

## The pH Studies of Recombinant Acetohydroxy Acid Synthase from Tobacco

Jung-Do Choi,<sup>†</sup> Bok-Hwan Kim,<sup>‡</sup> and Moon-Young Yoon\*

Department of Chemistry, Hanyang University, Seoul 133-791, Korea

<sup>†</sup>School of Life Sciences, Chungbuk National University, Cheongju 361-763, Korea

<sup>‡</sup>Department of Health Science, Korea National Open University, Seoul 110-791, Korea

Received February 12, 2003

The pH dependence of the kinetic parameters of recombinant acetohydroxy acid synthase catalyzed reaction was determined in order to obtain information about the chemical mechanism, particularly acid-base chemistry. The maximum velocity and  $V/K$  for pyruvate were bell-shaped with estimated pK values of 6.5-6.7 and 8.6-8.9, respectively. The maximum velocity and  $V/K$  for 2-ketobutyrate were also bell-shaped with estimated pK values of 6.6-7.0 and 8.4-8.6. The pH dependence of  $1/K_i$  for 3-bromopyruvate, a competitive inhibitor of pyruvate, was also bell-shaped, giving pK values almost identical with those obtained for pyruvate. Since the same pK values were observed in the  $pK_{i, 3\text{-bromopyruvate}}, V/K$  pH profiles and  $V_{\max}$  profiles, both enzyme groups must be in their optimum protonation state for efficient binding of reactants. These results reflect that two enzyme groups are necessary for binding of substrate and/or catalysis.

**Key Words :** Acetohydroxy acid synthase, pH study, Tobacco

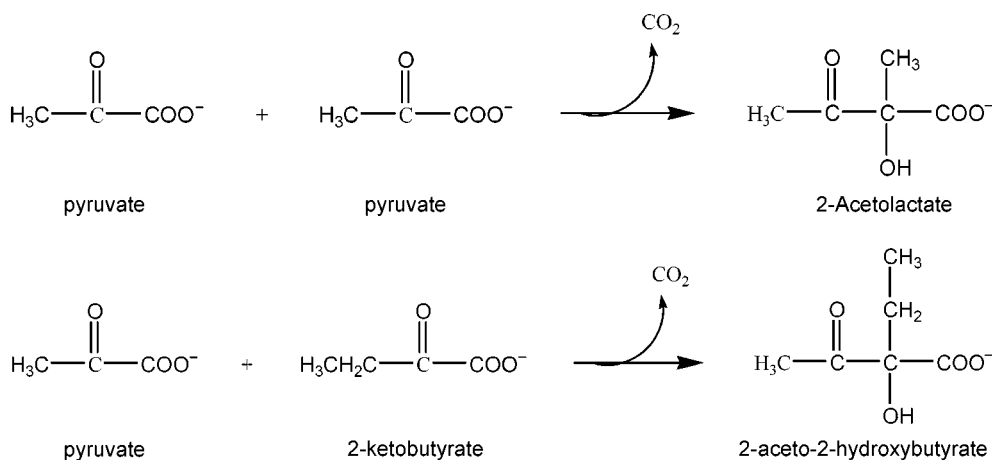
### Introduction

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 plant and microorganisms. 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 (Scheme 1).

AHAS, which catalyzes the decarboxylation of 2-ketoacids, requires thiamine diphosphate (ThDP) and a divalent metal ion as cofactors (that anchors ThDP in the active site). AHAS also requires FAD, which is unexpected, because the reaction involves no oxidation or reduction.<sup>1,2</sup> The first two

cofactors, ThDP and metal ion, 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 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 interest in AHAS was stimulated by the discovery that it is the target site of at least four structurally diverse families of herbicides; sulfonylureas, imidazolinones, triazolopyrimidines, and pyrimidinyl oxybenzoates.<sup>2</sup> These compounds bear no resemblance to 2-ketoacids 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 formation of acetolactate (AL)



Scheme 1

\*To whom correspondence should be addressed. Tel: +82-2-2290-0946; Fax: +82-2-2299-0762; e-mail: myyoon@hanyang.ac.kr

and acetohydroxybutyrate (AHB) have been determined simultaneously.<sup>2,5</sup> The ratio between the rates of production of the two alternative products and the concentrations of the substrates pyruvate and 2-ketobutyrate leads them to  $V_{\text{AHB}}/V_{\text{AL}} = R$  ( $[2\text{-ketobutyrate}]/[\text{pyruvate}]$ ). 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, suggesting a 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.*<sup>6</sup> 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 catalytic subunit<sup>7</sup> and AHAS enzyme<sup>8</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,  $\text{Mg}^{2+}$  and FAD. The structure, in combination with molecular modeling, also suggests 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). Although an abundance of information is available on many aspects of the acetohydroxy acid synthase reaction,<sup>15,16,17</sup> little is known of the chemical mechanism. This study presents an investigation of the pH dependence on the steady state kinetic parameters of recombinant AHAS from tobacco. Data will be discussed in terms of the reaction mechanism, particularly the acid-base chemistry.

## Materials and Methods

**Materials.** Thimine diphosphate, GSH, FAD,  $\alpha$ -naphthol and creatine were obtained 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 a modification of the procedure described by Chang *et al.* (1997).<sup>9</sup> *E. coli* DH5 $\alpha$  cells containing the expression vector pGEX-ALS were grown at 37 °C in Luria-Bertani (LB) medium containing 50  $\mu\text{g}/\text{mL}$  ampicillin to an  $\text{OD}_{600}$  of 0.7–0.8. Expression of the ALS gene was induced by adding 1.0 mM isopropyl-D-thiogalactoside (IPTG). Cells were grown for an additional 4 hours at 30 °C, and 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  $\text{MgCl}_2$ ) containing protease inhibitors (2  $\mu\text{g}/\text{mL}$  Leupeptin, 4  $\mu\text{g}/\text{mL}$  Aprotinin, 2  $\mu\text{g}/\text{mL}$  Pepstatin A). The cell suspension was lysed by sonication at 4 °C and the homogenate was

centrifuged at 20,000 rpm for 20 min. The supernatant was applied to a GSH-coupled Sepharose 6B column and unbound proteins were removed by extensive washing with the PBST buffer. GST-AHAS fusion protein was eluted 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 analyzed by SDS-PAGE 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>10</sup> with a modification as reported previously.<sup>11</sup> The standard reaction mixture contained 100 mM potassium phosphate buffer (pH 7.5), 1 mM ThDP, 10 mM  $\text{MgCl}_2$ , 20  $\mu\text{M}$  FAD, 75 mM pyruvate, 30 mM 2-ketobutyrate and the enzyme (0.1U) in the absence or presence of various concentrations of inhibitors. Assays were initiated by the addition of AHAS and incubated at 37 °C for 30 min, and terminated by the addition of 6 N  $\text{H}_2\text{SO}_4$ . 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. The temperature was maintained at 37 °C using a circulating water bath with the capacity to heat and cool the thermo spacers in the cell compartment. All reactions were carried out in a 1-mL cuvette with 1-light path. The absorbance of the reaction mixture was determined at 525 nm. One unit (U) of activity was defined as the amount of enzyme required to form 1  $\mu\text{mol}$  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.** To be certain that the kinetic mechanism is pH independent and to estimate the  $K_m$  values for pyruvate and 2-ketobutyrate, steady-state kinetic patterns in the absence of products were obtained at pH 6.0, 7.5 and 9.0. It was obtained by measuring the rate at different levels of pyruvate and several fixed levels of 2-ketobutyrate and at saturating concentration of cofactors. Once the measured kinetic patterns were shown to be pH independent, saturation curves for pyruvate and 2-ketobutyrate were obtained at a fixed saturating concentration of the cofactors as a function of pH. Buffers used at 100 mM concentration were MES at 5.5 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. Several of the 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 doubled in a separate assay to saturate. No significant rate change was detected. Inhibition data were obtained for 3-bromopyruvate, competitive inhibitor versus pyruvate. The pyruvate concentration varied, while the 2-ketobutyrate concentrations were maintained at saturation. Once the competitive nature of the inhibition was determined to be pH independent, Dixon experiments were performed in which the variable substrate was fixed at a concentration equal to its  $K_m$ , and the inhibitor concentration was varied from zero over the range where there was inhibition. 3-

bromopyruvate was unstable and precipitated above higher pH (> 8.0).

**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 of Cleland (1979).<sup>12</sup> Individual saturation curve were fit to Eq. (1). Data for the sequential kinetic patterns were fit using Eq. (2). Competitive inhibition data at each pH were fitted to Eq. (3). Data for pH profile that decreased with a slope of  $-1$  at low pH and a slope of  $-1$  at high pH were fit to Eq (4).

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

$$v = VAB/(K_{ia}K_b + K_aB + K_bA + AB) \quad (2)$$

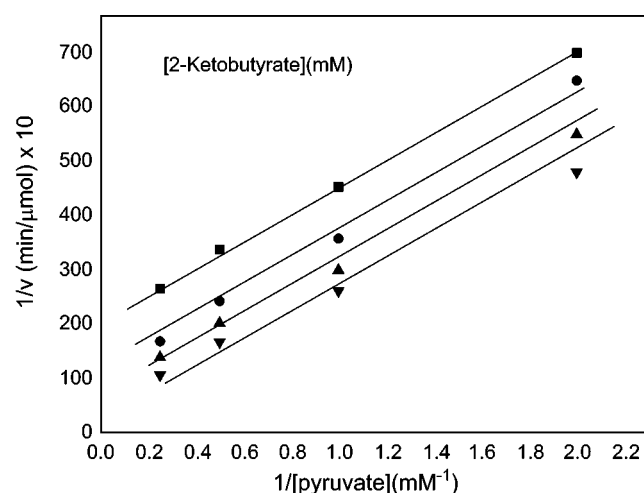
$$v = VA/\{K(1 + I/K_{is}) + A\} \quad (3)$$

$$\text{Log } y = \log\{C/(1 + [H^+]/K_1 + K_2/[H^+])\} \quad (4)$$

In Eqs. (1) and (2), A is the reactant concentration. V is the maximum velocity, and K is the Michaelis constant for the varied substrate. In Eq. (3), I is inhibitor concentration and  $K_{is}$  is inhibition constant for slope. In Eq (4), H is the hydrogen ion concentration,  $K_1$  and  $K_2$  represent dissociation constant for enzyme groups, y is V/K, and C is the pH-independent value of y.

## Results and Discussion

It is important to determine the stability of an enzyme over the pH range studied, since this could potentially give rise to incorrectly determined values of the kinetic parameters as well as the inhibition constants for inhibitors. In this study, acetohydroxy acid synthase was incubated with the buffer systems for up to 30 min. Aliquots were removed at various time points and assayed for activity at pH 7.5, a pH where



**Figure 1.** Initial velocity patterns for the acetohydroxy acid synthase at pH 9.0. The assays were carried out under standard conditions. The results fitted plots ( $\nabla$ ) for 0.5 mM, ( $\blacktriangle$ ) for 2 mM, ( $\bullet$ ) for 7 mM, ( $\blacksquare$ ) for 40 mM of 2-ketobutyrate, respectively. Points are experimental and the solid lines are from a fit using equation (1).

**Table 1.** Summary of pK values obtained from the pH dependence of kinetic parameters<sup>a</sup>

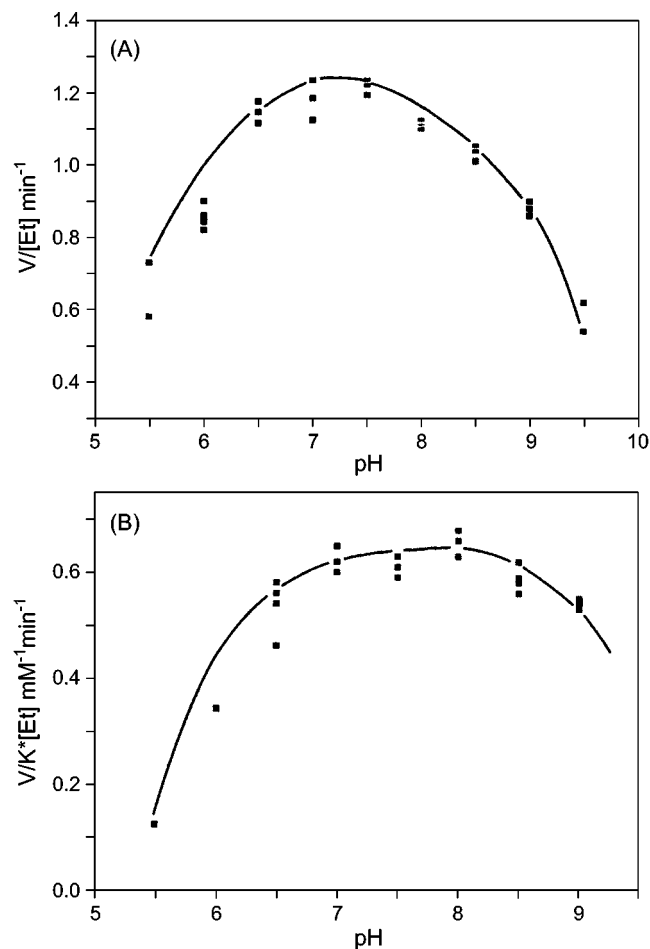
Parameters	Variable Substrate <sup>b</sup>	pK <sub>1</sub> ± SE	pK <sub>2</sub> ± SE
V <sub>max</sub>	pyruvate	6.5 ± 0.1	8.6 ± 0.1
V <sub>max</sub>	2-ketobutyrate	7.0 ± 0.1	8.4 ± 0.2
V/K <sub>pyruvate</sub>	pyruvate	6.7 ± 0.2	8.9 ± 0.1
V/K <sub>2-ketobutyrate</sub>	2-ketobutyrate	6.6 ± 0.1	8.6 ± 0.1
pK <sub>i</sub> <sup>c</sup>	pyruvate	6.5 ± 0.2	8.9 ± 0.2

<sup>a</sup>pK<sub>1</sub> indicates that the group must be protonated for enzyme activity and pK<sub>2</sub> indicated that the group must be deprotonated. <sup>b</sup>Substrate indicated was varied around K<sub>m</sub> and another substrate was saturated. <sup>c</sup>pK<sub>i</sub>s was obtained using only pyruvate substrate.

the enzyme is known to be stable.<sup>6</sup> No significant decrease in activity occurred at the pH extremes (pH 5.5 and pH 9.0) (data not shown). An estimate of the variation of kinetic mechanism over the pH range to be studied is required in order to ensure that enzyme complex is being titrated. Thus, steady state kinetic patterns were obtained at pH 5.5 and 9.0 by varying 2-ketobutyrate at fixed level of pyruvate. The results at pH 7.5 are shown in Figure 1. These results are in agreement with the ping pong mechanism of pyruvate prior to 2-ketobutyrate noted by Lee *et al.*<sup>6</sup>

The pH dependence of the kinetic parameters for acetohydroxy acid synthase was determined over the pH range 5.5 to 9.5 in the presence of both substrates pyruvate and 2-ketobutyrate. Each pH profile provides information concerning the ionizable groups of the enzyme and its substrates as well as information about binding and catalysis. A pH dependent decrease in V/K or V<sub>max</sub> profiles provide information regarding ionizable groups on substrates and/or the enzyme that must be in a given state of ionization to participate in either catalysis or binding.<sup>13</sup> Interpretation of these profiles requires the assumption that there is only one ionization state of active site residues that supports catalysis, and that all protonation/deprotonation equilibria are rapid. The observed pK values from each of the pH profiles generated are summarized in Table 1.

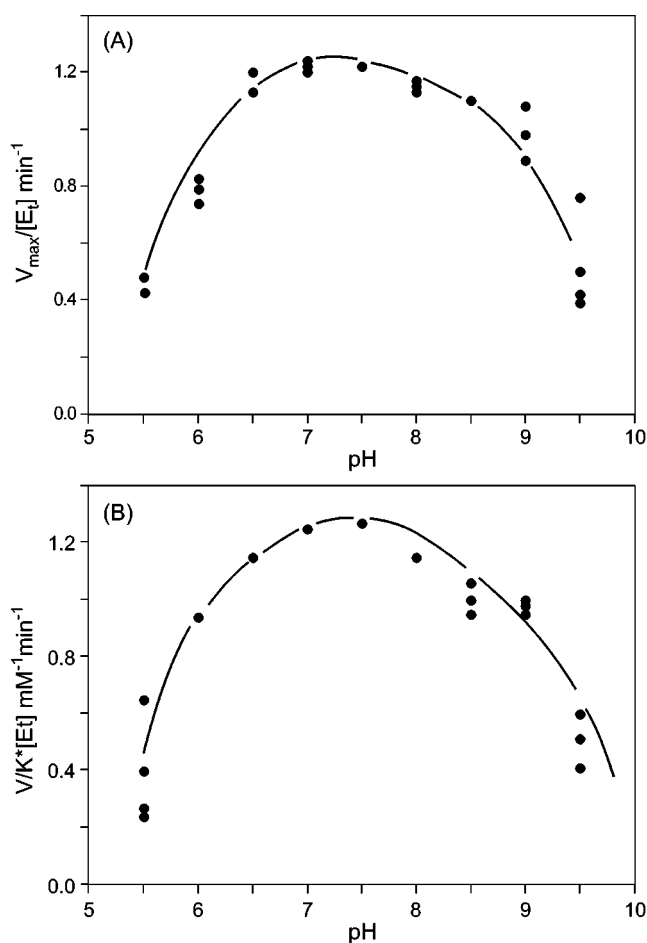
The V/K for a reactant is the second-order rate constant for conversion of free enzyme and free reactant to products. Thus, the pKs observed in the V/K pH profiles for pyruvate reflect acid-dissociable functional groups in E:2-ketobutyrate:ThDP:FAD:Mg. The pKs observed in the V/K pH profiles for 2-ketobutyrate reflect acid dissociable functional groups in E:pyruvate:ThDP:FAD:Mg. The V/K profiles for pyruvate and 2-ketobutyrate on either the acidic or the basic side decrease to a limiting slope of one, 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 V/K profiles for pyruvate and 2-ketobutyrate have the same maximum value at pH 7.5 and decrease on either side of this maximum (Figure 2B and 3B, respectively). However, the basic side of the V/K for pyruvate decreased slightly. The same activity observed near neutral pH in the V/K profiles might be related to no change in the substrate requirement of AHAS. The pK values for V/K<sub>pyruvate</sub> profile obtained on the acidic side are 6.4, while that on the basic side is 9.0. The pK



**Figure 2.** pH dependence of  $V_{\max}$  (A) and  $V/K$  (B) of pyruvate in the AHAS reaction. The concentration of pyruvate was varied around its  $K_m$  with 2-ketobutyrate concentration maintained at saturation. The points are experimental values. The curves for  $V_{\max}$  and  $V/K_{\text{pyruvate}}$  are theoretical from a fit of eq. (4) to the data.

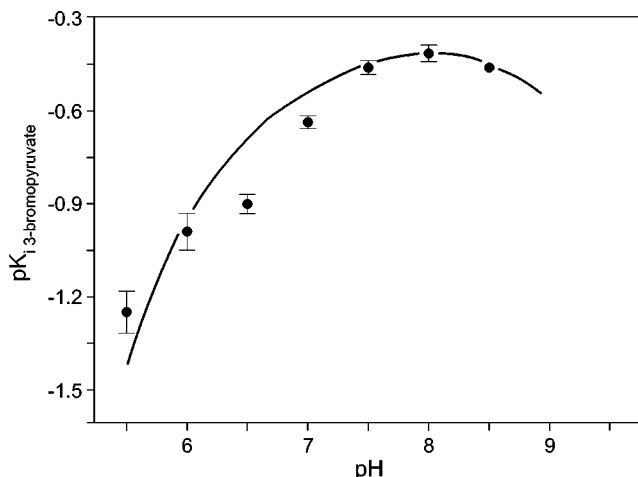
values for  $V/K_{2\text{-ketobutyrate}}$  profile are 6.4 on the acidic side and 8.4 on the basic side. The observed pK values from  $V/K_{\text{pyruvate}}$  and  $V/K_{2\text{-ketobutyrate}}$  must reflect enzyme groups since pyruvate and 2-ketobutyrate exhibit no pKs in the pH range 5.5-9.5. Qualitatively similar pH profiles are observed in the  $V/K$  profiles of pyruvate and 2-ketobutyrate. No change in the value of the acidic pKs was observed. However, there is an apparent perturbation in the value of the basic pK from 8.4 to 9.0. The bell-shaped nature of the pH rate profiles obtained for pyruvate and 2-ketobutyrate suggests that two groups on AHAS are responsible for the observed pKs function in catalysis. These groups are also required in their correct protonation state for the binding of 3-bromopyruvate since its dissociation constant increases above and below the same pKs observed in the  $V/K$  profiles (*vide infra*).

The  $V_{\max}$  profile contains information about the ionization states of the central enzyme-substrate complexes. The  $V_{\max}$  profiles show that substrate behaves similarly between the pyruvate and 2-ketobutyrate. The maximum velocity obtained under pyruvate concentrations and saturating 2-ketobutyrate decreased at low and high pH with pK values of 6.3 and 8.9,



**Figure 3.** pH dependence of  $V_{\max}$  (A) and  $V/K$  (B) of 2-ketobutyrate in the AHAS reaction. The concentration of 2-ketobutyrate was varied around its  $K_m$  with pyruvate concentration maintained at saturation. The points are experimental values. The curves for  $V_{\max}$  and  $V/K_{2\text{-ketobutyrate}}$  are theoretical from a fit of eq. (4) to the data.

respectively (Figure 2A). The maximum velocity for 2-ketobutyrate decreased at low and high pH with pK values of 6.5 and 8.7, respectively (Figure 3A). The maximum velocity profiles have an optimum at pH 7.5 under conditions pyruvate varying at saturating 2-ketobutyrate and 2-ketobutyrate varying at saturating pyruvate. A comparison of the maximum velocity of pyruvate and 2-ketobutyrate shows the binding of pyruvate and 2-ketobutyrate to the enzyme does not affect the ionization of the group on the both side of the pH profiles since nearly the same pK (approximate 6.3-6.5 on the acidic side and approximate 8.7-8.9 on the basic side) value was observed within error limits. The two groups likely function in catalysis as discussed above for  $V/K$  profiles. The group with a pK of 6.3-6.5 is required to be unprotonated for optimal activity and is responsible for a pH dependent activation, as it becomes unprotonated. The pK values of the acidic side (6.3-6.5) were similar to those observed in the  $V/K$  profiles, indicating that there was no perturbation as the pH increased from 5.5 to 7.5. The pK values of the basic side (8.7-8.9) were slightly different from those observed in the  $V/K$  profiles, indicating that there was some perturbation as

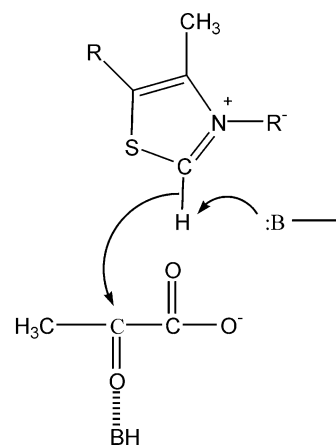


**Figure 4.** pH dependence of 3-bromopyruvate binding to AHAS. All data were obtained from Dixon plots varying the concentration of 3-bromopyruvate and pyruvate equal to its  $K_m$ . Data were obtained as discussed under Methods and Materials. The data were fitted to eq. (4).

the pH increased from 7.5 to 9.5. Both groups observed in the  $V_{max}$  profiles were observed in the  $V/K$  profiles, suggesting that these groups interact with pyruvate and 2-ketobutyrate, which are bound sequentially to the enzyme.<sup>6</sup>

In order to further test if the  $pK$  values obtained from the pH dependence of kinetic parameters are intrinsic, the pH dependence of the  $K_i$  for 3-bromopyruvate, a structural analogue (3-hydrogen of pyruvate replaced by bromine) and competitive inhibitor of pyruvate, was determined. Since this inhibition is competitive it is only observed under limited substrate conditions and where all steps prior to addition of substrate have come to thermodynamic equilibrium. Therefore, true  $pK$  values observed for the pH dependence of  $K_i$  values for competitive inhibitors can be used to check whether or not the observed  $pK$  values in the substrate profile and true  $pK$  values if the protonation state of these groups affects inhibitor binding.<sup>14</sup> The  $pK_i$  of 3-bromopyruvate was unstable at high pH, but decreased slightly above pH 7.5. These data were fitted to the bell-shaped pH profiles in which the limiting slopes were +1 and -1. The observed  $pK_i$  values for 3-bromopyruvate were 6.5 on the acidic side and 8.9 on the basic side (Figure 4). The  $pK$  values obtained from the  $pK_i$  profile of 3-bromopyruvate are essentially the same as the values observed from the  $V/K$  profiles of pyruvate and 2-ketobutyrate. Thus, the observed  $pK$  values in the  $V/K$  profiles of pyruvate and 2-ketobutyrate are indeed intrinsic.

The  $pK$ s values observed at pH 8.4 and 9.0 on the basic side in the  $V/K$  profile of AHAS activity were also seen in the  $pK_i$  profile with 3-bromopyruvate as competitive inhibitor against pyruvate. Since there are no common inhibitor or substrate  $pK$  values in this pH range, this decrease on the basic side in the  $pK_i$  profile indicates a loss of binding of this inhibitor as a result of ionization of a group on the enzyme. The  $pK$ s values seen on the acidic side of the  $V/K$  profile at pH 6.4, in either the presence of pyruvate or 2-ketobutyrate,



**Scheme 2.** Proposed acid-base chemical mechanism of AHAS.

and the  $pK_i$  seen on the acidic side of 3-bromopyruvate also indicate the ionization of a group on the enzyme.

A mechanism taking into account the above pH studies is shown in Scheme 2. Pyruvate binds to enzyme as the monoanion with a carboxyl group. A proton is abstracted from C-2 by an enzyme general base with a  $pH$  of 6.3-6.5. The  $pK$  for general base is observed in all of the  $V$  profiles, all of the  $V/K$  profiles, and the  $pK_i$  3-bromopyruvate profile. Considering the conventional view of the catalytic cycle, ThDP ionizes to the reactive ylide. The resulting carbanion is stabilized by delocalization of electrons into the carboxyl presumably with the assistance of a one or more positively charged enzyme residues in the vicinity of the carboxyl that then attacks a pyruvate molecule. The  $pK$  for the general acid is about 8.4-8.9. The general acid group is observed in all of the same pH rate profiles specified above for the general base, as well as the  $pK_i$  3-bromopyruvate profiles. Since the same  $pK$  values are observed in the  $pK_i$  3-bromopyruvate and  $V/K$  pH profile, both enzyme groups must be in their optimal protonation state for efficient binding of reactants. The general acid group is likely a neutral or acidic residue, since it would be repulsive to the oxygen of pyruvate if it were anionic. As a neutral acidic residue there is also the possibility of hydrogen bonding to the carbonyl of pyruvate. At the release of  $CO_2$ , the first reaction, both the general base and general acid groups might be in a protonation state opposite to that in which they started when pyruvate was bound.

**Acknowledgments.** This work was supported by the Korea Science and Engineering Foundation (R01-2000-00137).

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