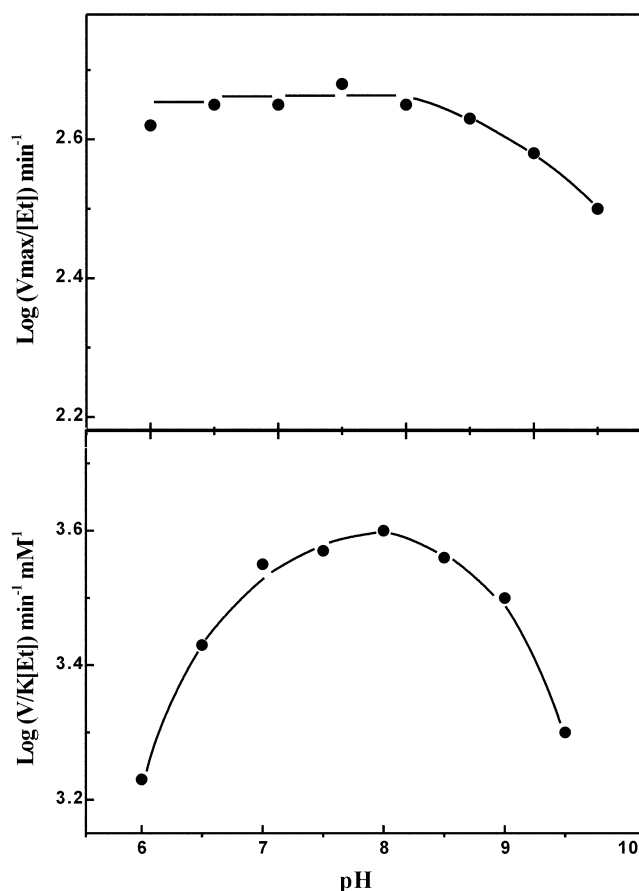
In Eq. (1), A is the reactant concentration. V is the maximum velocity, and K is the Michaelis constant for the varied substrate. In Eqs. (2) and (3), H is the hydrogen ion concentration, K<sub>1</sub> and K<sub>2</sub> represent dissociation constants for enzyme groups, y is V/K, and C is the pH-independent value of y.

### Results and Discussion

AHAS is a thiamine diphosphate-dependent enzyme about which there is a large body of structural and functional information. The active site contains several absolutely conserved ionizable groups and all of these appear to be important, as judged by the fact that mutation of these sites diminishes or abolishes catalytic activity. The pH dependence of kinetic parameters was determined in order to obtain information on the acid-base chemistry catalyzed by the catalytic subunit of AHAS. Previously, we have shown<sup>12</sup> that the activity is pH-dependent due to changes in K<sub>cat</sub> and V/K<sub>m</sub>. The chemical mechanism for AHAS was discussed in detail. In this paper, we studied the chemical mechanism (acid-base chemistry) of AHAS through the cofactors (ThDP, FAD, Mg<sup>2+</sup>) pH studies.

The kinetic parameters for ThDP are shown in Figure 2. The maximum velocity for ThDP decreased at high pH with a slope of -1. However, the V/K for ThDP was bell-shaped, decreasing at both low and high pH with slopes of 1 and -1. The pK of the V profile for ThDP was 9.5 ± 0.3 at high pH. The pKs of the V/K<sub>ThDP</sub> profile yielded pK values of 6.3 ± 0.2 at low pH and 9.0 ± 0.2 at high pH. The pH independent values of V/E<sub>t</sub> and V/(K<sub>ThDP</sub>)E<sub>t</sub> were 7.6 s<sup>-1</sup> and 66.35 mM<sup>-1</sup> s<sup>-1</sup>, respectively. The H392M mutant yielded an active enzyme that had similar specific activity (5.78 U/mg) to native AHAS. The pH profiles of ThDP, FAD and Mg<sup>2+</sup> for H392M showed the same pattern as the native AHAS profiles (data not shown), indicating that the ionizing group on each side of the profile is from the active site. The mutants H351 and H487 could not be pursued due to very low or no enzyme activity, respectively.

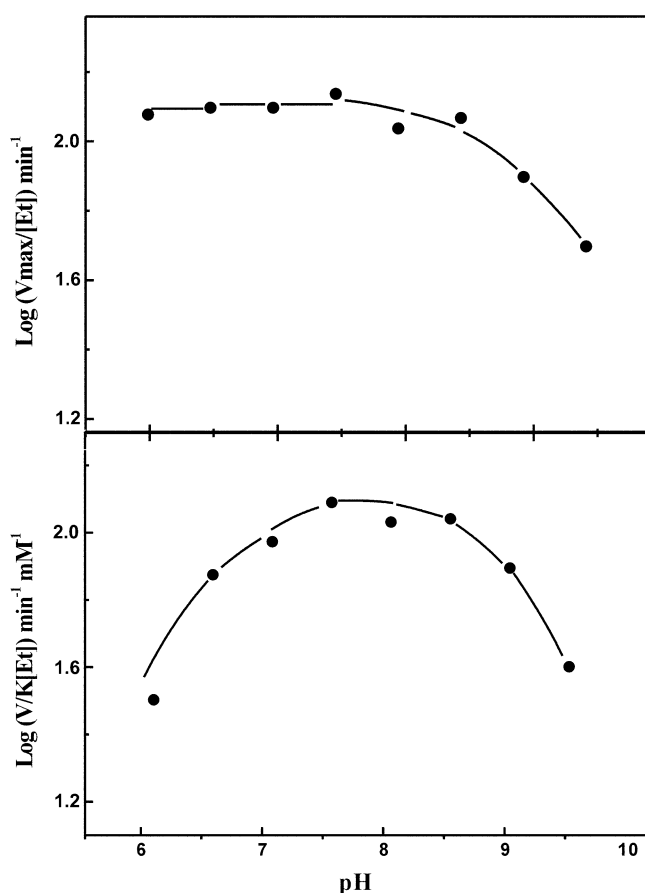
The kinetic parameters for FAD are shown in Figure 3. The maximum velocity for FAD also decreased at high pH with a slope of -1. However, the V/K for FAD was bell-shaped as in the V/K profile for ThDP, decreasing at both low and high pH with slopes of 1 and -1. The pK of the V



**Figure 2.** pH dependence of the kinetic parameters for ThDP in the AHAS reaction. The V/K value was obtained at saturating concentrations of pyruvate and cofactors (FAD and Mg<sup>2+</sup>) with the concentration of ThDP varied around its K<sub>m</sub>. Conditions for the experiment were described in Materials and Methods. The points shown are experimentally determined values. The curves are the theoretical value of V and V/K from a fit to the data using Eq. 2 and 3, respectively.

profile for FAD was 9.3 ± 0.2 at high pH. The pK of V/K<sub>FAD</sub> profile yielded pK values of 6.2 ± 0.3 at low pH and 9.0 ± 0.2 at high pH. The pH independent values of V/E<sub>t</sub> and V/(K<sub>FAD</sub>)E<sub>t</sub> were 2.1 s<sup>-1</sup> and 2.0 mM<sup>-1</sup> s<sup>-1</sup>, respectively. The kinetic parameters for Mg<sup>2+</sup> were also carried out. The maximum velocity and V/K for Mg<sup>2+</sup> were the same as the other cofactors within error. (see Table 1)

The V/K for a reactant is the second-order rate constant for conversion of free enzyme and free reactant to products. Thus, the pKs observed in the V/K<sub>ThDP</sub> pH profiles reflect acid-dissociable functional groups in free E : Pyruvate : Mg : FAD in the presence of other cofactors (Mg, FAD). The pKs observed in the V/K<sub>Mg</sub> pH profiles reflect acid-dissociable functional groups in free E : Pyruvate : ThDP : FAD in the presence of other cofactors (ThDP, FAD). Also, the pKs observed in the V/K<sub>FAD</sub> pH profiles reflect acid-dissociable functional groups in free E : Pyruvate : Mg : ThDP in the presence of other cofactors (Mg, ThDP). A bell-shaped pH profile is obtained for V/K<sub>ThDP</sub>. Both of the observed pK values must reflect enzyme groups or cofactors since pyruvate exhibits no pKs in the pH range 6.0-9.5. The group with a



**Figure 3.** pH dependence of the kinetic parameters for FAD in the AHAS reaction. The V/K value was obtained at saturating concentrations of pyruvate and cofactors (ThDP and Mg<sup>2+</sup>) with the concentration of FAD varied around its K<sub>m</sub>. Conditions for the experiment were described in Materials and Methods. The points shown are experimentally determined values. The curves are the theoretical value of V and V/K from a fit to the data using Eq. 2 and 3, respectively.

pK of 6.2-6.3 is most likely a base. A base catalysis is expected in the case of the AHAS reaction with a base accepting the proton from the C-2 of the thiazole ring of ThDP. A key step in thiamin-catalyzed reactions is the initial ionization of the thiazole C-2 proton. The pK for this step is very high (17-19),<sup>22</sup> however, it is possible that enzyme-bound ThDP might have a much lower pK consistent with the observation that enzyme-catalyzed reactions can be more than 10<sup>12</sup>-fold faster than their non-enzymatic counterparts.<sup>23</sup> Jordan *et al.*<sup>24</sup> have compared the ionization of the benzyl analog of hydroxyethyl-ThDP to yeast pyruvate decarboxylase (PDC) and found a pK shift from 15.4 in water to 6 in

PDC. They suggest that the pK for the ionization of the C2 proton of enzyme bound ThDP might also be shifted to a similar extent.

From the pH studies of substrate, pyruvate, the V<sub>max</sub> and the V/K<sub>pyruvate</sub> profiles were odd shaped and bell-shaped, respectively.<sup>12</sup> The pK of the V profile for pyruvate was 8.4 at high pH. The V/K<sub>pyruvate</sub> profile yielded pK values of 6.45 at low pH and 8.3 at high pH. The pK values from cofactors were the same as those of pyruvate, within error (see Table 1). The maximum velocity is pH dependent at high pH, which exhibits a similar behavior on the basic side of the V/K<sub>cofactors</sub> pH profiles. The pKs of the V profile for cofactors were increased to 1.0 pH unit, indicating a different affinity for the complex formation. *i.e.* when the cofactors (or the substrate) first fill under saturating conditions in the active site, the structural changes or the "stickiness" of the central complex derive the pK difference. Since the V profile is pH dependent at high pH, a mechanism in which reactants do not bind selectively to the correctly protonated form of the enzyme is suggested. For a mechanism of this type, intrinsic pK values are not observed for enzyme and substrate functional groups in the pH profiles.

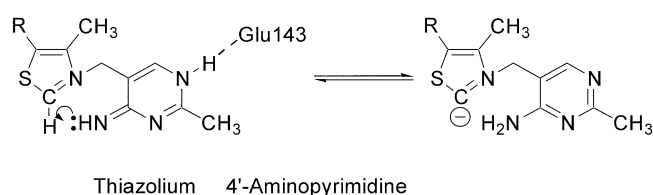
The pKs of the V/K profile for cofactors showed no shift (6.2-6.4 pK value) on the acidic side and 0.7 pH unit shift (9.0-9.1pK value) on the basic side. The pK for the acidic side of the V/K profile was not shifted, indicating that a general base might be not involved in any cofactors binding with enzyme directly (*vide ante*). In addition to this base, there is the requirement for an enzyme residue with a pK of 8.25 that must be protonated for activity. Yoon *et al.*<sup>12</sup> suggested that this enzyme residue is another catalytic group for pyruvate that may serve to orient it and donate a proton to the substrate for subsequent chemistry or the transient formation of an alcoholate anion ThDP. In the latter case, the protonation of an alcoholate anion ThDP is a required step in catalytic cycle. However, as a pK of 8.25 is lower for an alcoholate anion in water, the possibility of its acting as a general acid is vague. The pK of the V/K profile were increased to 0.7pH unit. These results suggest that the cofactors may pursue sequent chemistry when the substrate is saturated in the active site.

These data suggest a mechanism in which one group participates in catalysis with the reactant pyruvate. This is suggested by the cofactor pH studies together with the exclusive pH studies.<sup>12</sup> A mechanism in which an intermediate C-2 carbanion is formed with a short lifetime has been postulated by Kern *et al.*<sup>25</sup> based on thiamine-catalyzed reactions. Glu143, which is conserved in all AHASs, forms a

**Table 1.** Summary of pK values obtained from the pH dependence of V<sub>max</sub> and V/K<sub>m</sub><sup>a</sup>

|                  | ThDP                 |                      | FAD                  |                      | Mg <sup>2+</sup>     |                      |
|------------------|----------------------|----------------------|----------------------|----------------------|----------------------|----------------------|
|                  | pK <sub>1</sub> ± SE | pK <sub>2</sub> ± SE | pK <sub>1</sub> ± SE | pK <sub>2</sub> ± SE | pK <sub>1</sub> ± SE | pK <sub>2</sub> ± SE |
| V <sub>max</sub> | ND <sup>b</sup>      | 9.5 ± 0.3            | ND                   | 9.3 ± 0.2            | ND                   | 10.05 ± 0.1          |
| V/K <sub>m</sub> | 6.3 ± 0.2            | 9.0 ± 0.2            | 6.2 ± 0.3            | 9.0 ± 0.2            | 6.43 ± 0.1           | 9.12 ± 0.3           |

<sup>a</sup>pK<sub>1</sub> indicated that the group must be protonated for enzyme activity and pK<sub>2</sub> indicates that the group must be deprotonated. <sup>b</sup>ND, Not Determined.



**Figure 4.** Proposed catalytic mechanism of ThDP for AHAS reaction. The enamine/ $\alpha$ -carbanion reaction intermediate that is formed with a stabilizing hydrogen bond to the 4'-amino of ThDP. R represents the hydroxyethyl diphosphate group.

hydrogen bond with the N1' of the thiazolium ring of ThDP. The protonation of ThDP by Glu143 is a necessary first step for catalysis. This conserved interaction is suggested to facilitate the formation of the 4'-imino form of ThDP (Figure 4). A proton is abstracted from C-2 by the 4'-imino form of ThDP with a pK of 6.45. The pK for the general base is observed in the  $V/K_{\text{pyruvate}}$  and  $V/K_{\text{cofactors}}$  profiles.

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