Binding Properties of an Allosteric Activator Site of Aspartase from *Hafnia alvei*

Min-Sub Lee, Jeong-Rim Kim,† and Moon-Young Yoon*

Department of Chemistry, Hanyang University, Seoul 133-791, Korea
**Department of Chemistry, Hanyang University, Ansan 425-791, Korea*
Received June 1, 1999

Aspartase [L-aspartate ammonia-lyase, EC4.3.1.1] catalyzes the reversible conversion of L-aspartate to fumarate and ammonium ion in the following reversible reaction.

$$OOCCHCH_2COO \leftrightarrow OOCCH-CHCOO + NH_4^+$$

$$NH_3$$

Aspartase is a textbook example of a highly specific enzyme with no identifiable alternative substrates.[1] The enzyme is specific to aspartate and fumarate, but NH_2OH can be substituted for ammonia as a substrate.[2] A variety of divalent metal ions a such as Mg^{2+}, Mn^{2+}, Zn^{2+} and Co^{2+} activate the reaction.[3,11] Initial velocity data for the aspartase from *Hafnia alvei* are consistent with rapid equilibrium ordered addition of Mg^{2+} prior to aspartate but with completely random release of Mg^{2+}, NH_4^+ and fumarate.[4] Using an organic solvent perturbation method, a general base and a general acid for the catalysis of *Hafnia alvei* aspartase were predicted respectively as a histidine and a cysteine residue.[5,10,12]

The enzyme can be activated or inhibited when a certain substance binds to a certain site distant from its active site. Aspartase possesses an activator site that has the unusual specificity of binding a substrate, L-aspartate.[6,7] It has been observed that the occupation of this site with aspartate or with certain structural analogues of the substrate at pH 8.0 and above eliminates the reaction time lags that are observed in the amination direction and the nonlinear kinetics seen in the deamination direction.[6,7] No time lag was observed in the deamination direction under normal conditions since L-aspartate is already present in the activator site. Aspartase exists in a pH dependent equilibrium between two forms. The higher pH form of aspartase is activated by divalent metal ions and substrate analogues, while the lower pH form of aspartase does not require any effectors for catalytic activity.[6] Aspartase has been reported to be specific for its amino acid substrate L-aspartate, showing no activity with D-aspartic acid, crotonic acid, glycine, alanine, phenylalanine, leucine, methylsuccinate, tyrosine, the mono-and diethyl esters of fumarate, glutamine, maleic acid, or glutaconic acid.[8] Inhibition studies have reported that citrate, EDTA and pyrophosphate block the action of aspartase.[9]

Monod and Koshland have previously proposed the models to account for cooperative kinetics and the effects of modifiers on allosteric enzymes.[13,14] With the observation of time dependent changes in enzyme activities, these models have been extended to allow for the possibility of slow binding of substrates or modifiers and /or conformational changes to account for transient kinetics.[15] In our previous investigation,[7] a time lag was observed during the amination reaction of *Hafnia alvei* aspartase, which occurred before the final steady-state rate was attained. The time lag was shortened at lower pH, but lengthened at higher pH. Also, the time lag was shortened at higher temperature and lengthened at lower temperature. L-aspartate, D-aspartate and α-methyl-DL-aspartate markedly revealed the activating effects. The time lag and nonlinear kinetics in the amination reaction were eliminated when one of these effectors was added in the reaction mixture. These results suggested that these effectors occupy a distant allosteric activator site apart from the active site. In an effort to elucidate this allosteric activator site in aspartase from *Hafnia alvei*, we have further investigated the allosteric site properties by virtue of the activity and the time lag changes by using many amino acid analogues of L-aspartate. The enzyme used in this investigation was purified according to the method described by yoon *et al.*[11]

The observation of a time lag during the amination of fumaric acid catalyzed by aspartase has led to various chemicals in an attempt to provide an explanation for this phenomenon. The L-glutamate concentration dependence of the time lag is shown in Figure 1. L-glutamate, an acid amino

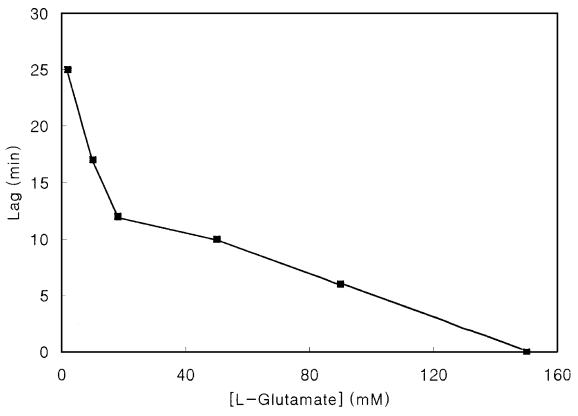

Figure 1. The D-aspartate concentration dependence of the aspartase time lag. The time lag was measured as the relaxation time moved from the initial to the final steady state rate. Assay mixtures contained 100 mM Ches (pH 8.5), 10 mM fumarate, 1 M $(NH_4)_2SO_4$, 2 mM $MgSO_4$ and the indicated concentrations of D-aspartate.

*To whom all correspondence should be addressed. Tel: 02-2290-0946, Fax: 02-2299-0762, E-mail: myyoon@email.hanyang.ac.kr

acid like L-aspartate substrate, appeared to completely eliminate the lag as the level was increased to 150 mM. The requirement of high concentration (> × 7) of L-glutamate in comparison to D-aspartate is due to a methyl group at the β position and a difference of D, L-stereo amino acid, indicating the residual composition of an allosteric site might be formed by fitting an acetic group rather than a propionic group.

Table 1 shows the effect of various chemicals on elimination of lag in the amination direction. The time lag was decreased as the concentration of D-aspartate increased. The time lag was slowly decreased from 20 min to 15 min until D-aspartate concentration increased to 12 mM, while a time lag tended rapidly to shorten from 15 min to 5 min as D-aspartate concentration increased to 17 mM. The results indicate that the highest acquisition of activity occurred rapidly rather than slowly. Saturating level (20 mM) of D-aspartate appeared to completely eliminate the lag under the conditions observed. Interestingly, D-aspartate at concentrations as high as 75 mM does not inhibit the aspartase catalyzed reaction, indicating that D-aspartate has low affinity for the active site.[6] An examination of substrate analogues can provide important information about the structural requirements that aspartase place on the amino acid substrate. D-alanine and L-alanine with only a methyl group at the β position appeared to completely eliminate the lag at 130 mM and 70 mM, respectively. Both D-alanine and L-alanine required relatively higher concentrations to eliminate the lag, and only two of concentrations are different. These results suggest that a crevice of the allosteric site of aspartase is larger than that occupied by a methyl group. Glycine, the smallest amino acid, appeared to completely eliminate the lag at 30 mM. The β position of glycine is composed of a hydrogen atom, while the β position of aspartate is composed of an acetic acid group. Interestingly, the required concentration for the time lag does not show a remarkable contrast. L-asparagine appeared to completely eliminate the lag at 0.1 mM. The required concentration of L-asparagine for the elimination of the lag was much lower (< × 200) than that of other amino acids examined in this study, indicating that the stereo position of a carbonyl and an amino group of amide group of L-asparagine is probably the most pertinent.

In the amination direction, it was observed that the addition of low concentrations (about 1 mM) of L-aspartate would eliminate the time lag.[7] When L-aspartate was present initially in the amination direction, no lag was detected, and the initial rate observed was identical to the final linear steady-state rate. Also, addition of L-aspartate during the lag phase of an amination reaction assay resulted in an immediate activation to the maximum steady-state rate. The relative activity of aspartase versus L-aspartate concentration is shown in Figure 2. As the concentration of L-aspartate increases, the maximum activity gradually increases in proportion. When the concentration of L-aspartate was 2.0 mM, the maximum activation of aspartase was revealed to be 173%. When the concentration of D-aspartate increased, the activity of enzyme was gradually increased. In the same manner, the time lag was gradually decreased by increasing the concentration of D-aspartate (*vide ante*). Table 2 shows the effect of various amino acids on aspartase activity in the amination reaction. α-Methyl-DL-aspartate, which is an uncommon amino acid, and L-glutamate of amino acids have shown similar phenomena. The maximum activity of α-methyl-DL-aspartate, which eliminated the time lag, was 205% with a concentration of 14 mM. Meanwhile, the maximum activity of L-glutamate, which eliminated the time lag, was 149% with a concentration of 150 mM. L-asparagine has been shown to have a much lower concentration (0.1 mM) for activation (196%). The results of the three amino acids above indicate that the allosteric site must be occupied to an acetyl group. D-alanine and L-alanine, which consist of a methyl group, have not shown activation even at higher concentrations, and they have unreliable results (*i.e.* mixed data). Similarly, the result of glycine, which has a hydrogen functional group, also shows no activation. These results indicate that the amino acids, having small size functional groups, do not affect the activation of allosteric sites.

Time lag and activation effects are correlative to each

Table 1. The effect of various amino acids on elimination of lag in the amination direction

	Concentration (mM)					
	Lag (min)					
D-Aspartate	5	6	11	12	17	20
	20	20	15	15	5	0
L-Glutamate	2	10	18	50	90	150
	25	17	12	10	6	0
D-Alanine	1	10	100	130		
	16	12	4.2	0		
L-Alanine	10	20	30	50	70	
	20	15	11	8	0	
Glycine	10	20	30			
	14	4	0			
L-Asparagine	0.01	0.1				
	27	0				

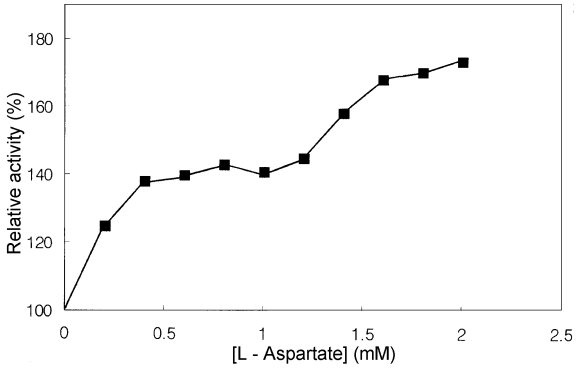

Figure 2. The L-aspartate concentration dependence of the aspartase activation. The activity was calculated by measuring the steady state rate that the reaction time course became a linear. Assay conditions were the same as in Figure 1 except for the indicated concentration of L-aspartate.

Table 2. The effect of various amino acids on aspartase activity in the amination direction

	Concentration (mM)									
	Relative activity (%)									
L-Aspartate	0.2	0.4	0.6	0.8	1.0	1.2	1.4	1.6	1.8	2.0
	125	138	140	143	141	145	158	168	170	173
D-Aspartate	2	4	6	8	10	12	14	16	18	20
	120	134	142	152	162	165	170	176	183	184
α-Methyl-	2	4	6	8	10	12	14			
DL-aspartate	116	120	150	170	187	195	205			
L-Asparagine	0.01	0.1	1							
	48	196	247							
L-Glutamate	2	10	50	90	150					
	105	114	121	143	149					
D-Alanine	1	10	50	100	130					
	48	50	93	80	95					
Glycine	10	30	50	80	100	150				
	94	59	51	48	45	26				
L-Alanine	10	70	100	150						
	101	64	92	63						

other as discussed above. The observation of time lag and activation during the amination of fumarate catalyzed by aspartase has led to a possible explanation of how activators bind to the allosteric site. As the concentration of effectors builds up, the reaction rate can increase in a highly cooperative manner until a linear steady-state rate is reached at the point where the activator site becomes fully occupied. L-asparagine among the amino acids tested was found to be the most effective activator. It is suggested that a carbonyl and an amino group of the amide group of L-asparagine position properly on allosteric site and lead to hydrogen bonds. In summary, the hydrogen bonds of the effectors on the allosteric site converted the enzyme into the activated form by inducing the conformational change, and then the enzyme attained to the maximum activation by the association of each subunit.

Acknowledgment. This research was supported by a research fund [HYU-99-033] of Hanyang University (1999).

References

1. Falzone, C. J.; Karsten, W. E.; Conley, J. D.; Viola, R. E. *Biochemisty* **1988**, *27*, 9089.
2. Karsten, W. E.; Viola, R. E. *Arch. Biochem. Biophys.* **1991**, *287*, 60.
3. Williams, V. R.; Lartigue, D. J. *J. Biol. Chem.* **1967**, *242*, 2973.
4. Nuiry, I. I.; Hermes, J. D.; Weiss, P. M.; Chen, C. Y.; Cook, P. F. *Biochemistry* **1984**, *23*, 5168.
5. Yoon, M. Y.; Cho, C. G. *Bull. Korean Chem. Soc.* **1998**, *19*, 31.
6. Karsten, W. E.; Gates, R. B.; Viola, R. E. *Biochemistry* **1986**, *25*, 1299.
7. Paik, T. J.; Lee, M. S.; Han, T. S.; Yoon, M. Y. *J. Korean Chem. Soc.* **1999**, *43*, 69.
8. Virtanen, A. I.; Ellfolk, N. *Methods Enzymol.* **1955**, *2*, 386.
9. Ellfolk, N. *Acta Chem. Scand.* **1953**, *7*, 1155.
10. Shim, J. B.; Kim, J. S.; Yoon, M. Y. *J. Biochem. Mol. Biol.* **1997**, *30*, 113.
11. Yoon, M. Y.; Park, J. H.; Choi, K. J.; Kim, J. M.; Kim, Y. O.; Park, J. B.; Kyung, J. B. *J. Biochem. Mol. Biol.* **1998**, *31*, 345.
12. Shim, J. H.; Kim, H. J.; Yoon, M. Y. *J. Biochem. Mol. Biol.* **1999**, *32*, 326.
13. Monod, J.; Wyman, J.; Changeux, J. P. *J. Mol. Biol.* **1965**, *12*, 88.
14. Koshland, D. E., Jr.; Nemethy, G.; Filmer, D. *Biochemistry* **1966**, *5*, 365.
15. Ainslie, G. R.; Shill, J. P.; Neet, K. E. *J. Biol. Chem.* **1972**, *247*, 7088.