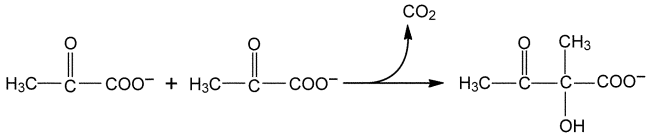
Lag Time of the Recombinant Acetohydroxy Acid Synthase from Tobacco

Jihyun Hwang, Jungmok Kim, Young-Tae Kim,† 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
‡Department of Microbiology, Bukyung National University, Busan 608-737, Korea
Received August 29, 2003

Key Words : Acetohydroxy acid synthase, Substrate activation, Lag time

The first enzymatic step common to the biosynthesis of branched-chain amino acids is catalyzed by acetohydroxy acid synthase (AHAS, EC 4.1.3.12; also known as acetolactate synthase). The enzyme catalyzes two parallel reactions, the condensation of two molecules of pyruvate to give rise to 2-acetolactate in the first step of the valine and leucine synthetic pathway (Scheme 1), and condensation of pyruvate and 2-ketobutyrate to yield 2-aceto-2-hydroxybutyrate in the second step of isoleucine biosynthesis.[1] AHAS requires three cofactors for its catalytic activity, thiamine diphosphate (ThDP), flavin adenine dinucleotide (FAD), and divalent metal ions, Mg^{2+} or Mn^{2+}. AHAS has attracted much interest since it was demonstrated to be the target of several classes of modern and potent herbicides, including the sulfonylureas,[2,3] the imidazolinones,[4] and the triazolopyrimidines.[5,6]



Scheme 1

AHAS activity is found in bacteria, yeast, and plants resulting from one or more isozymes. In bacteria, three AHAS isozymes have been studied extensively in term of their genetic regulation, kinetic properties, feedback regulation, and sensitivity to herbicidal inhibitors.[7-10] Each of the isozymes exists as a tetramer composed of two large catalytic subunits (59-60 kDa) and two small regulatory subunits (9-17 kDa).[7] In contrast to the bacterial enzyme, the structure and biochemical properties of AHAS from eukaryotes have not been well characterized since purification of eukaryotic AHAS is severely hampered by its extreme instability and very low abundance.[25,26] AHAS genes from *Arabidopsis thaliana*[11] and tobacco[12] have been functionally expressed in *E. coli*, and each of the enzymes has been purified. Various herbicide-resistant AHAS mutants from several plants have been obtained by spontaneous or induced mutation under field or laboratory conditions, and by site-directed mutagenesis (Summarized in Ref. 1).

The AHAS reaction in the absence of 2-ketobutyrate requires two moles of pyruvate and it might be expected that the substrate saturation curve would be sigmoidal. However, in the AHAS reaction, CO_2 release intervenes between the binding of the first and second pyruvate. Unless a very high CO_2 concentration is present, this step will be irreversible and the substrate saturation curve would be hyperbolic. This prediction agrees with the findings of the majority studies and K_m values in the range 1 to 20 mM are usually reported (summarized in Table 1 of Ref 1). There have been some reports of negative cooperativity in the pyruvate saturation curve. Phalip *et al.* (1995) reported a Hill coefficient of 0.84 for the catabolic AHAS from *Leuconostoc mesenteroides*.[14] AHAS from *Arabidopsis thaliana* has also been reported to display negatively cooperative kinetics (0.6 Hill coefficient) with respect to pyruvate concentration and there appeared to a short lag time of 1-2 min before full activity of the enzyme was attained.[15] AHAS activated with regulatory subunit displayed a hyperbolic substrate saturation curve[23] indicating that reconstitution with the regulatory subunit abolished the negative cooperativity. Pyruvate saturation curves from tobacco recombinant AHAS displayed Michaelis-Menten[24] and non-Michaelis-Menten kinetics.[17] However, the credibility of these results is lowered because the authors used the discontinuous assay methods.[24] In contrast, several papers have reported positive cooperativity kinetics for various forms of AHAS. These include the enzyme from barley,[18] *N. crassa*,[19] the bacterial anabolic AHAS from *L. lactis*,[20] *Serratia marcescens*,[21] and *M. aeolicu*..[22] Consequently, these differences of the kinetics might be due to different assay conditions and/or ignore the short lag time under higher substrate concentration. Since these earlier reports, there has been little additional work aimed at characterizing

Table 1. Summary of a lag time for cofactors[a]

Lag time (sec)		
ThDP	FAD	Mg
260 ± 40 (0.1 mM)	490 ± 50 (1 μM)	400 ± 40 (0.2 mM)
300 ± 60 (1 mM)	330 ± 55 (20 μM)	250 ± 30 (10 mM)

[a]The assays were conducted as described in Figure 1 with the reaction mixture containing 50 mM MOPS (pH 8.0), 20 μM FAD, 1 mM ThDP, 10 mM $MgCl_2$, 100 mM pyruvate. Concentrations of each cofactor were modified as indicated above.

*To whom correspondence should be addressed. Tel: +82-2-2290-0946, Fax: 182-2-2299-0762, e-mail: myyoon@hanyang.ac.kr

the non-classical kinetics exhibited by AHAS or at examining the metabolic role and regulatory behavior of this enzyme. The question remains as to whether non-Michealis-Menten kinetics might be a general feature of plant AHAS. We report here a lag time during the production of 2-acetolactate from pyruvate and cofactors that affects AHAS regulation.

A continuous assay based on the decrease in absorbance at 333 nm due to pyruvate (ε_{M} = 17.5 M^{-1} cm^{-1}) is approximately 1000-fold less sensitive than the discontinuous assay and is only suitable for purified enzymes that are available in large amounts. Nevertheless, it is the only assay that is reliable for studying the kinetics of herbicide inhibition and cofactor activation.[1] A lag in a reaction time course can be described[13] by the equation:

$$v_t = v_f + (v_i - v_f)e^{-kt}$$

where v_i and v_f are the initial and final velocities, respectively, v_t is the velocity at time t, and k is the rate constant for the transition. The lag times for each reaction are calculated by drawing tangents to the initial and final portions of the reaction time courses to determine v_i and v_f. The relaxation time for the transition from the initial to the final velocity is the point of intersection of these tangents. The reciprocal of this relaxation time is the apparent rate constant for the transition.

A time lag was observed during subsequent studies with the native enzyme (Figure 1). This time lag has been observed to be 400 seconds at 40 mM pyruvate concentration before a linear, steady state rate was attained. A lag time was not exhibited without prior incubation with cofactors. To test whether this lag time is eliminated or not, AHAS concentration was increased for 50-200 μg. As AHAS concentration was increased 50, 100 and 200 μg, the lag time was decreased 280, 180 and 130 seconds, respectively. The lag time decreasing was not a linear relation to AHAS concentration, indicating that pyruvate or cofactors affect the lag time. As mentioned the above, the results from the pyruvate saturation curve would be expected to be hyperbolic (*vide ante*). The sigmoidal shape of the pyruvate saturation curve will indicate that the catalytic activity of this enzyme is regulated by pyruvate. AHAS in our study showed a sigmoidal dependence of catalytic activity on pyruvate concentration at pH 8.5 (Figure 2). The K_m for pyruvate was 6.39 = 0.39 mM. When the data from the pyruvate saturation curve were fitted to a Hill plot, we observed a Hill coefficient of 1.58 = 0.14, indicating that pyruvate is converted to acetolactate cooperatively. These results suggest that slow binding of pyruvate or ThDP and/or conformational changes may possibly account for the transient kinetics. We are presently pursuing these possibilities.

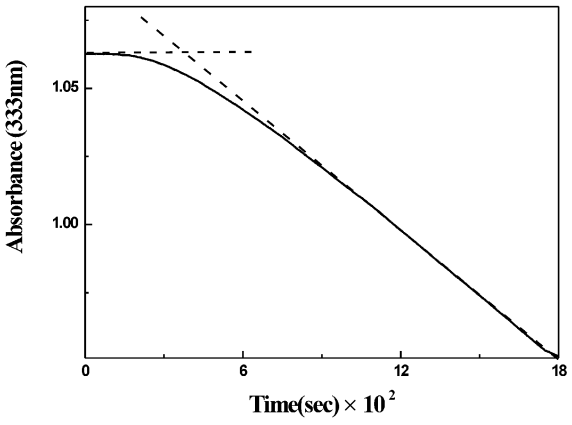


Figure 1. Time course of the AHAS catalyzed reaction. Assays were initiated by the addition of pyruvate to the reaction mixtures that incubated at 37 °C for 5 min. Pyruvate consumption was monitored at 333 nm. The assay mixture contained 50 mM MOPS (pH 8.0), 40 mM pyruvate, 20 μM FAD, 5 mM ThDP, 10 mM $MgCl_2$, AHAS (52.6 μg). The tangents drawn to the initial and final segments of the time course are used for calculation of the relaxation time for the transition.

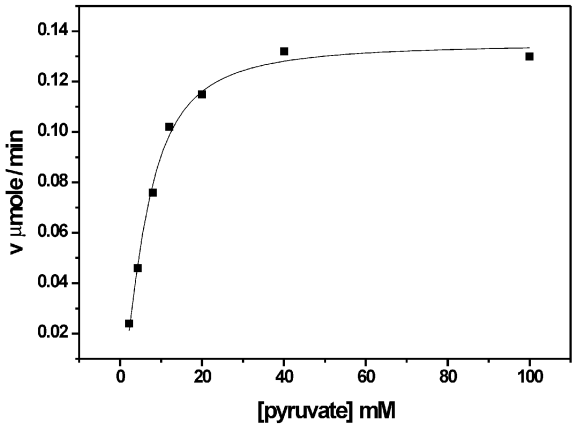


Figure 2. Rate plot of the AHAS catalyzed reaction. The assays were conducted as described in Figure 1 with the reaction mixture containing 50 mM MOPS (pH 8.5), 20 μM FAD, 1 mM ThDP, 10 mM $MgCl_2$, and the indicated concentration of pyruvate. The points are experimental values and repeated at least three times. The curve is theoretical from a fit of v = VA/(K + A) to the data.

The lag time was also found to be dependent on substrate (pyruvate) and cofactor (ThDP, FAD, Mg^{2+}) concentrations (see Table 1). Lower levels (non-saturating concentrations) of pyruvate, FAD, Mg^{2+} resulted in longer lag times before the time course of the reaction became linear. Higher levels (saturating concentrations) of pyruvate, FAD, Mg^{2+} tended to shorten lag times. Interestingly, lower levels of ThDP resulted in shortened lag times (260 sec) while higher concentrations (5 mM) of ThDP resulted in longer lag times (340 sec).

In detail, when the concentration of FAD varied in the reaction mixture over the range of 1-20 μM, the lag time decreased 490 to 330 seconds. The K_d obtained from FAD was 0.72 μM. The FAD at the saturated condition (over 99%) had about 0.67 fold lag time. Even at extremely high concentration (1 mM) of FAD, the lag phase in product formation was not eliminated entirely for AHAS. When the concentration of Mg^{2+} varied in the reaction mixture over the range of 0.2-10 mM, the lag time decreased 400 to 250 seconds. The K_d obtained from Mg^{2+} was 0.3 mM. The Mg^{2+} at the saturated condition (over 99%) had about 0.63 fold lag

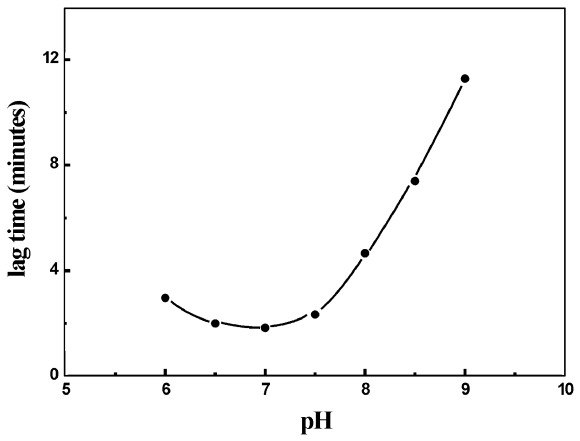


Figure 3. pH dependence of the AHAS lag time. The time lag was measured as the relaxation time ongoing from the initial to the final steady-state rate. Assay conditions were the same as in Figure 1, except that the pyruvate concentration was changed to 75 mM.

time. Even at extremely high concentration (10 mM) of Mg^{2+}, the lag phase in product formation was also not eliminated entirely for AHAS. Interestingly, at the extreme conditions (over 99% saturation), both of FAD and Mg^{2+} had decreased the same range of the lag time (0.63-0.67 fold). However, when the concentration of ThDP varied in the reaction mixture over the range of 0.1-5 mM, the lag time had a dramatic effect (260 to 400 sec) on the length of the lag phase. Even at high concentrations (5 mM) of ThDP, the lag phase in product formation was extended to 400 seconds. The K_d obtained from ThDP was 0.29 mM. The ThDP at the saturated condition (over 95%) had about 1.5-fold lag time, indicating that ThDP in higher concentration might have inhibitory effects.

Further investigation revealed that the time lag was pH dependent with little lag observed between pH 6.5 and pH 7.5, while the relaxation time for attainment of the final steady-state rate increased longer at higher pH (Figure 3). At lower pH (< pH 7.5) the time lag was revealed similar range (2-3 minute). At higher pH, with low concentrations of pyruvate and cofactors, a lag time as long as 30 minutes has been observed before a linear, steady-state rate was attained (data not shown). We also investigated whether the time lag is temperature dependence or not (Figure 4). The time lag of temperature dependence was obtained between 15 °C to 53 °C. The lag time at 37 °C was 250 seconds which is lower lag time. As the temperature was increased or decreased from 37 °C, the lag time was increased. However, when the temperature was decreased to 15 °C or increased to 53 °C, the lag time was undetectable due to the enzyme activity is diminished.

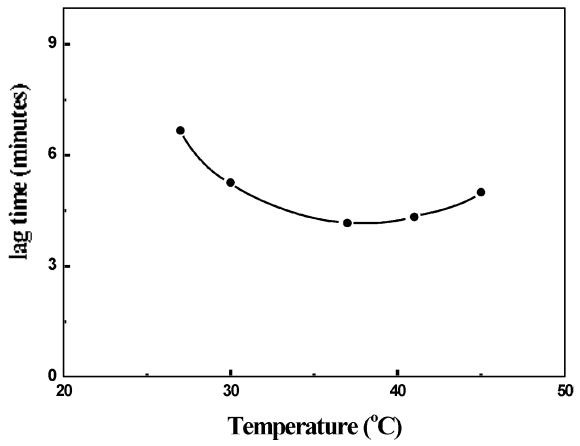


Figure 4. Temperature dependence of the AHAS lag time. The time lag was measured as the relaxation time ongoing from the initial to the final steady-state rate. Assay conditions were the same as in Figure 1, except that the pyruvate concentration was changed to 100 mM and the buffer was changed to Mops (pH 7.5).

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

References

1. Duggleby, R. G.; Pang, S. S. *J. Biochem. Mol. Biol.* **2000**, *33*, 1.
2. LaRossa, R. A.; Schloss, J. V. *J. Biol. Chem.* **1984**, *259*, 8753.
3. Ray, J. B. *Plant Physiol.* **1984**, *75*, 827.
4. Shaner, D. L.; Anderson, P. C.; Stidham, M. A. *Plant Physiol.* **1984**, *76*, 545.
5. Gerwick, B. C.; Subramanian, M. V.; Loney-Gallant, V.; Chander, D. P. *Pestic. Sci.* **1990**, *29*, 357.
6. Namgoong, S. K.; Lee, H. J.; Kim, Y. S.; Shin, J.-H.; Che, J.-K.; Jang, D. Y.; Kim, G. S.; Yoo, J. W.; Kang, M.-K.; Kil, M.-W.; Choi, J.-D.; Chang, S.-I. *Biochem. Biophys. Res. Commun.* **1999**, *258*, 797.
7. Schloss, J. V.; Dyk, K. E. V.; Vasta, J. F.; Kutny, B. M. *Biochemistry* **1985**, *24*, 4952.
8. Barak, Z.; Chipman, D. M.; Gollop, N. *J. Bacteriol.* **1989**, *169*, 3750.
9. Eoyang, L.; Silverman, P. M. *Methods Enzymol.* **1988**, *166*, 435.
10. Hill, C. M.; Duggleby, R. G. *Biochem. J.* **1998**, *335*, 653.
11. Ott, K.-H.; Kwagh, J.-G.; Stockton, G. W.; Sidrov, V.; Kekefuva, G. *J. Mol. Biol.* **1996**, *263*, 359.
12. Mazur, B. J.; Chui, C.-F.; Smith, J. K. *Plant Physiol.* **1987**, *75*, 1110.
13. Frieden, C. *Annu. Rev. Biochem.* **1979**, *48*, 471.
14. Phalip, V.; Schmitt, P.; Divies, C. *Curr. Microbiol.* **1995**, *31*, 316.
15. Chang, A. K.; Duggleby, R. G. *Biochem. J.* **1997**, *327*, 161.
16. Chang, A. K.; Duggleby, R. G. *Biochem. J.* **1998**, *333*, 765.
17. Kil, M. W.; Chang, S. I. *J. Biochem. Mole. Biol.* **1998**, *31*, 287.
18. Miflin, B. J. *Arch. Biochem. Biophys.* **1971**, *146*, 542.
19. Kuwana, H.; Date, M. *J. Biochem.* **1975**, *77*, 257.
20. Snoep, J. L.; Teixeira de Mattos, M. J.; Starrenburg, M. J. C.; Hugenholtz, J. *J. Bacteriol.* **1992**, *174*, 4838.
21. Yang, J. H.; Kim, S. S. *Biochim. Biophys. Acta* **1993**, *1157*, 178.
22. Xing, R.; Whitman, W. B. *J. Bacteriol.* **1994**, *176*, 1207.
23. Lee, Y. T.; Duggleby, R. G. *Biochemistry* **2001**, 6836.
24. Chang, S. I.; Kang, M. K.; Choi, J. D.; Namgoong, S. K. *Biochem. Biophys. Res. Commun.* **1997**, *234*, 549.
25. Lee, B.; Choi, J.; Yoon, M. *Bull. Korean Chem. Soc.* **2002**, *23*, 765.
26. Choi, J.; Kim, B.; Yoon, M. *Bull. Korean Chem Soc.* **2003**, *24*, 1.