Steady-State Kinetics of the Recombinant Acetohydroxy Acid Synthase from Tobacco

Bong-Whan Lee, Jung-Do Choi,[†] and Moon-Young Yoon[*]

Department of Chemistry, Hanyang University, Seoul 133-791, Korea
[†]School of Life Sciences, Chungbuk National University, Cheongju 361-763, Korea
Received January 22, 2002

Introduction

Acetohydroxyacid synthase (AHAS; EC 4. 1.3.18, also referred to as acetolactate synthase) catalyzes the first reaction in the metabolic pathway leading to the biosynthesis of the branched-chain amino acids.[1,2] AHAS catalyzes the condensation of two molecules of pyruvate to form aceto-lactate 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).

Considerable interest in this enzyme has resulted from the discovery that it is the site of action by several different commercial herbicides.[3] A common pathway in micro-organisms and plants synthesize valine, leucine and iso-leucine. The common precursor for these amino acids is the central metabolite pyruvate, hence these form a subset of the pyruvate-derived amino acids. In addition, isoleucine also requires a second precursor, 2-ketobutyrate. AHAS cata-lyzes the first of the parallel steps and is at a critical branch point in the pathway because its reaction will determine the extent of carbon flow through to the branched-chain amino acids. The reaction involves the irreversible decarboxylation of pyruvate and the condensation of the acetaldehyde moiety with a second molecule of pyruvate to give 2-acetolactate, or with a molecule of 2-ketobutyrate to yield 2-aceto-2-hydroxybutyrate.

AHAS has been studied in steady state kinetic experiments in which the rates of acetolactate (AL) and acetohydroxy-butyrate (AHB) have been determined simutaneously.[2,4] The ratio between the rates of production of the two alternative products and the concentrations of the substrates pyruvate and 2-ketobutyrate leads to them, R, $V_{AHB}/V_{AL}=R$ ([2-keto-butyrate]/[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 mean that they have the presence of at least one AHAS activity with high specificity for aceto-hydroxybutyrate 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. In spite of the exten-sive studies done with AHAS, little kinetic investigation has been reported to date.[2,5,6] In this paper, we describe the steady-state kinetic studies of tobacco AHAS. The kinetic parameters for catalysis were compared with other source AHAS.

Materials and Methods

Chemicals. Pyruvic acid sodium salt, tris(hydroxymethyl)-aminomethane, flavin adenine dinucleotide (FAD), thiamin pyrophosphate (TPP), α-naphtol, creatine, glutathione, iso-propy-β-D-thiogalactoside (IPTG), NaCl, TritonX-100, and $MgCl_2$ were all purchased from Sigma Chemical Co. (St. Louis, USA). 2-Ketobutyric acid sodium salts and 3-bromo-pyruvic acid were purchased from TCI Co. (Tokyo, Japan). Bacto-tryptone, yeast extract, and bacto-agar were obtained from Difco Laboratories (Detroit, MI). Epoxy-activated sepharose 6B was purchased from Pharmacia Biotech. (Uppsala, Sweden). All other chemicals were of pure or extra pure grade and commercially available.

Expression and purification of the recombinant tobacco AHAS. Expression and purification of GST-AHAS were performed as described by Chang *et al.*[7] Cells, *E. coli* DH5α, were incubated at 37 $^{\circ}$C, 150 rpm until OD_{600} = 0.8-0.9 and induced with 0.5 mM IPTG for 5 hours and

pyruvate   +   2-ketobutyrate

2-aceto-2-hydroxybutyrate

Scheme 1

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

harvested by centrifugation at 4000 rpm. The cell pellets were resuspended with PBST (150 mM NaCl, 20 mM Sodium phosphate buffer (pH 7.4), 1% Triton X-100, 2 mM EDTA, 0.1% β-Mercaptoethanol) buffer and lysis by sonication at 4 °C. The homogeneity was centrifuged at 14,000 rpm for 20 min. The supernatant applied on a GSH-coupled Sepharose 6B resin and washed unbounded proteins by a sufficient volume of the PBST buffer. The GST-AHAS fusion protein was then eluted by elution buffer (20 mM GSH, 50 mM Tris-HCl (pH 8.0). The isolated proteins were identified by SDS-PAGE analysis and the protein concentration was determined by the Bradford method.

Enzyme assay. The enzyme activities of the purified AHAS were measured according to the method of Wester-ford.[8] The reaction mixture (200 μL) contained 50 mM potassium phosphate buffer (pH 7.5), 1 mM TPP, 20 μM FAD, 10 mM MgCl$_2$ and various concentrations of pyruvate and 2-ketobutyrate in either the presence or absence of 2-ketobutyrate. Assays were initiated by adding the AHAS (0.15 μM) and terminated by adding 20 μL of 6 N H$_2$SO$_4$ after incubating at 37 °C for 1 hr. The acetolatate was allowed to decarboxylate at 67 °C for 15 min. The converted acetoin was incubated with 200 μL of 0.5% creatine at 67 °C for 15 min and colorized with 200 μL of 5% α-naphtol at 67 °C for 15 min. The absorbance of the reaction mixture was determined at 525 nm (ε = 6,500 M^{-1}cm^{-1}). The activity was calculated by the different absorbance of the reaction mixture between 2-ketobutyrate absence (OD$_i$) and presence (OD$_f$). One unit (U) of activity was defined as the amount required to form 1 μmol of acetohydroxybutyrate per minute under the assay conditions described above.

Initial velocity study. The conditions of initial velocity pattern in the reaction at pH 7.5 were the same as in the standard enzyme assay conditions, and the initial velocity pattern was obtained by varying the concentrations of 2-ketobutyrate at different fixed levels of pyruvate. The various concentrations of substrates around K_m were corrected for the complexation with divalent metal. All the chemicals were corrected for the metal-chelating effect.[9]

Dead-end inhibition study. Dead-end inhibition patterns were obtained by varying the concentrations of substrate with the second fixed at K_m and at three different levels of concentrations of the inhibitor (0, 1K_i, and 2K_i). The dead-end inhibitor was 3-bromopyruvate as a substrate analog of pyruvate. Velocity was measured as a function of enzyme concentration around the K_m value of reactants and around the K_i value of inhibitor. In all cases, the plots were linear.

Data processing. Reciprocal initial velocities were plotted as a function of reciprocal substrate concentrations. Data were fitted using the appropriate rate equations and Fortran programs of Cleland.[17] Data conforming to a ping-pong mechanism were fitted using equation (1). Data for competitive, uncompetitive, and noncompetitive inhibition were fitted using equations (2)-(4). In equations (1)-(4), v and V represent initial and maximum velocities; K_a and K_b are K_m values of substrate A and B, respectively.

$$\nu = \frac{VAB}{K_a B + K_b A + AB} \tag{1}$$

$$\nu = \frac{VA}{K_a(1 + I/K_{is}) + A} \tag{2}$$

$$\nu = \frac{VA}{K_a + A(1 + I/K_{ii})} \tag{3}$$

$$\nu = \frac{VA}{K_a(1 + I/K_{is}) + A(1 + I/K_{ii})} \tag{4}$$

K_{is} and K_{ii} are slope and intercept inhibition constants, while A, B, and I represent reactants and inhibitor concentrations, respectively.

Results and Discussion

The kinetic studies of most authors have confined themselves to study the effect of pyruvate alone.[2] The most detailed kinetics study is that described by Gollop *et al.* (1989) who examined the effect of simultaneous variation of the concentration of pyruvate and 2-ketobutyrate using *E. coli* AHAS III.[4] In the absence of 2-ketobutyrate, it might be expected that the substrate saturation curve would be sigmoidal due to CO$_2$ release intervening between the binding of the first and second pyruvate. Likewise, most plant enzymes, recombinant tobacco and recombinant *A. thaliana* AHAS have confirmed that the enzyme does not follow Michaelis-Menten kinetics in the absence of 2-ketobutyrate.[10,11] The negative cooperativity for these plant enzymes was ascribed to interactions between the active sites of the dimer. In the presence of 2-ketobutyrate, the substrate saturation curve of the AHAS reaction was shown to be hyperbolic (data not shown). Such behavior implies that there is an effectively irreversible step between the addition of the first and second pyruvate to the enzyme.

Evaluation of the kinetic mechanism and the kinetic parameters of an enzyme-catalyzed reaction can often be best approached through initial velocity studies. Double reciprocal plots of the initial velocity data allow the differentiation of the kinetic mechanism; Ping pong mechanisms result in one or more parallel lines in double reciprocal plots, whereas sequential mechanisms exhibit non-parallel lines. The results of an experiment, in which the pyruvate concentration was varied at different fixed concentrations of 2-ketobutyrate, are presented in double reciprocal form in Figure 1. The lines are parallel. This behavior is characteristic of the Ping Pong mechanism in which one or two products are released before the introduction of a final substrate. When the initial velocity data of Figure 1. were plotted with 2-ketobutyrate as the variable substrate, the results again yielded families of parallel lines, suggesting that the pyruvate-2-ketobutyrate interaction in the AHAS catalyzed reaction might be a Ping Pong mechanism. The data fitted well to the initial velocity equation and the true values for the kinetic constants were obtained as followed: V$_{max}$, 0.0016 ± 0.0002 μmole/min; K$_{pyruvate}$, 3.66 ± 0.78 mM; K$_{2-ketobutyrate}$, 1.37 ± 0.29 mM; V/K$_{pyruvate}$, 4.37 × 10^{-4} min^{-1};

Notes

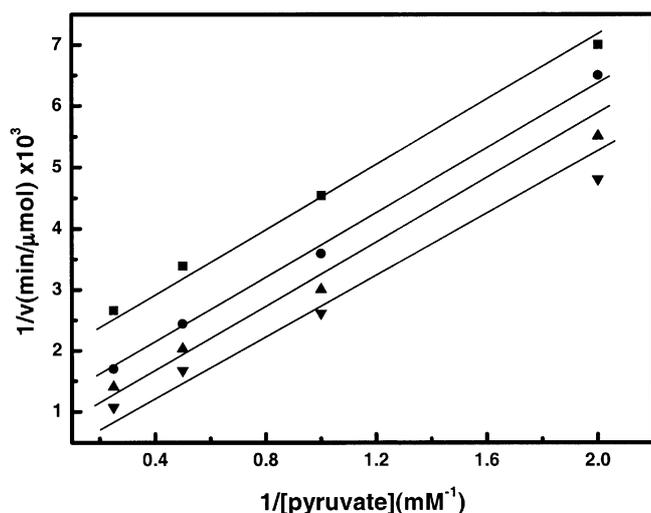*Bull. Korean Chem. Soc.* **2002**, Vol. 23, No. 5   767

Figure 1. Initial velocity pattern for the acetohydroxyacid synthase at pH 7.5. The assays were carried out under standard conditions. The results fitted plots for 0.5 mM (■), for 2 mM (●), for 7 mM (▲), for 40 mM (▼) of 2-ketobutyrate, respectively. Points are experimental and the solid lines are from a fit using equation (1).

$V/K_{2\text{-ketobutyrate}}$, 1.17×10^{-3} min^{-1}.

In addition to the initial velocity data, we examined dead-end inhibition studies in order to conform the most likely pathway of substrate addition to the enzyme. The most commonly used method involves holding one substrate at non-saturating concentration while varying the second substrate and inhibitor concentrations. The initial velocity data are then plotted in a double reciprocal form, and the inhibition patterns can be used to limit the possible kinetic pathways. 3-bromopyruvate is a dead-end analog of pyruvate. Through varying the concentration of pyruvate at several fixed concentration of 3-bromopyruvate, the dead-end inhibition patterns showed an intersection on the ordinate, indicating that 3-bromopyruvate is competitive *vs.* pyruvate (data not shown). The diagnosis of the Ping Pong mechanism by dead-end inhibition is that the analog of substrate A is uncompetitive *vs.* varied substrate B. Through varying the concentration of 2-ketobutyrate at several fixed concentrations of 3-bromopyruvate, the dead-end inhibition patterns showed a parallel lines in Figure 2, indicating that 3-bromopyruvate is uncompetitive *vs.* 2-ketobutyrate.

Taken along with the result from the initial velocity experiments, these inhibition patterns indicate that substrate A (the first substrate to bind) is pyruvate and substrate B is 2-ketobutyrate. Therefore, a Uni Uni Ping Pong mechanism is the most reasonable kinetic pathway taken by the acetohydroxy acid synthase reaction. The 2-bromopyruvate for the inhibition constants has a K_i value of 6.47 ± 1.1 mM.

The dependence of 2-acetolactate formation in the absence of 2-ketobutyrate for AHAS III from *E. coli* does not obeys Michaelis-Menten kinetics having the K_m of 6 mM.[4,12] The K_m of pyruvate for AHAS I and AHAS II was 1.5 mM and 11 mM, respectively.[13,14] The K_m of pyruvate for AHAS from yeast was in the range 8.2 to 18.1 mM.[2] The K_m of pyruvate for the plant was in the range 1 to 11 mM.[2] For
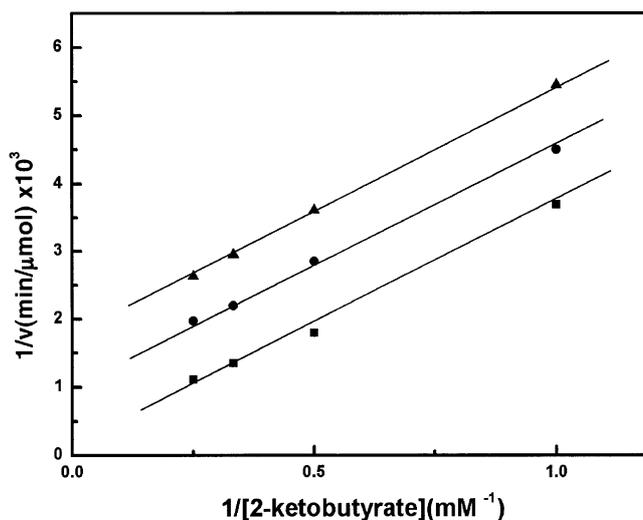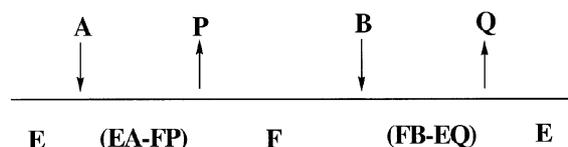

Figure 2. Dead-end inhibition pattern for the acetohydroxyacid synthase at pH 7.5. The assays were carried out under standard conditions. The 3-bromopyruvate concentrations were 0 mM (■), 2 mM (●) and 4 mM (▲) as a dead-end inhibitor of pyruvate. Points are experimental and the solid lines are from a fit using equation (3).

purified AHAS from tobacco, the K_m of pyruvate was 6.5 to 9.6 mM. However, for recombinant AHAS from tobacco, the K_m of pyruvate was 12.1 to 20.8 mM.[7,15,16] The K_m values of recombinant AHAS are about 2 fold higher than that of native. Initial velocity experiments to obtain the above mentioned values in which the concentration of pyruvate (one substrate reaction) was varied without any consideration of metal-substarte and metal-cofactor chelate (so-called, apparent K_m) was carried out. In this case, initial velocity patterns were obtained for the overall reaction in which the concentration of pyruvate was varied in the presence of different levels of 2-ketobutyrate (two substrates reaction) (so-called, true K_m). In this paper, the true K_m for pyruvate was obtained. The value of 3.66 mM and this value is 3 to 5 fold lower than other results (*vide ante*). Conclusively, this result may imply that there is a difference of the experiment condition and/or the reflection of recombinant one.

The results from initial velocity and dead end inhibition patterns described above support the Uni Uni Ping Pong Bi Bi system for the acetohydroxyacid synthase reaction. As a consequence, we propose that recombinant tobacco AHAS catalyzes the reaction in the manner of a Uni Uni Ping Pong Bi Bi mechanism as follows:

$$\begin{array}{cccccc}
& \text{A} & \text{P} & & \text{B} & \text{Q} \\
& \downarrow & \uparrow & & \downarrow & \uparrow \\
\hline
\text{E} & \text{(EA-FP)} & \text{F} & & \text{(FB-EQ)} & \text{E}
\end{array}$$

Where A. B, P, Q, E and F represent pyruvate, 2-ketobutyrate, CO_2, 2-aceto-2hydroxybutyrate, AHAS and central complex, respectively.

References

1. Umbarger, H. E. *Annu. Rev. Biochem.* **1978**, *47*, 533-606.
2. Duggleby, R. G.; Pang, S. S. *J. Biochem. Mol. Biol.* **2000**, *33*, 1-36.
3. Schloss, J. V.; Ciskanik, L. M.; VanDyk, D. E. *Nature* **1988**, *331*, 360-362.
4. Gollop, N.; Damri, B.; Barak, Z.; Chipman, D. M. *Biochemistry* **1989**, *28*, 6310-6317.
5. Joo, H. S.; Kim, S. S. *J. Biochem. Mol. Biol.* **2001**, *34*, 244-249.
6. Shin, Y. S.; Chong C. K.; Choi, J. D. *J. Biochem. Mol. Biol.* **1999**, *32*, 37-43.
7. Chang, S. I.; Kang, M. K.; Choi, J. D.; Namgoong, S. K. *Biochem. Biophys, Res. Commun.* **1997**, *234*, 549-553.
8. Oh, K. J.; Park, E. J.; Yoon, M. Y.; Han, T. R.; Choi, J. D. *Biochem. Biophys. Res. Commun.* **2001**, *282*, 1237-1243.
9. Park, S. H.; Kiick, D. M.; Harris, B. G.; Cook, P. F. *Biochemistry* **1984**, *23*, 5446-5453.
10. Kil, M. W.; Chang, S. I. *J. Biochem. Mol. Biol.* **1998**, *31*, 287-293.
11. Lee, Y. T.; Chang, A. K.; Duggleby, R. G. *FEBS Lett.* **1999**, *452*, 341-345.
12. Barak, Z.; Calvo, J. M.; Schloss, J. V. *Methods Enzymol.* **1988**, *166*, 455-458.
13. Grimminger, H.; Umbarger, H. E. *J. Bacteriol.* **1979**, *137*, 846-853.
14. Schloss, J. V.; Van Dyk, D. E.; Vasta, J. F.; Kutny, R. M. *Biochemistry* **1985**, *24*, 4952-4959.
15. Kim, H. J.; Chang, S. I. *J. Biochem. Mol. Biol.* **1995**, *28*, 265-270.
16. Chong, C. K.; Shin, H. J.; Chang, S. I.; Choi, J. D. *Biochem. Biophys. Res. Commun.* **1999**, *259*, 136-140.
17. Cleland, W. W. *Methods Enzymol.* **1979**, *63*, 103-143.