

# The active site and mechanism of action of recombinant acetohydroxy acid synthase from tobacco

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**Abstract** Acetohydroxy acid synthase (AHAS) is one of several enzymes that require thiamine diphosphate and a divalent cation as essential cofactors. Recently, the three-dimensional structure of the enzyme from yeast has been determined [Pang et al., *J. Mol. Biol.* 317 (2002) 249–262]. While this structure sheds light on the binding of the cofactors and the reaction mechanism, the interactions between the substrates and the enzyme remain unclear. We have studied the pH dependence of kinetic parameters in order to obtain information about the chemical mechanism in the active site. Data are consistent with a mechanism in which substrate selectively catalyzed to the enzyme with an unprotonated base having a  $pK$  of 6.48, and a protonated group having a  $pK$  of 8.25 for catalysis. The temperature dependence of kinetic parameters was pH-dependent, and the enthalpies of ionization,  $\Delta H_{ion}$ , calculated from the slope of  $pK_1$  and  $pK_2$  are both pH-independent. The solvent perturbation of kinetic parameters was pH-dependent, and the  $pK_1$  from the acidic side and the  $pK_2$  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. The data are discussed in terms of the acid–base chemical mechanism.

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**Key words:** Acetohydroxy acid synthase; Enzyme kinetics; pH study; Chemical mechanism

## 1. 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 [1,2]. 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, which catalyzes the decarboxylation of 2-ketoacids,

uses thiamine diphosphate (ThDP) as a cofactor. The enzyme also requires a divalent metal ion that anchors ThDP in the active site. AHAS has an essential requirement for flavin adenine dinucleotide (FAD), which is unexpected because the reaction involves no oxidation or reduction. The first two cofactors are typical for enzymes that catalyze the decarboxylation of 2-ketoacids, as occurs in the first stage of the AHAS reaction. The requirement for FAD is not unprecedented and has also been described for glyoxylate carboligase [3], which is structurally related to AHAS, as well as the unrelated enzyme chorismate synthase [4]. Much interest in AHAS 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 [2]. 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.

AHAS has been studied in steady-state kinetic experiments in which the rates of acetolactate and acetohydroxybutyrate formation have been determined simultaneously [5]. The ratio between the rates of production of the two alternative products and the concentrations of the substrates pyruvate and 2-ketobutyrate leads to  $V_{AHB}/V_{AL} = R$  ([2-ketobutyrate]/[pyruvate]). Among the three enterobacterial enzymes, only AHAS I has a relatively low  $R$  factor of 2. However, AHAS II and III have high  $R$  values of 65 and 40, respectively, which means that they have the presence of at least one AHAS activity with high specificity for acetohydroxybutyrate formation. This is consistent with the fact that the intracellular concentration of the major metabolic intermediate pyruvate is higher than that of 2-ketobutyrate, indicating that the mechanism involves an irreversible and rate-determining reaction of pyruvate. Lee et al. [6] carried out steady-state kinetic studies of recombinant tobacco AHAS using pyruvate and 2-ketobutyrate as substrates. They proposed that recombinant tobacco AHAS catalyzes the reaction in the manner of a Uni Uni Ping Pong Bi Bi mechanism.

The crystallization of the catalytic subunit [7] and AHAS enzyme [8] from yeast was recently reported at 2.6 Å resolution. This structure revealed the location of several active site features, including the position and the conformation of the cofactors ThDP,  $Mg^{2+}$  and FAD (Fig. 1, showing only ThDP). The structure, in combination with molecular modeling, also suggested the geometry and location of the binding

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**Abbreviations:** AHAS, acetohydroxy acid synthase; ThDP, thiamine diphosphate; FAD, flavin adenine dinucleotide; IPTG, isopropyl- $\beta$ -D-thiogalactoside; PDC, pyruvate decarboxylase

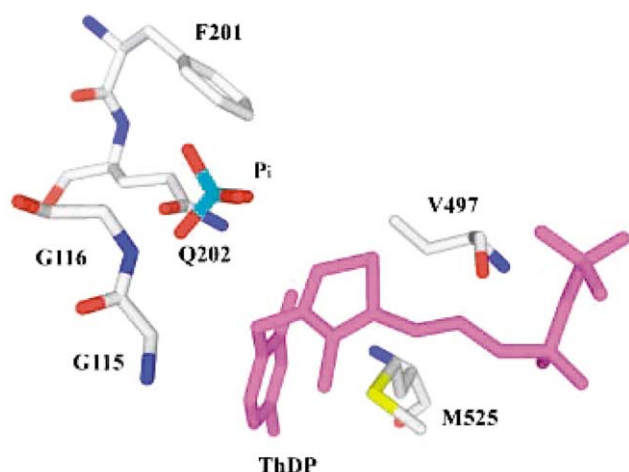


Fig. 1. Structure of the active site of *Saccharomyces cerevisiae* AHAS. The position of  $P_i$  is that occupied by substrates. ThDP is shown in gray. Amino acid residues are side chain atoms. All of the residues are shown in the A subunit with the exception of V497 which is in the B subunit. The figure was created using the Chimera package from the Computer Graphics Laboratory, UCSF.

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 studies have revealed that Trp490 [9], Cys411 [10], His487 [11] and Lys219 [12] residues are essential for the catalytic function in tobacco AHAS. Although some information is available on the aspects of the AHAS reaction [13–16], little is known of the chemical mechanism. In an attempt to determine the ionization state of the enzyme during catalysis, we have carried out an investigation of the pH dependence by substrates, deuterium solvent effects as well as temperature- and solvent-dependent activity changes in recombinant and site-directed mutants of AHAS from tobacco. The data will be discussed in terms of the reaction mechanism for AHAS, particularly acid–base chemistry.

## 2. Materials and methods

### 2.1. Chemicals

Pyruvic acid sodium salt, tris(hydroxymethyl)aminomethane, FAD, ThDP,  $\alpha$ -naphthol, creatine, glutathione, isopropyl- $\beta$ -D-thiogalactoside (IPTG), NaCl, Triton X-100, GSH and  $MgCl_2$  were all purchased from Sigma (St. Louis, MO, 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.

### 2.2. Site-directed mutagenesis

Site-directed mutagenesis of tobacco AHAS was performed on the plasmid pGEX-2T containing tobacco AHAS cDNA, using the polymerase chain reaction megaprimer method. All DNA manipulations were carried out using techniques reported previously [11,12].

### 2.3. Enzyme purification

Expression and purification of the recombinant AHAS was performed with modification as described by Chang et al. [29]. Briefly, *Escherichia coli* DH5 $\alpha$  cells containing the expression vector pGEX-ALS were grown at 37°C in Luria–Bertani medium containing 50  $\mu$ g/ml ampicillin to an OD<sub>600</sub> of 0.7–0.8. Cells were induced by the addition of 1.0 mM IPTG and grown for an additional 4 h at 30°C. Cells were harvested by centrifugation at 6000 rpm for 15 min. The cell pellet for the purification was resuspended in PBST buffer (150 mM Tris–HCl, pH 7.5, 1 mM pyruvate, 10% (v/v) ethylene glycol, 10 mM

$MgCl_2$ ) 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 20000 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 sodium dodecyl sulfate–polyacrylamide gel electrophoresis analysis and the protein concentration was determined by the method of Bradford.

### 2.4. Enzyme assay

Enzyme activities of the purified AHAS were measured according to the method of Westerfeld [17] with a modification as reported previously [18]. The standard reaction mixture contained 100 mM potassium phosphate buffer (pH 7.5), 1 mM ThDP, 10 mM  $MgCl_2$ , 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 30 min and terminated by the addition of 6 N  $H_2SO_4$ . The reaction product acetohydroxy acid was allowed to decarboxylate at 60°C for 15 min. The acetoin formed by acidification was incubated and colored with 0.5% creatine and 5%  $\alpha$ -naphthol at 60°C for 15 min. All data were collected using a Shimadzu spectrophotometer. Assays of temperature dependence were performed at 20°C, 30°C and 37°C. Reaction mixtures for experiments involving organic solvent perturbation contained 15% ethanol. 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. [19]. 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.

### 2.5. 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  $K_m$  values for pyruvate, 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 different concentrations of substrate and at saturating concentrations of cofactors unless otherwise mentioned. The saturation curve for pyruvate was obtained at a fixed saturating concentration of the cofactors as a function of pH. Buffers used at 100 mM concentration were 2-(N-morpholino)ethanesulfonic acid at 6.0–6.5, 4-morpholinopropanesulfonic acid at pH 6.5–8.0 and 3-[[tris(hydroxymethyl)methyl]-amino]propanesulfonic acid at pH 8.0–9.5. 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. At the pH extremes, the concentration of cofactors was identified in a separate assay to be sure that they were still saturated.

The pH profiles were repeated in  $D_2O$ . Enzyme stability and the pD dependence of the kinetic mechanism were also determined at the pD extremes with results qualitatively similar to those obtained in  $H_2O$ . All reagents, except enzyme, were made up in  $D_2O$  and titrated to the desired pD with KOD. The final concentration of  $D_2O$  was approximately 95%. The pH of the solution was measured and the pD adjusted by adding 0.4 to the pH reading as a result of the isotope effect on the electrode [20].

### 2.6. Data analysis

Reciprocal values of the steady-state rate were plotted as a function of the reciprocal of the substrate concentrations. Data were analyzed according to the appropriate rate equations using the Fortran programs of Cleland [22]. 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 11 varied substrate. In Eqs. 2 and 3,  $H$  is the hydrogen ion concentration,  $K_1$  and  $K_2$  represent dissociation constants for enzyme groups,  $y$  is  $V/K$ , and  $C$  is the pH-independent value of  $y$ .

### 3. Results

In order to obtain information on the acid–base chemistry catalyzed by the catalytic subunit of AHAS, the pH dependence of kinetic parameters was determined. However, prior to a determination of the pH dependence of the kinetic parameters, the pH stability and the pH dependence of the kinetic mechanism must first be obtained. As pointed out in Section 2, the enzyme, which is stored at pH 7.5, is sufficiently stable over the pH range 6.0–9.5 for the time necessary to collect the velocity data.

Efforts to identify the amino acid or other residue(s) involved in the catalytic function of an enzyme on the basis of the  $pK$  values of ionizing groups derived from kinetic measurements are valid only when no conformational changes occur in the enzyme under the conditions of the experiment. If such changes occur, then it can be argued that an ionization

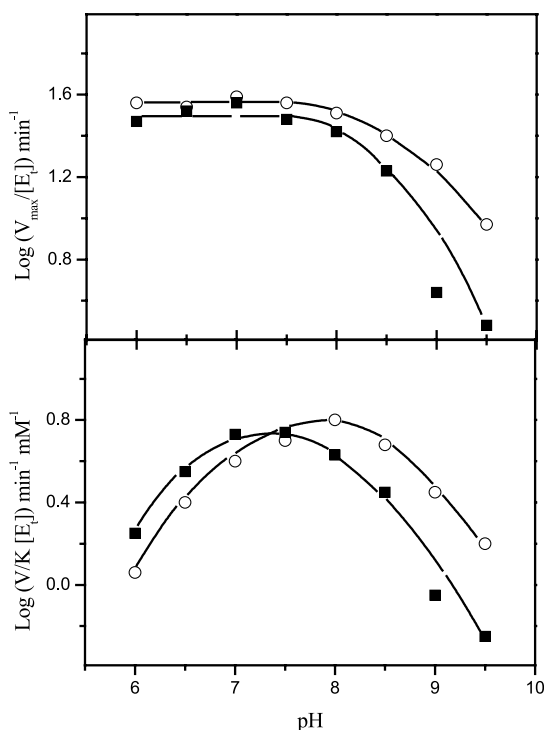


Fig. 2. pH dependence (■) and pD dependence (○) of the kinetic parameters for pyruvate in the AHAS reaction. The  $V/K$  value was obtained at saturating concentrations of cofactors with the concentration of pyruvate varied around its  $K_m$ . Conditions for the pD experiment were described in Section 2. All reagents were prepared in  $D_2O$ . 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 Eqs. 2 and 3, respectively.

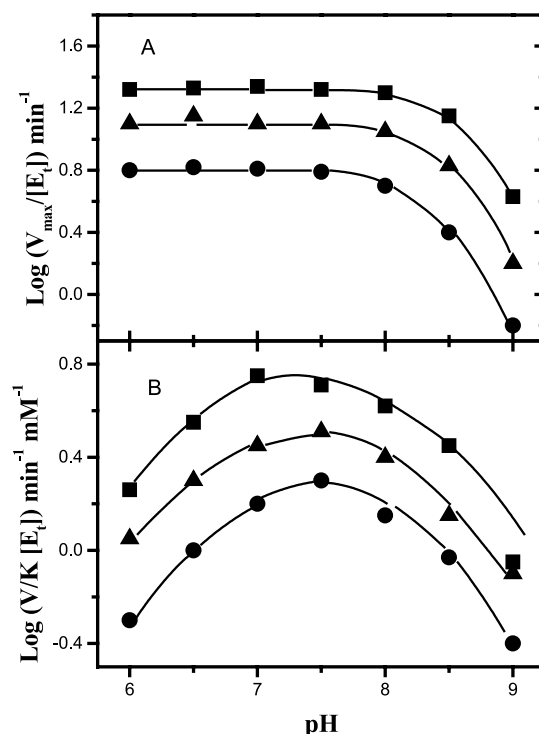


Fig. 3. pH dependence of the kinetic parameters for AHAS on the temperature effect. A: pH dependence of  $V_{max}$ . (■) Kinetic parameters at 37°C; (▲) kinetic parameters at 30°C; (●) kinetic parameters at 20°C. B: pH dependence of  $V_{max}/K_m$ . (■) Kinetic parameter at 37°C; (▲) kinetic parameters at 25°C; (●) kinetic parameters at 20°C. 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 Eqs. 2 and 3, respectively.

that affects  $V_{max}$  or  $K_m$ , or both, does so by changing the conformation of the enzyme and does not involve any residue which directly participates in the catalytic process. No such conformational changes have been shown to occur in the case of AHAS in the pH range 6.0–9.5. Absorption spectra of the enzyme in the 225–330 nm range under these conditions (data not shown) have been found to be identical and superimposable, indicating no alteration either in the environment of aromatic residues or in the peptide backbone structure of the enzyme. The absence of the environmental disturbance of aromatic chromophores in 15% ethanol shifts in  $pK$  values at the active site and suggests that no denaturation occurs in the presence of organic solvents.

The kinetic parameters are shown in Fig. 2. The maximum velocity for pyruvate decreases at high pH with a slope of  $-1$ . However, the  $V/K$  for pyruvate is bell-shaped, decreasing at both low and high pH with slopes of 1 and  $-1$ . The  $pK$  of the  $V$  profile for pyruvate is  $8.44 \pm 0.12$  at high pH. The  $V/K_{pyruvate}$  profile yielded  $pK$  values of  $6.48 \pm 0.2$  at low pH and  $8.25 \pm 0.15$  at high pH. The pH-independent values of  $V/E_t$  and  $V/(K_{pyruvate})E_t$  are  $5.27 \times 10^{-1} \text{ s}^{-1}$  and  $9.16 \times 10^{-2} \text{ mM}^{-1} \text{ s}^{-1}$ , respectively. The H392M mutation yielded an active enzyme that had similar specific activity (5.78 U/mg) to native AHAS. The pH profiles for H392M have shown a similar pattern to the native AHAS profiles, 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 and no enzyme activity, respectively.

Some of the data obtained for  $V$  and  $V/K_{pyruvate}$  (not shown

in Fig. 2) indicated a scattering in the parameter at the low and the high pH due to instability of the enzyme. Since this scattering gives a large standard error, it becomes important to determine the true parameters. It is known that the  $pK$  values of nitrogen and oxygen bases increase by 0.4–0.6 pH units in  $D_2O$  [20]. A determination of the  $V$  and  $V/K_{\text{pyruvate}}$  pH dependence in  $D_2O$  should then decrease at lower pH giving more accurate  $pK$  values. The  $pK$  values act as an internal control as to whether the method works. The  $pK$  of the  $V$  profile for pyruvate is  $8.90 \pm 0.17$  at high pD. The  $V/K_{\text{pyruvate}}$  profile yielded  $pK$  value of  $6.83 \pm 0.13$  at low pD and  $8.65 \pm 0.10$  at high pD. The pD-independent values of  $V/E_1$  and  $V/(K_{\text{pyruvate}})E_1$  are  $6.34 \times 10^{-1} \text{ s}^{-1}$  and  $1.05 \times 10^{-1} \text{ mM}^{-1} \text{ s}^{-1}$ , respectively. The ratio of the pH-independent values in  $H_2O$  and  $D_2O$  gives no significant solvent isotope effects.

One method for identifying the functional group of a residue at the active site of an enzyme is based on the temperature dependence of  $pK$  values [21]. Fig. 3A shows the log  $V$  versus pH plots for the enzyme at 37°C, 30°C and 20°C. The  $pK_2$  values of the basic side did not change in proportion to the decrease of temperature within error. The  $pK_2$  values at 37°C, 30°C and 20°C were  $8.44 \pm 0.12$ ,  $8.40 \pm 0.20$  and  $8.16 \pm 0.14$ , respectively. Fig. 3B shows the log  $V/K_{\text{pyruvate}}$  versus pH plots for the enzyme at 37°C, 30°C, and 20°C. The log  $V/K_{\text{pyruvate}}$  plots indicate the effects of temperature on the  $pK$  values of the ionizing groups on the free enzyme. The pH-dependent hydrolysis over the entire temperature range was shown by a kinetic scheme requiring minimally two critical ionizations of the free enzyme ( $EH_2$  and  $E$ ). The  $pK_1$  value of  $V/K_{\text{pyruvate}}$  increases in proportion to the decrease of temperature. The  $pK_1$  values at 37°C, 30°C and 20°C were  $6.48 \pm 0.2$ ,  $6.57 \pm 0.15$  and  $6.61 \pm 0.1$ , respectively. On the other hand, the  $pK_2$  values of  $V/K_{\text{pyruvate}}$  did not change in proportion to the decrease of temperature within error. The  $pK_2$  values at 37°C, 30°C and 20°C were  $8.25 \pm 0.15$ ,  $8.30 \pm 0.11$  and  $8.30 \pm 0.1$ , respectively. The  $V/K$  profile decreases on both the acidic and basic side to a limiting slope of 1, indicating that a change in the ionization state of a single group on each side of the profile is involved in the loss of activity.

The enthalpy of ionization was obtained from the van't Hoff equation,  $(\text{dln}k)/\text{d}T = \Delta H_{\text{ion}}/RT^2$ . The value of  $\Delta H_{\text{ion}}$  is determined from a plot of  $pK$  versus reciprocal absolute temperature, which has a slope of  $\Delta H_{\text{ion}}/2.303R$ . These  $pK$  values did not vary within error to the reciprocal of absolute temperature. The enthalpies of ionization,  $\Delta H_{\text{ion}}$ , calculated from the slope of  $pK_1$  and  $pK_2$ , are both near zero kcal/mol (data not shown).

One method for identifying the charge types at the active site of an enzyme is based on the use of organic solvent per-

turbation [21]. The solvent perturbation method depends on the different behavior of neutral and cationic acids when organic solvents are added to water. The  $pK$  values were changed between the cationic acid buffer in water and 15% ethanol (see Table 1). The  $pK_2$  value for the  $V$  of pyruvate was  $8.71 \pm 0.22$  in the cationic acid buffer in 15% ethanol. The  $pK$  values for the  $V/K$  of pyruvate were  $6.14 \pm 0.13$  and  $8.91 \pm 0.36$  in the cationic acid buffer in 15% ethanol.

## 4. Discussion

### 4.1. Interpretation of the pH dependence of kinetic parameters

The  $V/K$  for a reactant is the second-order rate constant for conversion of free enzyme and free reactant to products. Thus, the  $pK$ s observed in the  $V/K_{\text{pyruvate}}$  pH profiles reflect acid-dissociable functional groups in free E:ThDP:Mg:FAD in the presence of cofactors (ThDP, Mg, FAD). A bell-shaped pH profile is obtained for  $V/K_{\text{pyruvate}}$ . Both of the observed  $pK$  values must reflect enzyme groups since pyruvate exhibits no  $pK$  in the pH range 6.0–9.5. The group with a  $pK$  of 6.48 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 thiamine-catalyzed reactions is the initial ionization of the thiazole C-2 proton. The  $pK$  for this step is very high (17–19) [25], 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  $10^{12}$ -fold faster than their non-enzymatic counterparts [27]. Jordan et al. [30] 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 C-2 proton of enzyme-bound ThDP might also be shifted to a similar extent.

In addition to this base, however, there is clearly the requirement for an enzyme residue with a  $pK$  of 8.25 that must be protonated for activity. 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. Although a  $pK$  of 8.25 is very low for an alcoholate anion in water (see above), the environment in the active site of AHAS may be quite different from that of water. The bell-shaped nature of the pH profile obtained for pyruvate suggests that the two enzyme groups responsible for the observed  $pK$ s function in catalysis. The maximum velocity is pH-dependent at high pH, which exhibits a similar behavior on the basic side of the  $V/K_{\text{pyruvate}}$  pH profiles. The basic group of the  $V$  profile has a  $pK$  value similar to that of the  $V/K_{\text{pyruvate}}$  profile in catalysis as discussed above. Since  $V$  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-rate profiles.

The method of temperature dependence of  $pK$  values depends on the different enthalpy of ionization ( $\Delta H_{\text{ion}}$ ) values of a functional group of the residues involved in catalysis and/or binding [21]. As long as the ionizations are not accompanied by conformational changes in the enzyme which have large  $\Delta H_{\text{ion}}$  values that are often 15–25 kcal/mol, the  $\Delta H_{\text{ion}}$  can

Table 1  
Summary of  $pK$  values obtained from the pH dependence of  $V_{\text{max}}$  and  $V_{\text{max}}/K_m$  in water and ethanol

Pyruvate	Solvent <sup>a</sup>	$pK_1 \pm \text{S.E.M.}^b$	$pK_2 \pm \text{S.E.M.}^b$
$V_{\text{max}}$	Water	ND <sup>c</sup>	$8.44 \pm 0.12$
	Ethanol	ND	$8.71 \pm 0.22$
$V_{\text{max}}/K_m$	Water	$6.48 \pm 0.20$	$8.25 \pm 0.15$
	Ethanol	$6.14 \pm 0.13$	$8.91 \pm 0.36$

<sup>a</sup>Organic solvent was used to 15% ethanol.

<sup>b</sup> $pK_1$  indicates that the group must be protonated for enzyme activity and  $pK_2$  indicates that the group must be deprotonated.

<sup>c</sup>ND, not determined.



distinguish the groups. The carboxyl group shows almost no temperature dependence. On the other hand, the imidazole group and sulfhydryl group show 6–7.5 kcal/mol [21]. The  $V$  profiles at 20°C and 30°C are also pH-dependent at high pH ranges. The  $V$  profile implies the effect of these temperatures on the  $pK$  values of the ionizing groups on the enzyme–substrate complex responsible for its catalytic activity. The  $V/K_{\text{pyruvate}}$  values at 20°C and 30°C are also pH-dependent over the entire pH range. The  $V/K_{\text{pyruvate}}$  profile implies the effect of these temperatures on the  $pK$  values of the ionizing groups on the free enzyme responsible for its catalytic activity. Two groups were observed in  $V/K_{\text{pyruvate}}$  profiles. The  $\Delta H_{\text{ion}}$  is  $0.0 \pm 0.8$  kcal/mol from the slope of  $pK_1$  and  $0.0 \pm 0.3$  kcal/mol from the slope of  $pK_2$ , respectively. The enthalpies of ionization that control a general base ( $pK_1$ ) and a general acid ( $pK_2$ ) are similar to that of a carboxyl residue of a free amino acid ( $\Delta H_{\text{ion}} = \pm 1.5$  kcal/mol) within error.

The solvent perturbation method depends on the different behavior of neutral and cationic acids when organic solvent is added to water. The  $pK$  values were changed in the neutral acid and in the cationic acid in the presence of 15% ethanol. The log  $V$  plots represent the effect of these solvents on the  $pK$  values of the ionizing groups on the enzyme–substrate complex responsible for its catalytic activity. Two groups were observed in the  $V/K$  profiles. The  $pK_1$  of the acidic side was shifted down 0.4 pH unit as water was replaced by 15% ethanol; however, the  $pK_2$  of the basic side was shifted up 0.6 pH unit as water was replaced by 15% ethanol. In general, changes of 0.2 units are not considered significant, but changes of 0.4 units should be seen in the case where buffer and catalytic group are of opposite types [21]. This follows from the fact that neutral acids either increase or have a constant  $pK$  as water is replaced by an organic solvent such as ethanol, while cationic acids either decrease or have a constant  $pK$  under similar conditions [21]. These results suggest that the enzyme group ( $pK_1$ ) for the acidic side and the enzyme group ( $pK_2$ ) for the basic side are of the cationic and/or neutral type, respectively, but not anionic.

Recently, crystallization and preliminary X-ray diffraction analysis of the catalytic subunit of *Saccharomyces cerevisiae* AHAS have been reported [7,8]. In detail, centered approximately 3.7 Å from the C-2 of ThDP a prominent, tetrahedrally shaped island of electron density was observed in each monomer. Based on its shape and intensity, together with the composition of the crystallization buffer, the authors proposed that this is a phosphate ion. The oxygen atoms form hydrogen bonds with the main chain nitrogen atom of Gly116, the side chain of Gln202 and a water molecule. They speculate that the position of the phosphate ion is normally occupied by the carboxyl group of pyruvate and makes non-covalent interactions with the side chains of His126 and His599' (another subunit residue), and the main chain nitrogen atom of Thr598.

The group with a  $pK$  of 8.25 is most likely the general acid that must be protonated for activity. The group with a  $pK$  of 8.25 could certainly play a role in stabilization of the bound pyruvate conformation by hydrogen bonding the pyruvate carbonyl. The simplest explanation is that this enzyme residue is a proton donor group for the pyruvate. If so, there are only a few reasonable possibilities in terms of the functional group on the enzyme. Thr130 (corresponding residue of His126 from *S. cerevisiae* AHAS), His585 (corresponding residue of His599

from *S. cerevisiae* AHAS), and the main chain nitrogen atom of Thr586 (corresponding residue of Thr598 from *S. cerevisiae* AHAS) are required for tight binding and represent the initial interaction of the carboxyl group of pyruvate [8]. This presumably occurs via electrostatic interactions with the enzyme residues as discussed above. As a result, these residues are already anchored in the active site. There are several groups in the vicinity of the phosphate ion and any one of these could be the group that controls  $V/K_{\text{pyruvate}}$ . Three possible residues are the main chain nitrogen atom of Gly120 (corresponding residue of Gly116 from *S. cerevisiae* AHAS), the side chain of Gln206 (corresponding residue of Gln202 from *S. cerevisiae* AHAS) and a water molecule, any or all of which could become the group with a  $pK$  of 8.25. The water is ruled out since its ionization is very slight and it will be displaced as substrate enters the active site. If one assumes that enzyme catalysis reactions can usually occur on the side chain of the enzyme residue, but not on the enzyme backbone [28], then the nitrogen atom of the backbone of the Gly120 can be eliminated. The amino group of the side chain of Gln206 remains possible to bind to the enzyme due to 8.0 Å distance from the oxygen of phosphate ion in case of *S. cerevisiae* AHAS. However, the  $pK$  of the amino group of the side chain of Gln206 is very high ( $> 17$ ), making Gln206 a poor candidate for the general acid.

The roles of three well-conserved histidine residues (His351, His392, His487) in tobacco AHAS were determined using site-directed mutagenesis [11]. Both H487F and H487L mutations abolished the enzymatic activity as well as the binding affinity for the cofactor FAD. These results suggested that the His487 residue is located near the active site of the enzyme and is likely involved in the binding of cofactor FAD in tobacco AHAS. The mutation of H392 did not have any significant effect on the kinetic parameters [11]. In order to test the possibility of the general base, we tested the pH dependence of  $V/K$  for H392M mutants. The kinetic parameters were unaffected, indicating that this mutant is not involved in any catalytic reaction.

All ThDP-dependent enzymes are believed to follow a similar reaction mechanism [23]. PDC from *Zymomonas mobilis* also uses ThDP as a cofactor and is believed to follow a similar reaction mechanism to AHAS. The mutagenesis study from PDC has suggested that upon substrate binding, His113 is placed close to the C-2 of the thiazole ring and acts as a base to assist ionization of ThDP [24]. A molecular modeling study of this enzyme suggested that His113 is protonated and has been implicated in interactions with the alcoholate ions of lactyl-ThDP and he-ThDP [26]. In the case of tobacco AHAS, we could speculate on the possibility of the well-conserved His142 as a general base (corresponding residue of His113 from *S. cerevisiae* AHAS), even if it is located 14.7 Å from the oxygen of phosphate ion in the crystal structure of *S. cerevisiae* AHAS. Assuming that His142 is a general base, when a substrate binds the enzyme, the optimum conformation change displaces His142 toward the C-2 of the thiazole ring. Therefore, the possibility of His142 acting as the general base may be low since it would require a significant conformational change. We propose that a general base with a  $pK$  of 6.48 could possibly be the 4'-amino group of the pyrimidine ring of ThDP. The possibility of the 4'-amino group of ThDP acting as a general base is being pursued presently.

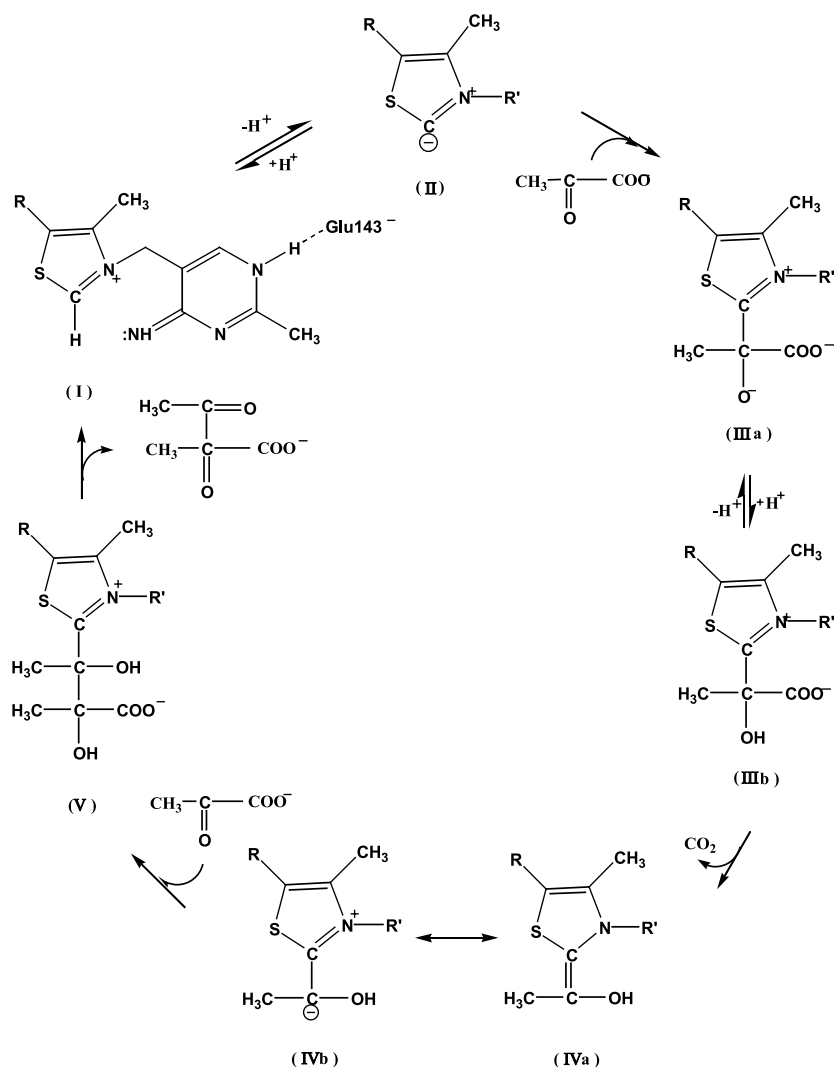


Fig. 4. Proposed catalytic mechanism of AHAS. Intermediates I to V are discussed in the text. Double arrows are ionization, single arrows are steps involving reactant addition or release, and the double-headed arrow represents resonance. The single arrows indicate the prevailing reaction direction when pyruvate is converted to acetaldehyde and CO<sub>2</sub>, and are not intended to imply that the step is irreversible. R represents the hydroxyethyl diphosphate group while R' is the methyl aminopyrimidine ring of ThDP.

#### 4.2. Chemical mechanism

The data suggest a mechanism in which two groups participate in catalysis with the reactant pyruvate. This is suggested by the pH dependence of the  $V$  profiles, the  $V/K$  profiles, the pD profiles, the temperature dependence profiles and the organic solvent perturbation profiles (we will not address in detail ThDP and FAD catalysis involvement in the enzyme residues in this study). A mechanism in which an intermediate C-2 carbanion is formed with a short lifetime has been postulated by Kern et al. [23] based on thiamine-catalyzed reactions. A mechanism taking into account the carbanionic intermediate and the above pH studies is shown in Fig. 4. Glu143, which is conserved in all AHASs, forms a 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. A proton is abstracted from C-2 by the 4'-imino form (a general base) of ThDP with a pK of 6.48. The pK for the general base is observed in the  $V/K_{\text{pyruvate}}$  profile. Pyruvate binds to the en-

zyme as the monoanion with its carboxyl groups. The pK for a general acid is 8.25. The general acid group is observed in both of the  $V$  profiles and  $V/K_{\text{pyruvate}}$  profiles.

Considering the conventional view of the catalytic cycle, ThDP (I) is ionized to the reactive ylide (II). The resulting carbanion attacks a pyruvate molecule stabilized by delocalization of electrons into the carboxyl probably with the assistance of enzyme residues in the vicinity of carboxyl. This yields the lactyl-ThDP intermediate (IIIa) via the transiently formed alcoholate anion (IIIb). After decarboxylation, the enamine of hydroxyethyl-ThDP (IVa) undergoes charge separation giving the α-carbanion (IVb) that can now react with a second pyruvate to give the product complex (V). Finally, the product is released and ThDP is regenerated.

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