# Kinetics and Mechanism of Acetohydroxy Acid Synthase Isozyme III from *Escherichia coli*†

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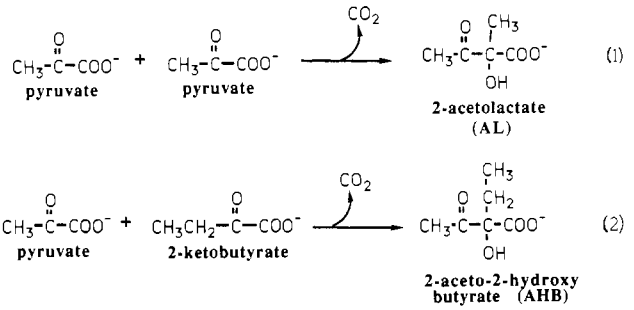
ABSTRACT: Acetohydroxy acid synthase (AHAS, EC 4.1.3.18) isozyme III from *Escherichia coli* has been studied in steady-state kinetic experiments in which the rates of formation of acetolactate (AL) and acetohydroxybutyrate (AHB) have been determined simultaneously. The ratio between the rates of production of the two alternative products and the concentrations of the substrates pyruvate and 2-ketobutyrate (2KB) leading to them, $R$, $V_{AHB}/V_{AL} = R([2KB]/[pyruvate])$, was found to be 40 ± 3 under a wide variety of conditions. Because pyruvate is a common substrate in the reactions leading to both products and competes with 2-ketobutyrate to determine whether AL or AHB is formed, steady-state kinetic studies are unusually informative for this enzyme. At a given pyruvate concentration, the sum of the rates of formation of AL and AHB was nearly independent of the 2-ketobutyrate concentration. On the basis of these results, a mechanism is proposed for the enzyme that involves irreversible and rate-determining reaction of pyruvate, at a site which accepts 2-ketobutyrate poorly, if at all, to form an intermediate common to all the reactions. In the second phase of the reaction, various 2-keto acids can compete for this intermediate to form the respective acetohydroxy acids. 2-Keto acids other than the natural substrates pyruvate and 2-ketobutyrate may also compete, to a greater or lesser extent, in the second phase of the reaction to yield alternative products, e.g., 2-ketovalerate is preferred by about 2.5-fold over pyruvate. However, the presence of an additional keto acid does not affect the relative specificity of the enzyme for pyruvate and 2-ketobutyrate; this further supports the proposed mechanism. The substrate specificity in the second phase is an intrinsic property of the enzyme, unaffected by pH or feedback inhibitors. The model for the enzyme that is suggested by the data is one in which the site for the competing second substrate has a specific and rather rigid nonpolar cavity of appropriate size to bind the $CH_3CH_2$ group of 2-ketobutyrate. Two other isozymes of AHAS from *E. coli* have also been studied and follow the same reaction mechanism. While AHAS II is similar to AHAS III in its substrate specificities, AHAS I has a much lower preference for 2-ketobutyrate ($R$ = 2) and reacts very little with larger keto acids.

Acetohydroxy acid synthase (AHAS, EC 4.1.3.18, also known as acetolactate synthase) catalyzes the condensation of an acetaldehyde moiety derived from pyruvate either with another molecule of pyruvate to form 2-acetolactate (AL, eq 1) or with 2-ketobutyrate to form 2-aceto-2-hydroxybutyrate (AHB, eq 2).




The synthesis of these acetohydroxy acids is the first of a series of common steps in the biosynthesis of the branched-chain amino acids (Umbarger, 1978, 1987). The two AHAS reactions are key steps in these pathways, as they are irreversible and committed steps toward the synthesis of two different sets of products, valine (and leucine) or isoleucine. Considerable interest in this enzyme has also arisen recently from the discovery that it is a good target for herbicides (La Rossa & Schloss, 1984; Shaner et al., 1984; Ray, 1986).

At least three different AHAS isozymes, differing in genetic loci and control as well as in physiological and biochemical parameters, have been recognized in the enteric bacteria. The genes *ilvBN*, *ilvGM*, and *ilvIH*, which code for the isozymes known as AHAS I, II, and III, respectively, have been cloned and sequenced (Friden et al., 1985; Wek et al., 1985; Lawther et al., 1981; Squires et al., 1983). By use of bacterial clones producing large quantities of a single isozyme it is possible to isolate each enzyme in significant quantities. AHAS I (Eoyang & Silverman, 1984), AHAS II (Schloss et al., 1985), and AHAS III (Barak et al., 1988) have been purified essentially to homogeneity. The availability of isolated enzymes, together with a gas chromatographic method for the simultaneous quantitative assay of AL and AHB (Gollop et al., 1987), allowed us to study the substrate specificity of the various AHAS isozymes and to propose a quantitative picture of their physiological roles under different conditions (Barak et al., 1987). This method has also made it possible to study the steady-state kinetics of an AHAS isozyme in the presence of both pyruvate and 2-ketobutyrate. Because pyruvate is a common substrate in both reactions, as well as an alternative substrate (to 2-ketobutyrate) in one of them (eq 1 and 2), the steady-state kinetics can provide considerable information about the mechanism of the enzyme.

In this paper, we describe in detail steady-state kinetic studies of purified *Escherichia coli* AHAS III. Comparison of these data with less extensive data on AHAS I and II indicates that all three isozymes have similar mechanisms,

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despite significant quantitative differences among them.

## Materials and Methods

*Materials.* Sodium pyruvate, sodium 2-ketovalerate, sodium glyoxylate, flavin adenine dinucleotide, thiamin pyrophosphate, 2,3-butanedione, amino acids, and buffer compounds were obtained from Sigma Chemical Co., St. Louis, MO. Ethyl 2-acetoxy-2-methylacetoacetate, sodium 2-ketobutyrate, 2,3-pentanedione, and 2,3-hexanedione were obtained from Aldrich Chemical Co., Milwaukee, WI. 3,4-Hexanedione was obtained from Merck, Darmstadt, FRG. Other materials were of analytical grade.

Aqueous solutions of acetolactate were prepared by saponification of ethyl 2-acetoxy-2-methylacetoacetate as described by Krampitz (1948).

*Enzymes.* AHAS III was purified as described elsewhere (Barak et al., 1988), from *E. coli* K-12 CB88-432, and had a specific activity of 2.7 units/mg. Purified AHAS II, from a clone of *Salmonella typhimurium ilvGM* in *E. coli*, was a gift of Dr. J. V. Schloss (Schloss et al., 1985). AHAS I, purified from a clone of *E. coli ilvBN* (Newman et al., 1982) to more than 90% homogeneity by the criteria of polyacrylamide gel electrophoresis, was the generous gift of Dr. J. V. Schloss (Aulabaugh and Schloss, unpublished results).

*Enzyme Assay.* The enzymatic reaction was carried out as previously described. The standard reaction mixture contained 10 mM $MgCl_2$, 20 μg/mL flavin adenine dinucleotide, and 30 μg/mL thiamin pyrophosphate in 0.1 M potassium phosphate buffer (pH 7.6). The formation of 2-acetolactate in the absence of 2-ketobutyrate was followed by the colorimetric method of Bauerle et al. (1964). The gas chromatographic (GLC) method (Gollop et al., 1988) was employed when both substrates of AHAS were present in the reaction mixture. In a typical experiment, 40 μg of AHAS III was added to 20 mM pyruvate and varying amounts of 2-ketobutyrate, in 5 mL of the standard reaction mixture, and the enzymatic reaction was allowed to proceed for 6 min at 37 °C. In experiments at low pyruvate concentration, the reaction volume was increased to 10 mL to obtain readily detectable amounts of products without exhaustion of the substrates. In this method, the reaction was quenched by addition of an amount of $H_3PO_4$ that lowered the pH to 4.0 ± 0.2. The acetohydroxy acid products were converted to the corresponding 2,3-diketones by iron salt catalyzed oxidative decarboxylation (Gollop et al., 1987), and the mixture of volatile diketones was then transferred to methanol and analyzed by GLC with electron capture detection. The chromatograms were acquired and analyzed by using the Chromaset 401 PC-based chromatographic data handling system (Barspec Corp., Rehovoth, Israel).

The authentic diketones were processed through the entire analytical scheme to calibrate response factors (chromatographic peak area per mole of diketone). Since the diketones are susceptible to degradation during storage, forming compounds that are not readily detected by GLC using electron capture detection, it was necessary to check the purity of the standards by another method, such as proton NMR. Use of a sample of, e.g., pentanedione as a standard, which had a lower than assumed weight percent purity, would lead to overestimation of the amount of AHB in a reaction mixture. The reliability of the GLC assay technique for AL was proven by comparison of the measured rate of AL formation in the presence of pyruvate alone with that determined by the colorimetric method (Bauerle et al., 1964) and the continuous spectrophotometric assay of pyruvate disappearance (Schloss et al., 1985). The results of the three methods were in good agreement (Gollop et al., 1987). The assay for AHB was tested by analyzing successive aliquots of reaction mixtures containing 5 mM pyruvate, 0.1 or 0.2 mM 2-ketobutyrate, and AHAS III. In such reaction mixtures, the measured amount of AHB reached a plateau with time, which was 95–107% of the amount expected from conversion of all the 2-ketobutyrate present to AHB, while the sum of the products AL and AHB increased linearly for at least 1 h.

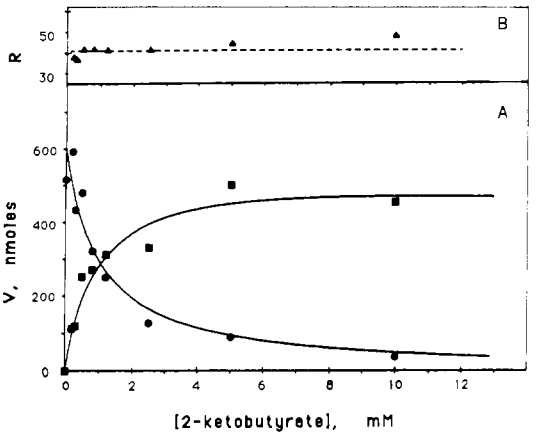



FIGURE 1: (A) Dependence of rate of product formation by purified AHAS III on the 2-ketobutyrate concentration. The reaction was carried out in the presence of 40 mM pyruvate in 5 mL of the standard reaction mixture, quenched after 6 min, and analyzed by GLC as described under Materials and Methods for acetolactate (AL, ●) and 2-aceto-2-hydroxybutyrate (AHB, ■). $V$ is given in nanomoles per reaction mixture. The lines are the best simultaneous fit of the data to eq 8 and 9. (B) Calculated values of $R = (V_{AHB}[Pyr])/(V_{AL}[2KB])$ for each reaction mixture (▲). The dashed line is at the average $R$ for the experiment.

*Analysis of the Kinetic Data.* The steady-state kinetic data were fit to the appropriate equations by using the program ENZFITTER, written by R. J. Leatherbarrow (1987), for a PC-compatible microcomputer. Simple weighting was used, with the "robust weighting" algorithm of Mosteller and Tukey (1977) to deal with outliers. Explicit weighting by standard errors was used for replots of derived parameters.

## Results

For purified AHAS III, the dependence of AL formation on the pyruvate concentration obeys Michaelis–Menten kinetics in the absence of 2-ketobutyrate ($K_m$ = 6 mM at pH 6.5–8.5; data not shown), even though two molecules of pyruvate participate in the reaction. Similar results have previously been reported for AHAS III, for both crude extracts and purified enzyme (De Felice et al., 1978; Barak et al., 1988). The same behavior has also been observed with AHAS I ($K_m$ = 1.5 mM) (Grimminger & Umbarger, 1979) and AHAS II ($K_m$ = 11 mM) (Schloss et al., 1985). As Schloss et al. (1985) have pointed out, such behavior implies that there is an effectively irreversible step between the addition of the first and second pyruvate to the enzyme.

*Substrate Competition.* In the presence of pyruvate and 2-ketobutyrate, the enzyme catalyzes the formation of both AL and AHB. Figure 1 shows a typical experiment carried out with AHAS III at a constant pyruvate concentration (40 mM) and varying concentrations of 2-ketobutyrate. The amount of AHB produced increased as the 2-ketobutyrate concentration increased, while the amount of AL produced decreased (Figure 1A). The *total* rate of product formation remained nearly constant within experimental error. The ratio of the two products remained in the same constant proportion to the substrate concentration, over a wide range of keto-

Table I: Attempts To Detect the Formation of EHOP from Two Molecules of 2-Ketobutyrate

| enzyme | [pyruvate] (mM) | [2KB] (mM) | products detected[a] (nmol/reaction) AL | AHB | EHOP |
|---|---|---|---|---|---|
| AHAS III | 20 | 0.2 | 1130 | 602 | nd |
| | 0 | 0.2 | nd | nd | nd |
| | 0 | 5 | nd | nd | nd |
| AHAS I | 20 | 5 | 1070 | 670 | nd |
| | 0 | 5 | nd | nd | 60 |

[a] Reactions were carried out in the standard reaction mixture at pH 7.6 and 37 °C in a total volume of 10 mL for 20 min. The quenched reaction mixtures were analyzed by the GLC method. nd indicates that no peak could be detected above background noise.

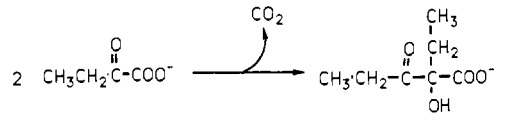
Table II: Definition of the Parameters Used in the Fit of Data to Eq 8–11[a]

$$V_0 = k_{cat}[E][Pyr]/([Pyr] + a)$$
$$V_1 = k_{cat}R[E][Pyr]/(b[Pyr] + c)$$
$$K = ([Pyr]^2 + a[Pyr])/(b[Pyr] + c)$$
$$K_{is} = d/(b[Pyr] + c)$$
$$V_m = k_{cat}[E]$$
$$V_2 = k_{cat}[E]R[2KB]$$
$$F = a + b[2KB]$$
$$G = c[2KB] + d[2KB]^2$$

[a] The constants $k_{cat}$, $R$, $a$, $b$, $c$ and $d$ are those in eq 5 and 6. $V_0$, $V_1$, $K$, and $K_{is}$ should be constant at given enzyme and pyruvate concentrations (eq 8 and 9). $V_2$, $F$, and $G$ should be constant at given enzyme and 2-ketobutyrate concentrations (eq 10 and 11), while $V_m$ should be dependent only on the enzyme concentration.

butyrate levels (Figure 1B). In the experiment shown here this proportion was 41 ± 3.

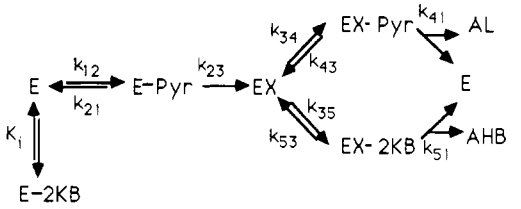
The synthesis of 2-ethyl-2-hydroxy-3-oxopentanoate (EHOP), the alternative product that might be formed by decarboxylation of 2-ketobutyrate and condensation of the resulting "active propionaldehyde" moiety with a second molecule of 2-ketopyruvate (eq 3), was not detected in these



(3)

experiments. Formation of EHOP by AHAS III could not be detected even in reactions carried out with 0.2 or 5 mM 2-ketobutyrate in the absence of any competing pyruvate (Table I). The sensitivity of the GLC method was such that we could have detected about 10 nmol of EHOP, which is less than 0.5% of the total product formation in the presence of both substrates in this experiment. We have assumed, therefore, that pathways involving 2-ketobutyrate as the first substrate can be ignored in the analysis of the kinetics of AHAS III. It is noteworthy, however, that we were able to detect the formation of small amounts of EHOP by AHAS I (Table I); in the presence of 5 mM 2-ketobutyrate alone, EHOP was formed by AHAS I at about 3% of the rate of total product formation in the presence of saturating pyruvate.

From the results presented above, we can postulate a kinetic mechanism for the enzyme (eq 4). In this mechanism, pyruvate and 2-ketobutyrate compete for the intermediate EX formed irreversibly from the first pyruvate. We have included in the mechanism a dead-end complex of the enzyme with 2-ketobutyrate, to explain the inhibition of AHB formation observed at very high 2-ketobutyrate concentrations without significant formation of EHOP (data not shown, see below).



(4)

The steady-state kinetic equations for formation of AL and AHB derived from this mechanism are given in eq 5 and 6, respectively (details are given in the supplementary material). These two equations predict that the ratio of the rates of AHB and AL formation will be given by eq 7. The factor $R$ therefore characterizes the specificity of the enzyme for AHB formation relative to AL formation and should be constant at all substrate concentrations.

$$V_{AL} = k_{cat}[E][Pyr]^2/([Pyr]^2 + a[Pyr] + b[Pyr][2KB] + c[2KB] + d[2KB]^2) \quad (5)$$

$$V_{AHB} = k_{cat}R[E][Pyr][2KB]/([Pyr]^2 + a[Pyr] + b[Pyr][2KB] + c[2KB] + d[2KB]^2) \quad (6)$$

$$V_{AHB}/V_{AL} = R[2KB]/[Pyr] \quad (7)$$

At a constant pyruvate concentration, eq 5 and 6 simplify to eq 8 and 9, in which the parameters $V_0$, $V_1$, $K$, and $K_{is}$ depend on the pyruvate concentration (as shown in Table II).

$$V_{AL} = V_0K/([2KB] + K + K_{is}[2KB]^2) \quad (8)$$

$$V_{AHB} = V_1[2KB]/([2KB] + K + K_{is}[2KB]^2) \quad (9)$$

The results of experiments carried out at different pyruvate concentrations, above and below $K_m$, all obey eq 8 and 9 (see, e.g., Figure 1). The parameters for the best fit to these equations are summarized in Table III. Several aspects of these parameters are significant: (a) $R$ is close to 40 in each experiment. (b) At a given pyruvate concentration, the rate of AL formation in the absence of 2-ketobutyrate, $V_0$, is nearly equal to the extrapolated rate of AHB formation at saturation

Table III: Summary of Steady-State Experiments with AHAS III, at Constant Pyruvate and Varying 2-Ketobutyrate Concentrations[a]

| [pyruvate] (mM) | parameters[b] $V_1/V_0$[c] | $K$ (μM) | $K_{is}$ (mM$^{-1}$) | $R$(fitted)[d] | $R$(average)[e] |
|---|---|---|---|---|---|
| 0.5 | 0.64 ± 0.06 | 9.5 ± 1.4 | 2.3 ± 0.8 | 34 ± 5 | 38 ± 17 |
| 1 | 1.11 ± 0.08 | 27 ± 3 | 0.09 ± 0.09 | 42 ± 6 | 39 ± 9 |
| 2 | 1.03 ± 0.07 | 58 ± 5 | 3.3 ± 0.6 | 35 ± 4 | 38 ± 3 |
| 5 | 0.77 ± 0.06 | 122 ± 16 | 0.11 ± 0.03 | 32 ± 5 | 31 ± 12 |
| 20 | 0.67 ± 0.04 | 297 ± 27 | 0.08 ± 0.02 | 45 ± 5 | 42 ± 4 |
| 40 | 0.92 ± 0.10 | 970 ± 170 | 0.009 ± 0.005 | 38 ± 8 | 41 ± 3 |

[a] Experiments were carried out at pH 7.6 and 37 °C. [b] The parameters are those for the best simultaneous fit of eq 8 and 9 to the rates of AL and AHB formation, respectively, as a function of [2KB], in a single experiment. An experiment at 40 mM pyruvate is shown in Figure 1. Uncertainties are standard errors of estimate of the fit. [c] The fit of the data gives $V_1$ and $V_0$ separately. These are not directly comparable in experiments with different enzyme concentrations, and only their ratio is relevant here. [d] $R$(fitted) is derived from $R = V_1[Pyr]/V_0K$ (see Table II), with appropriate propagation of standard errors of estimate. [e] $R$(average) is the average for $R = V_{AHB}[Pyr]/V_{AL}[2KB]$ calculated for each reaction mixture. Values of $R$ become highly biased by systematic errors of measurement when $V_{AHB}$ or $V_{AL}$ is very low, and in some cases such values have been eliminated.

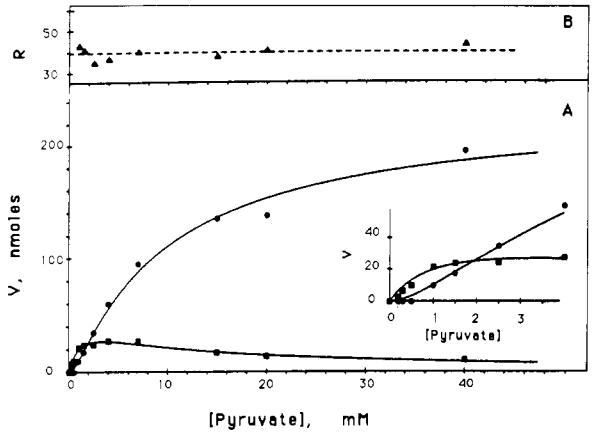



FIGURE 2: Dependence of AL (●) and AHB (■) formation by purified AHAS III on the pyruvate concentration, in the presence of 0.05 mM 2-ketobutyrate. (A) Reactions were carried out as for Figure 1, except that 10-mL reaction mixtures were used. (Inset) Data at low [pyruvate] on an expanded scale. The lines are the best simultaneous fit of the data to eq 10 and 11. (B) Calculated $R$ values (▲).

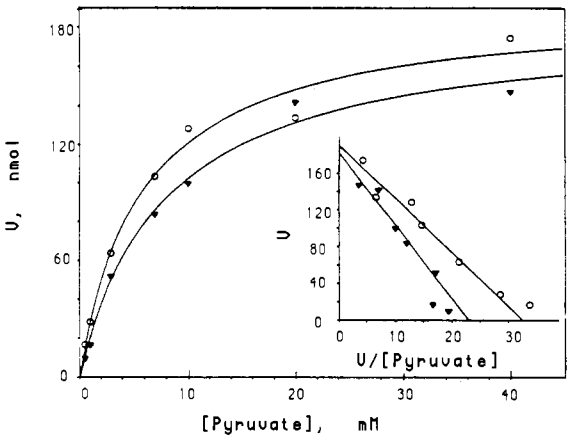



FIGURE 3: Dependence of the total rate of product formation (AL + AHB) by purified AHAS III on [pyruvate], in the presence (○) and absence (▼) of 0.1 mM 2-ketobutyrate. The lines are the best fit to the Michaelis–Menten equation. (Inset) Eadie–Hofstee plots for the same data.

with 2-ketobutyrate, $V_1$ (i.e., $V_1/V_0$ is close to unity). (c) $K$ increases with the pyruvate concentration as expected from Table II. (d) $K_{is}$, the parameter reflecting inhibition by 2-ketobutyrate at very high concentrations (eq 8 and 9), is poorly determined and not very significant in these experiments.

At a constant 2-ketobutyrate concentration, eq 5 and 6 simplify to eq 10 and 11, in which the parameters $V_2$, $F$, and $G$ depend on the 2-ketobutyrate concentration and $V_M$ does not (see Table II). Figure 2 shows the results of an experiment

$$V_{AL} = V_M[Pyr]^2/([Pyr]^2 + F[Pyr] + G) \quad (10)$$

$$V_{AHB} = V_2[Pyr]/([Pyr]^2 + F[Pyr] + G) \quad (11)$$

carried out with varying pyruvate concentrations in the presence of 0.05 mM 2-ketobutyrate. The data were fit very well by eq 10 and 11. It can be seen that the rate of AL formation showed sigmoidal dependence on pyruvate in the presence of 2-ketobutyrate (note inset to Figure 2A), as expected from eq 10. $R$ was constant (40 ± 3) in this experiment over a wide range of pyruvate concentrations (Figure 2B) and similar to the value determined in other experiments with AHAS III (see Table III).

Figure 3 shows the total rate of formation of the two products, AL and AHB, in the presence of 0.1 mM 2-ketobutyrate, as a function of pyruvate concentration, and compares this with the rate of AL formation in the absence of 2-ketobutyrate. The reaction in the absence of 2-ketobutyrate obeyed Michaelis–Menten kinetics, as expected, with $K_m$ = 5.9 mM. It can be seen that the total rate in the presence of 0.1 mM 2-ketobutyrate could also be fit rather well to Michaelis–Menten kinetics, with $K_m$(app) = 8 ± 1 mM. The apparent $V_{max}$ values for both sets of data were almost the same, as expected from the fact that $V_M$ is independent of the 2-ketobutyrate concentration.

We examined the significance of product inhibition in the presence of 5 mM pyruvate and 0.5 mM 2-ketobutyrate. Racemic AL (up to 0.25 mM), $HCO_3^-$ (up to 0.1 mM), or both together had no detectable effect on AHB formation under these conditions (data not shown). The effect of these products on AL formation could not be accurately measured in these experiments because the amount of exogenous AL added was much greater than the amount of AL formed in the controls. Reversal of the reaction under similar conditions was examined by incubation of the enzyme with 0.3 mM AL and 0.1–0.5 mM 2-ketobutyrate. AHB formation under these

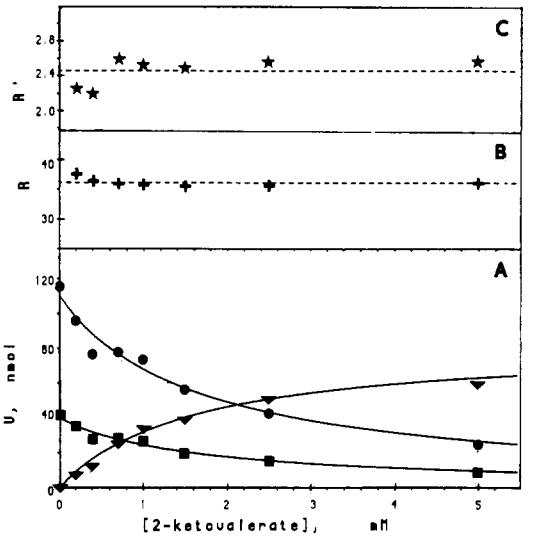



FIGURE 4: Dependence of product formation by purified AHAS III on [2-ketovalerate], in the presence of 5 mM pyruvate and 0.05 mM 2-ketobutyrate. Reactions were carried out as in Figure 2. (A) Rates of formation of AL (●), AHB (■), and AHV (▼). (B) Calculated $R$ values for each reaction mixture (+). (C) Calculated values of $R' = (V_{AHV}/V_{AL})([Pyr]/[2KV])$ (★).

conditions was less than the limits of detection [<3 nmol (mg enzyme)$^{-1}$ min$^{-1}$]. Product inhibition or reversal of the reaction can thus be ignored at reasonable extents of substrate conversion.

*Substrate Analogues.* Since we assume that pyruvate and 2-ketobutyrate compete for the enzyme intermediate at the same site, it was of interest to examine the possible reaction of the intermediate with other 2-keto acids. The next higher homologue of 2-ketobutyrate, 2-ketovalerate, reacted with pyruvate to yield a product, presumably 2-aceto-2-hydroxyvalerate (AHV), that was detected as 2,3-hexanedione in the GLC method we used. The specificity constant $R'$ for AHV formation relative to AL formation (eq 12) was about 2.5 in

$$R' = V_{AHV}[Pyr]/V_{AL}[2KV] \quad (12)$$

this experiment (data not shown). Figure 4, which shows an experiment with varying 2-ketovalerate concentrations and constant pyruvate (5 mM) and 2-ketobutyrate (0.05 mM) concentrations, demonstrates that 2-ketovalerate competes with the two other substrates for a single intermediate. The production of AL and AHB decreased in exactly the same

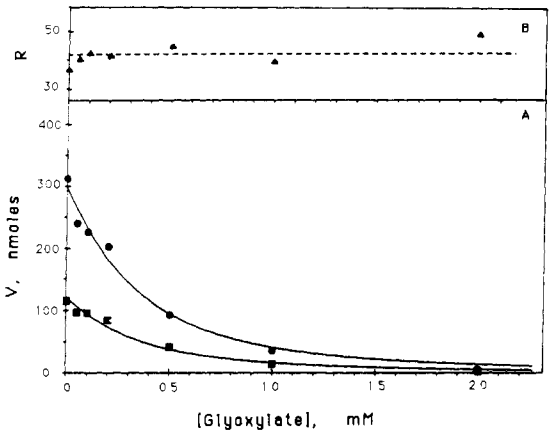



FIGURE 5: Inhibition of AHAS III by glyoxylate. The reaction was carried out as in Figure 2, in the presence of 5 mM pyruvate, 0.05 mM 2-ketobutyrate, and varying concentrations of glyoxylate as indicated. (A) Rates of formation of AL (●) and AHB (■). The lines are the best fits to $V = V_0K_i/(K_i + I + I^2/K_{i2})$. (B) Calculated values of $R$ for each reaction mixture (▲).

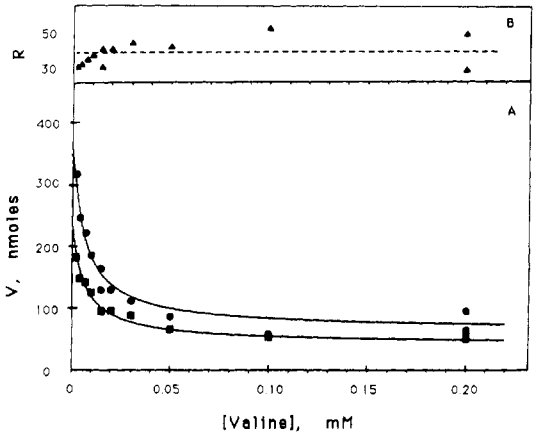



FIGURE 6: Influence of valine on the activity of AHAS III. The reaction was studied by the GLC method in the presence of 4 mM pyruvate and 0.07 mM 2-ketobutyrate, in the standard reaction mixture at pH 7.6. (A) Rates of formation of AL (●) and AHB (■). (B) Calculated values of $R$ for each reaction mixture (▲).

manner, and the calculated $R$ and $R'$ each remained essentially constant (36.1 ± 0.6 and 2.26 ± 0.15, respectively) over a wide range of 2-ketovalerate concentrations.

The branched-chain keto acid 2-oxo-3-methylbutyrate ("ketoisovalerate", which is the immediate precursor of valine) competes very poorly, if at all, with the normal substrates of the enzyme and is not an effective inhibitor (data not shown). On the basis of the concentrations of substrate used and the accuracy of the method, its apparent affinity for the intermediate EX must be at least 10-fold lower than that of pyruvate.

The lower homologue of pyruvate and 2-ketobutyrate, glyoxylate, is also an inhibitor of AL and AHB formation by AHAS III (Figure 5). It is possible that a product of glyoxylate condensation with one of the substrates was formed in this experiment. However, the GLC method we used was not expected to detect such a product, since the result of its oxidative decarboxylation, a 2-ketoaldehyde, would have been further oxidized to the original keto acid under the conditions of the analysis (e.g., pyruvate + glyoxylate → 2-hydroxy-3-ketobutyrate → pyruvaldehyde → pyruvate). The rates of formation of both AL and AHB were inhibited to the same extent, so that the specificity of the enzyme remained constant (Figure 5B). Since glyoxylate is a lower homologue of pyruvate, it is possible that it competes with pyruvate at the first site, as well as with the substrates for the intermediate at the second site. The data of Figure 5, as well as that of experiments with pyruvate alone as substrate (data not shown), were best fit by the assumption that glyoxylate competes at *both* stages of the reaction, although more detailed experiments are required to prove this assumption. At one of these stages, the enzyme has higher apparent affinity for glyoxylate than for pyruvate, since 50% inhibition was obtained at glyoxylate concentrations 10-fold lower than the pyruvate concentration.

*Effect of Mediators of AHAS Activity.* Valine, isoleucine, and leucine (in decreasing order of effectiveness) are inhibitors of AHAS III, with valine playing a major role in physiological control of the activity (Umbarger, 1978). Figure 6 shows the effect of valine on the rates of production of AL and AHB when both pyruvate and 2-ketobutyrate were present. The two reactions were similarly inhibited, with $R$ remaining constant, as previously shown (Barak et al., 1987). It is of interest that the inhibition by valine was not complete and reached saturation at about 70% under these conditions (4 mM pyruvate and 0.07 mM 2-ketobutyrate). The residual activity at saturation with the inhibitor, or the valine concentration required for a given fractional inhibition, increased with increasing pyruvate concentration, but did not seem to be affected by 2-ketobutyrate (data not shown). Similar behavior was observed with the other branched-chain amino acids (Barak et al., 1987). Details of the effects of feedback inhibitors on AHAS isozymes will be presented elsewhere.

Table IV: Substrate Specificity of AHAS III at Various pHs

| pH | buffer | $R^a$ |
|---|---|---|
| 7.60 | 0.1 M potassium phosphate | 40 ± 4 |
| 7.15 | 0.1 M Mops | 46 ± 3 |
| 7.50 | 0.1 M Tricine hydrochloride | 43 ± 3 |
| 8.45 | 0.1 M Tricine hydrochloride | 37 ± 4 |

[a] $R$ was determined in experiments at 5 mM pyruvate with 0.02–0.4 mM 2-ketobutyrate, in the appropriate buffer.

Table V: Effect of DMSO on Activity and Specificity of AHAS III

| vol % DMSO | relative activity[a] | $R^b$ |
|---|---|---|
| 0 | 100 | 40 ± 7 |
| 20 | 130 | 36 ± 3 |
| 30 | 133 | 25 ± 4 |
| 40 | 44 | 22 ± 1 |

[a] Activity was measured by the GLC method in 0.1 M Tricine buffer at pH 7.60 ± 0.05 (measured in the final reaction mixture), in the presence of 20 mM pyruvate alone. [b] $R$ is the average $R$ calculated at three to five different 2-ketobutyrate concentrations from 0.03 to 1 mM.

*Influence of Other Factors.* The pH dependence of the activity of purified AHAS III shows a maximum at pH 8.3–9.0 (De Felice et al., 1978). The type of buffer also influences the activity of AHAS III; at pH 7.5, for instance, the relative activities of the enzyme in phosphate, Tricine, Hepes, Mops, and Bis-tris propane buffers are 1.0, 0.8, 0.7, 0.6, and 0.3, respectively. Table IV shows that the specificity of the enzyme is apparently affected rather little by the pH [see also Barak et al. (1987)] or the buffer species. The $K_m$ for pyruvate is also independent of pH (data not shown).

In addition, the influence of other factors that can affect the tertiary structure of proteins and protein–ligand binding forces was studied. Table V shows that DMSO did have some influence on the specificity of AHAS III, although the effect was not large. The enzyme was shown to be stable over at least 15 min in relatively high DMSO concentrations. The specificity of AHAS III was also affected by temperature. To avoid ambiguities that might be caused by the effect of tem-

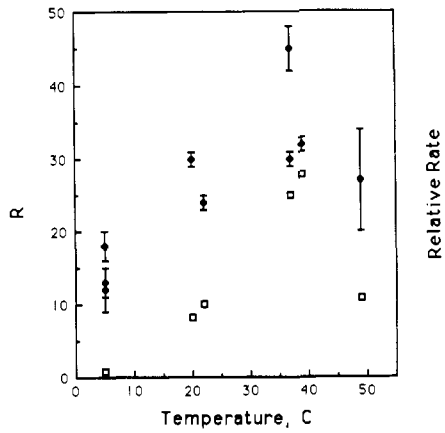



FIGURE 7: Influence of temperature on activity (sum of the rates of product formation, □) and specificity (◆) of AHAS III. The reaction was studied by the GLC method under the standard conditions, except that the buffer was 0.1 M Tricine, pH 8.4. The reaction mixtures contained 25 mM pyruvate and 0.1–0.8 mM 2-ketobutyrate. Reactions were carried out for 10 min at 37–50 °C. At temperatures of 22 °C and below, reactions were carried out for 20 min with increasing enzyme concentrations.

perature on ionization constants, we examined the reactions at the pH optimum, pH 8.4, in Tricine buffer. There was clearly a significant effect of temperature on $R$ (Figure 7), and $R$ decreased with decreasing temperature. On the other hand, the specificity of AHAS II was unaffected by temperature: $R = 70 \pm 6$ in the range from 5 to 37 °C (data not shown).

*Other Isozymes.* We have also examined in detail the steady-state kinetics of the two other isozymes of AHAS from the enterobacteria, AHAS I and II, which are now available in purified form. The experiments we carried out included determination of the dependence of the rates of product formation on the concentrations of the substrates and the effects of substrate homologues, branched-chain amino acids, and pH on the activity and specificity. Some of these experiments have been reported in preliminary and previous publications (Gollop et al., 1986, 1987, 1988; Barak et al., 1987). Both of these enzymes follow the mechanism demonstrated here for AHAS III (eq 4), and each shows a characteristic specificity that is unaffected by modulators or pH. The isozymes differ from one another in their steady-state kinetic parameters $K_m$, $R$, and $R'$ (Table VI) as well as pH optimum and sensitivity to valine inhibition (De Felice et al., 1978; Gollop et al., 1983).

## Discussion

*Implications of the Kinetics.* The steady-state kinetic experiments with AHAS isozymes from the enterobacteria described above provide strong support for the kinetic mechanism proposed in eq 4. Several alternative mechanisms can be ruled out, since they would not be expected to lead to a ratio of rates of product formation that is in a constant proportion to the ratio of substrate concentrations (i.e., obey eq 7). A mechanism in which the reactions leading to AL and AHB take place at separate sites would hardly be likely to yield the two products with the same specificity ratio $R$ under widely varying conditions that lead to large changes in the total activity: pyruvate concentrations above and far below $K_m$, a range of pHs, the presence and absence of inhibiting concentrations of valine, and the presence of additional competing substrate homologues. An alternative mechanism, in which the two substrate molecules bind to the enzyme in random order and no irreversible step occurs before both reacting molecules are bound, would also not obey eq 7. In addition, a mechanism

Table VI: Kinetic Parameters for Three AHAS Isozymes from Enterobacteria

| parameter[a] | AHAS I[b] | AHAS II[c] | AHAS III[d] |
|---|---|---|---|
| $K_m$ (pyruvate) (mM) | 1.5 | 10.5 | 6 |
| $R$[e] | 2 | 65 | 40 |
| $R'$[f] | <0.1 | 2.4 | 2.3 |

[a] Summary of the best values of the parameters, determined in multiple experiments of the kinds reported explicitly here for AHAS III. [b] AHAS I purified to >90% homogeneity from MF2361 (Newman et al., 1982) by Schloss and Aulabaugh (unpublished results). [c] Purified *S. typhimurium* AHAS II (Schloss et al., 1985). [d] Purified *E. coli* AHAS III (Barak et al., 1988). [e] Relative specificity constant for 2-ketobutyrate relative to pyruvate (eq 7). Uncertainty, ±10%. [f] Relative specificity constant for 2-ketovalerate relative to pyruvate (eq 12). Uncertainty, ±10%.

involving reversible binding of both reacting molecules, whether ordered or random, would not lead to Michaelis–Menten kinetics for the reaction of pyruvate alone. To fit the data, the mechanism must include an irreversible step between the binding of the first pyruvate and the binding of the second keto acid to the enzyme. However, the irreversible step need not necessarily be decarboxylation (see below).

The steady-state experiments also provide further information about the proposed kinetic mechanism. The sum of the rates of formation of the two alternative products of AHAS remains nearly constant at a given pyruvate concentration, over a range of 2-ketobutyrate concentrations for which the product distribution changes from AL alone to AHB almost exclusively (see Figure 1 and the calculated values of $V_1/V_0$ in Table III). This requires that the rate-determining and product-determining steps in the mechanism be different. This requirement would be met in the proposed mechanism (eq 4) if the first steps, leading to the formation of the intermediate EX, were rate determining, and the subsequent steps, including binding and reaction of one of the competing second substrates, were all faster. An alternative but less likely explanation is that the rate of the last step in the mechanism, formation and release of an acetohydroxy acid, is rate determining and *independent of which product* is formed, so that the specificity of the enzyme is expressed solely in its equilibrium affinity for the second substrate. (These two possibilities are analyzed mathematically in the supplementary material).

The fact that inhibitors such as valine, or changes in pH, do not influence the specificity of the enzyme lends additional support to the idea that the first steps in the mechanism, preceding the binding of the second substrate, are rate determining. Modulation of these first steps would be expected to affect the turnover rate of the enzyme without affecting the choice of products.

*Catalytic Mechanism.* A detailed catalytic mechanism (Figure 8), which is in accord with the kinetic mechanism for AHAS demonstrated above, can be proposed on the basis of the known chemistry of other thiamin pyrophosphate dependent enzymes (Holzer & Kohlhaw, 1961). The intermediate EX can be assumed to be the complex of the enzyme with (hydroxyethyl)-TPP (HETPP), formed after steps 1 and 2 of Figure 8. Ciskanik and Schloss (1985) isolated HETPP from the reaction of AHAS II with [3-$^{14}$C]pyruvate and found a stoichiometry of 0.01 mol of HETPP/mol of catalytic protomer at saturating concentrations of pyruvate. The low stoichiometry supports the conclusion that formation of the enzyme–HETPP complex is rate determining. On the basis of the small carbon isotope effect observed in the release of $CO_2$ from [1-$^{13}$C]pyruvate by AHAS II, Abell et al. (1985) suggested that reaction 1 in the mechanism of Figure 8 is a committed step in the formation of HETPP. If this step is

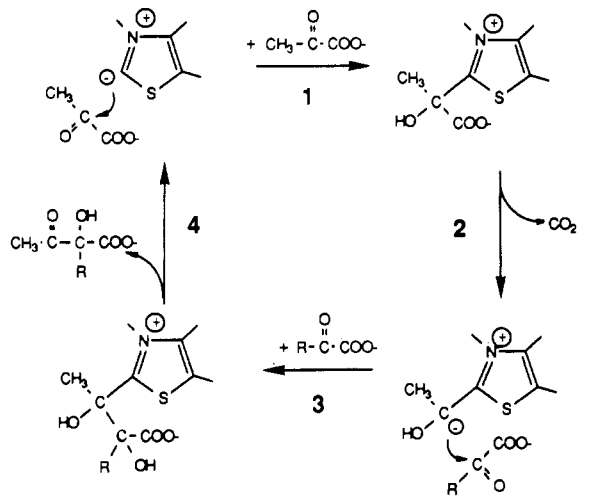


FIGURE 8: Catalytic mechanism for acetohydroxy acid synthase.

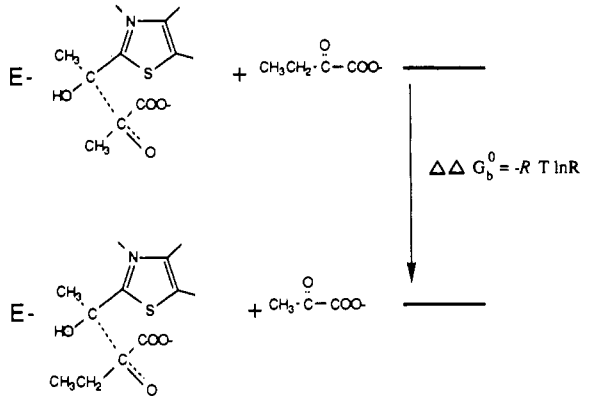


FIGURE 9: Relation between the observed specificity of an acetohydroxy acid synthase and the interactions of the enzyme with alternative substrates.

in fact effectively irreversible, it could be the rate-determining step, and decarboxylation (step 2) might conceivably occur *after* the binding of the second keto acid substrate.

*Enzyme–Substrate Interactions.* The specificity of AHAS isozymes II and III is unusually large. The specificity ratio ($R$) measured by competition between two substrates is a direct measure of the difference between the interaction energy of the enzyme with the transition state for the formation of one alternative product and that for its interaction with the alternative transition state (Fersht, 1984) (Figure 9). The observed specificity of AHAS III ($R$ = 40) implies that the interaction of the additional methylene group of 2-ketobutyrate with the enzyme contributes about −2.2 kcal/mol toward the stabilization of the transition state for formation of AHB relative to that for formation of AL ($\Delta\Delta G_b$ = −2.2 kcal/mol; see Figure 9). For AHAS II ($R$ = 65), $\Delta\Delta G_b$ is −2.5 kcal/mol. These interaction energies are too large to be due to "hydrophobic binding" alone. In fact, they are not very far from the interaction energy of about −3.4 kcal/mol (leading to a specificity ratio of about 250 in favor of the larger substrate) that has been suggested is the maximum possible for the interaction of a methylene group with a "precisely tailored active site" (Tsui & Fersht, 1981).

It is not possible to determine, of course, whether the interactions between the enzyme and the additional methylene group are fully expressed before the transition state. The specificity of the enzyme may be due to the noncovalent affinities of the second substrates [$(k_{43} + k_{41})k_{35}/k_{34}(k_{53} + k_{51})$; see eq 4] or to differences in the rates of the alternative bond-forming steps ($k_{51}/k_{41}$) or to both.

The specificity ratio for formation of AHV relative to AL, $R'$, is about 2.4 for both AHAS III and II. This means that the addition of one more methylene group to 2-ketobutyrate produces a substrate which is poorer by a factor of 15 or 25, respectively; $\Delta\Delta G_b$ for the additional group is +1.7–2 kcal/mol. Note that 2-ketovalerate is still a better substrate than pyruvate for these enzymes. On the other hand, addition of a methyl group at C-3 of 2-ketobutyrate (to yield the branched keto acid 2-oxo-3-methylbutyrate) leads to a $\Delta\Delta G_b$ of at least +3 kcal/mol.

The model for the site for the competing second substrates that emerges from this data for AHAS III (and II) is one with a rather rigid nonpolar cavity of the appropriate size to bind the $CH_3CH_2$ group of 2-ketobutyrate, so that many stabilizing van der Waals interactions are sacrificed when pyruvate is bound in place of 2-ketobutyrate. The site can be opened to accommodate 2-ketovalerate, but this significantly raises the energy of the transition state.

The substrate site for the first keto acid molecule, which is attacked by TPP and undergoes decarboxylation, is quite specific for pyruvate. On the basis of data presented here and elsewhere (Barak et al., 1988) AHAS III reacts with 2-ketobutyrate in the absence of pyruvate at least 100 times more slowly than with pyruvate. For AHAS II, pyruvate is favored over 2-ketobutyrate in the "first" site by about 20-fold (Abell et al., 1985), but it has also been reported (Schloss & Van Dyk, 1988) that in the absence of pyruvate the reaction of 2-ketobutyrate is only 5 times slower than that of pyruvate. It is likely that at least part of the specificity of the first site is due to simple steric exclusion, since glyoxylate appears to compete effectively with pyruvate in both stages of the reaction of AHAS III.

In AHAS I, the site for the binding of the second keto acid substrate must be somewhat different from that of AHAS II or III (Table VI). The additional methyl group of 2-ketobutyrate contributes only about −0.4 kcal/mol to enzyme–transition-state binding forces in this case. However, this methyl group of 2-ketobutyrate does not simply hang loosely out of the site, since the addition of one more carbon to the chain is at least as unfavorable in the case of AHAS I as it is for AHAS II or III; 2-ketovalerate is a far poorer substrate than pyruvate for this enzyme.

All of the "biosynthetic" isozymes from enterobacteria, including AHAS I, appear to have active sites optimized for the synthesis of acetohydroxybutyrate in preference to other products. Biosynthetic enzymes from other bacteria (Eggeling et al., 1987; Szentirmai et al., 1970; unpublished data), as well as from yeast and algae (unpublished data), have similar properties. On the other hand, enzymes of the class known as "pH 6" or "degradative" acetolactate synthases (Halpern & Umbarger, 1959; Holtzclaw & Chapman, 1975), which apparently have rather different physiological roles and molecular properties, have a strong preference for the synthesis of acetolactate ($R$ < 0.1; Huseby & Stormer, 1971; unpublished data). It might be biochemically appropriate to distinguish between these two classes of enzymes on the basis of their specificities and to refer to the former class as acetohydroxy acid synthases and to the latter as acetolactate synthases.

## Supplementary Material Available

Explicit solution of the rate equation in terms of constants of eq 4, analysis of the implications of the observation that $V_1/V_0$ = 1, and estimation of the values of the parameters of eq 5 and 6 from the data (4 pages). Ordering information is

given on any current masthead page.

**Registry No.** AHAS, 9027-45-6; DMSO, 67-68-5; 2KB, 600-18-0; pyruvic acid, 127-17-3; glyoxylate, 298-12-4; L-valine, 72-18-4.

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