Kinetic Mechanism in the Absence of Metal of *Hafnia alvei* Aspartase in the Amination Direction

Im-Jung La, Hyo-Joon Kim, † and Moon-Young Yoon *

Department of Chemistry, Hanyang University, Seoul 133-791, Korea
†Department of Biochemistry and Molecular Biology, Hanyang University, Ansan 425-791, Korea
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The kinetic mechanism of *Hafnia alvei* aspartase in the amination direction has been determined in the absence of metal. The initial velocity pattern obtained by varying the concentration of fumarate at several fixed concentrations of NH4⁺, shows an intersection on the left of the ordinate at pH 7.0, indicating that the kinetic mechanism is a sequential mechanism in which substrate inhibition by fumarate is observed. The dead-end inhibition pattern by varying the concentration of NH4⁺ at several fixed concentration of succinate shows an intersection on the left of the ordinate. These data are consistent with random addition of NH₄⁺, or fumarate. The Haldane relationship gives a $K_{eq}$ of $1.18 \times 10^{-3}$ M at pH 7.0, which is in agreement with the values obtained from the direct determination of reaction concentrations at equilibrium ($6.0 \pm 0.2 \times 10^{-3}$ M).

**Keywords**: Kinetic, Aspartase, Hafnia alvei.

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**Introduction**

L-aspartase [L-aspartate ammonia-lyase] from *Hafnia alvei* catalyzes the reversible deamination of L-aspartic acid to fumarate and ammonia.

![Reaction Mechanism](image)

This enzyme represents a case of absolute substrate specificity since no alternative substrates have been found to substitute for L-aspartate. Aspartase has been regarded as a catabolic enzyme in both bacteria and plants, but its reaction is reversible and favors aspartate formation with $\Delta G^o = 3.2$ kcal/mol for aspartate deamination. Gawron and Fondy showed that ammonia is added trans-across the double bond of fumarate. The enzyme is specific for L-asparate and fumarate, but NH₂OH can substitute for ammonia as a substrate. A variety of divalent metal ions can activate the reaction including Mg²⁺, Mn²⁺, Zn²⁺ and Co²⁺, but not Ca²⁺. Mn²⁺ has 80% the maximum rate of activation of Mg²⁺ prior to aspartate, but a completely random release of Mg²⁺, NH₄⁺, or fumarate. Yoon, et al., has studied the pH dependence of the kinetic parameters in both direction reactions. Two enzyme groups with $pK$ values of 6.6 and 7.2 are necessary for catalysis. An enzyme group that must be deprotonated has been identified. Another enzyme group must be protonated for substrate binding. Both the general base and general acid groups are in a protonation state opposite that in which they started when the aspartate was bound. A proton is abstracted from C-3 of the monoanionic form of the aspartate by the general base with a $pK$ value of 6.3-6.6 in the absence and presence of Mg²⁺. Ammonia is then expelled with the assistance of a general acid group, leaving NH₄⁺ as the product.

Aspartase originally catalyzes the divalent metal dependent deamination of L-aspartate to yield fumarate and ammonia in bacteria and some plants. Aspartase reaction takes place in vitro without the divalent metal. The role of metal is not known with regard to *in vivo* regulation. Because of our interest in the mechanism of these aspartate-utilizing enzymes, we have undertaken many studies. Although abundant information is available on many aspects of the aspartase reaction, little is known of the kinetic mechanism. In these studies, we present data on the kinetic mechanism in the amination direction of aspartase from *Hafnia alvei* in the absence of metal and equilibrium constant.

**Material and Methods**

**Chemicals.** Hafnia alvei (ATCC 9760) was purchased from ATCC (American Type Culture Collection). L-aspartic acid (L-aspartate), 4-(2-hydroxyethyl)-piperazine-1-ethanesulfonic acid (PIPES) were purified by recrystallization. 

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*To whom correspondence should be addressed. Tel: +82-2-2290-0946; Fax: +82-2-2299-0763; e-mail: myyoon@email.hanyang.ac.kr*
acid (HEPES), ethylenediaminetetraacetic acid (EDTA), and fumaric acid were purchased from Sigma Chemical Co. (St. Louis, USA). Ammonium sulfate, N,N-methylene-bis-acrylamide, acrylamide and N,N,N,N-tetramethylenediamine (TEMED) were purchased from Bio-Rad (Richmond, USA). All other chemicals were commercially available pure or extra pure grade.

**Enzyme purification.** Aspartase was purified from *Hafnia alvei* as previously described. Briefly, aspartase was obtained from a combination of diethylaminoethyl cellulose (DEAE cellulose), Red A-agarose, and Sepharose 6B chromatography. The purified enzyme was divided into aliquots and stored at -70 °C until use. The catalytic function of the enzyme remained stable for at least 1 month at 4 °C without appreciable loss of enzymatic activity. Protein concentration was determined by Bradford method.

**Enzyme assay.** The activity of aspartase was determined by measuring the decrease of fumarate following the decrease in absorbance at 240 nm \( (ε_{240}=2,255 \text{ M}^{-1}\text{ cm}^{-1}) \) at 25 °C, with a Hewlett Packard 8452 Diode Array spectrophotometer equipped with a constant-temperature cell housing. All reactions were carried out in a 1 mL cuvette with a 1 cm light path, or 0.5 mL cuvette with a 0.5 cm light path, which were incubated for at least 10 min in the cell compartment prior to initiation of the reaction by the addition of aspartase. The standard assay mixture contained 100 mM HEPES (pH 7.0) buffer, 1 mM EDTA, variable concentrations of fumarate at fixed concentrations of an ammonium ion, and the enzyme. One unit of enzyme activity was defined as the amount of 1 µmol of fumarate consumed per minute under the standard assay conditions.

**Initial velocity studies.** For the initial velocity studies of *Hafnia alvei* aspartase in the amination direction in the absence of metal, reactions were carried out in cuvettes of 1 cm path length in a final volume of 0.5 mL which contained the following: 100 mM HEPES, pH 7.0, 1.0 mM EDTA, variable concentrations of fumarate at several fixed concentrations of NH₄⁺ and an appropriate amount of the enzyme. Rates were calculated using an extinction coefficient for fumarate at 240 nm of 2,255 M⁻¹cm⁻¹. The temperature was maintained at 25 °C using a circulating water bath to heat the thermospacers of the cuvette compartments.

**Production inhibition studies.** The production inhibition patterns were obtained by measuring the initial rate of the reaction mixture containing 100 mM HEPES (pH 7.0) and 1.0 mM EDTA with varying the concentrations of one substrate and using several fixed concentrations of the product at a constant concentration of the other substrate. For the inhibition patterns with respect to fumarate, the constant concentration of the other substrate was 120 mM of NH₄⁺.

Conversely, for the inhibition patterns with respect to NH₄⁺, it was 16 mM of fumarate. Rates were determined in the same manner as described in the initial velocity studies.

**Dead-end inhibition studies.** The dead end inhibition patterns for succinate were measured in a constant concentration of one substrate, while varying the concentrations of the other substrate and using several fixed concentrations of succinate. Conditions were as described above, with the exception of the substitution of succinate for aspartate. For studies of succinate as an inhibitor, the constant concentration of the substrate was 5 mM of fumarate.

**Equilibrium constant determination.** The equilibrium constant (\( K_{eq} \)) for aspartase in the absence of metal was determined at pH 7.0 (100 mM HEPES) as follows. Reaction mixtures were prepared at different ratios of [fumarate]/[aspartate] and a constant concentration of NH₄⁺ at 100 mM. The enzyme was added and, after a 2-hr incubation period, aspartase was denatured by vortexing the mixture with a few drops of perchloric acid. The final fumarate concentration was determined enzymatically as described by Qamar, et al.\(^{17} \) The change in the fumarate concentration is plotted against the initial [fumarate]/[aspartate] ratio, with \( K_{eq} \) being taken as the point where the resulting curve crosses the Δfumarate axis. The point at which the curve crosses the abscissa is equal to the equilibrium constant.

**Data processing.** Reciprocal initial velocities were plotted as a function of reciprocal substrate concentrations. The data were fitted using the appropriate rate equations and Fortran programs of Cleland.\(^{18} \) Data conforming to a sequential mechanism were fitted using Eq. (1). Data for competitive and noncompetitive inhibition were fitted using Eqs. (2) and (3), respectively.

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\begin{align*}
\frac{v}{V} &= \frac{VA}{1 + \frac{I}{Ko}} \quad (1) \\
\frac{v}{V} &= \frac{VA}{1 + KA / Ko} \quad (2) \\
\frac{v}{V} &= \frac{VA}{1 + KA / Ko + LA / Ko} \quad (3)
\end{align*}
\]

In Eqs. (1)-(3), \( v \) and \( V \) represent initial and maximum velocities, respectively; \( K_a \) and \( K_i \) are \( K_{eq} \) values for \( A \) and \( B \), respectively. \( K_a \) and \( K_i \) are slope and intercept inhibition constants, respectively while \( A, B, \) and \( I \) represent reactant and inhibitor concentrations.

**Results and Discussion**

In the direction of aspartate deamination, when Mg²⁺ is varied and aspartate is maintained at a fixed concentration, apparent complete substrate inhibition is observed. A reciprocal experiment results in a hyperbolic increase in rate with an increase in aspartate concentration. The lag in the time course was alleviated in all cases by the addition of 1 mM aspartate to the reaction mixture. These data suggested that Mg²⁺ and aspartate are reactants but not the Mg-aspartate complex (i.e. not as the chelate complex). In the direction of fumarate amination in the absence of metal, time courses obtained at varied fumarate concentrations and saturated concentrations of NH₄⁺ were complicated by the appearance of a lag in the time course followed by a linear steady state rate (data not shown). This behavior is similar to that reported for the presence of Mg²⁺.

When fumarate in the direction of fumarate amination is varied at different fixed levels of NH₄⁺, the initial velocity pattern shown in Figure 1 is obtained. When fumarate is varied and NH₄⁺ is maintained at a fixed concentration, appar-
ent substrate inhibition is observed. Intersection on the left of the ordinate is indicative of a sequential mechanism (if a parallel line is observed, the mechanism represents ping-pong). The kinetic mechanism observed in the absence of metal could be either random or ordered. On the other hand, when uncomplexed aspartate is varied at different fixed levels of uncomplexed Mg$^{2+}$, the initial velocity pattern intersects on the ordinate. This latter is diagnostic for an equilibrium-ordered mechanism where Mg$^{2+}$ must bind to the enzyme prior to the aspartate. The kinetic parameters are listed in Table 1. A maximum velocity in the absence of metal is 250% of that in the presence of metal. It is ascribed that optimum pH in the absence of metal and in the presence of metal is pH 7.0 and pH 8.0, respectively. When the metal is present in comparison with absence, the $K_m$ for aspartate and fumarate both decrease by a factor of 3. The $K_m$ for aspartate and fumarate decreased when the metal is used as a pseudo-substrate, interaction of the metal with aspartate and fumarate is suggested (vide ante). This may be a coordination of the divalent metal with the $\beta$-carboxyl group. On the other hand, the $K_m$ for NH$_4^+$ increased by a factor of 3. Occupancy of the metal binding site on the aspartase by Mg$^{2+}$ results in an increase (about 3 fold) in affinity of the enzyme for aspartate and fumarate. These results indicate that this increase in affinity is obtained when the Mg$^{2+}$ bound to the $\beta$-carboxyl of aspartate as well as to residues on the enzyme.

Aspartate is tested as a product inhibitor against the two possible substrates, fumarate and NH$_4^+$. An inhibition pattern for aspartate vs. fumarate and aspartate vs. NH$_4^+$ is shown in Figures 2A and 2B. Product inhibition patterns obtained using aspartate, were competitive vs. fumarate at nonsaturating NH$_4^+$ with $K_i$ values of 7.95 ± 1.20 mM, and were competitive vs. NH$_4^+$ at nonsaturating fumarate with $K_i$ values of 8.00 ± 1.82 mM. Inhibition by the aspartate products of the reaction was competitive with both fumarate and NH$_4^+$, indicating that the products combine to the same enzyme form,

Table 1. Kinetic parameters for aspartase in the presence of metal$^a$ and in the absence of metal at pH 7.0

<table>
<thead>
<tr>
<th></th>
<th>Mg$^{2+}$</th>
<th>No metal</th>
</tr>
</thead>
<tbody>
<tr>
<td>$V_{max}$ (µmol/min)</td>
<td>0.02 ± 0.001</td>
<td>0.05 ± 0.005</td>
</tr>
<tr>
<td>$K_{aspartate}$ (mM)</td>
<td>0.58 ± 0.05</td>
<td>1.73 ± 0.51</td>
</tr>
<tr>
<td>$K_{fumarate}$ (mM)</td>
<td>0.2 ± 0.06</td>
<td>0.76 ± 0.24</td>
</tr>
<tr>
<td>$K_{NH4^+}$ (mM)</td>
<td>98 ± 20</td>
<td>31.82 ± 10.59</td>
</tr>
<tr>
<td>$K_i$ (mM)</td>
<td>0.165 ± 0.06</td>
<td>2.83 ± 1.29</td>
</tr>
<tr>
<td>$K_i$ (mM)</td>
<td>2.20 ± 0.04</td>
<td>1.19 ± 0.5</td>
</tr>
<tr>
<td>$V/K_{fumarate}$ (min$^{-1}$)</td>
<td>0.1 ± 0.02</td>
<td>0.007 ± 0.002</td>
</tr>
<tr>
<td>$V/K_{NH4^+}$ (min$^{-1}$)</td>
<td>0.0002 ± 0.00005</td>
<td>0.0002 ± 0.00004</td>
</tr>
</tbody>
</table>

$^a$Data were obtained by using under Materials and Methods and fitted as discussed under data processing. $^b$Data were from Yoon et al.$^{12}$

Figure 1. Initial velocity pattern in the direction of fumarate amination in the absence of metal. Fumarate was varied at several fixed levels of [NH$_4^+$] including 50 ( ■ ), 67 ( ● ), 100 ( ▲ ), and 200 ( ▼ ) mM. Assay were carried out in 100 mM HEPES (pH 7.0) at 25 °C. The points are experimental values, while the solid lines are from a fit of the data using Eq. 1.

Figure 2. Product inhibition by aspartate with respect to fumarate and NH$_4^+$ as the variable substrate. (panel A) Fumarate is competitive with [aspartate] of 0 ( ▼ ), 4 ( ▲ ), 8 ( ● ), and 12 ( ■ ) mM, and (panel B) NH$_4^+$ is competitive with [aspartate] of 0 ( ▲ ), 4 ( ● ), and 8 ( ■ ) mM in the direction of fumarate amination. All other conditions are as described under “Materials and Methods”. The points are experimental values, and the solid lines are from a fit of the data using Eq. 2.
the free enzyme (E). These results suggest that no significant concentrations of E-aspartate accumulate in the steady-state (if the complex even form) and the off-rate for the product is much faster than the catalytic rate.

Succinate is tested as a dead-end analog against the substrate, fumarate. The dead-end inhibition pattern for succinate vs. NH$_4^+$ is shown in Figure 3. The dead-end inhibition pattern by varying the concentration of NH$_4^+$ at several fixed concentrations of succinate shows an intersection on the left of the ordinate. This means that succinate is noncompetitive vs. NH$_4^+$ with a $K_i$ of 10.9 ± 5.95 mM and a $K_{ii}$ of 29.15 ± 14.90 mM. The noncompetitive inhibition pattern obtained with the succinate vs. NH$_4^+$ is diagnostic for a random mechanism with both pathways allowed, i.e. the pathway in which fumarate binds to the enzyme prior to NH$_4^+$ and the pathway in which NH$_4^+$ binds to the enzyme prior to fumarate. In order to show that the inhibition data are quantitatively internally consistent and thus support the qualitative interpretation suggested above, the observed $K_i$ values were corrected for the concentration of the fixed reactant to give an estimate of the true $K_i$ values. The apparent $K_i$ value, obtained from the noncompetitive inhibition of the succinate vs NH$_4^+$, can be corrected using the equation $appK_i = K_i(1 + fumarate/K_{fumarate})$ and $appK_{ii} = K_{ii}(1 + fumarate/K_{fumarate})$, which gives corrected $K_i$ values of 4.56 mM and 4.65 mM, respectively. These values are in agreement with $K_{sucinate}$ obtained from the succinate vs fumarate inhibition.

Therefore, the mechanism in the direction of fumarate amination appeared to be a random addition as shown in Scheme 1.

where A, P, and Q represent aspartate, fumarate, and NH$_4^+$, respectively. Dougherty, et al., postulated a uni-bi rapid equilibrium random mechanism with the metal ion at a saturating concentration. The overall kinetic mechanism for aspartase which includes the divalent metal ion as a pseudo reactant is consistent with the rapid equilibrium ordered addition of Mg$^{2+}$ prior to the aspartate but a completely random release of Mg$^{2+}$, NH$_4^+$, or fumarate.$^9$ The enzyme β-methylaspartase and fumarase have an identical kinetic mechanism to that catalyzed by aspartase in the amination reaction.$^{20,21}$

The equilibrium constant for the reaction catalyzed by the aspartase has been determined using the enzymatic methods: varying the [fumarate]/[aspartate] ratio at a fixed NH$_4^+$ concentration and measuring the change in fumarate concentration after equilibrium is reached as shown in Figure 4. The change in the fumarate concentration is plotted against the initial [aspartate]/[fumarate] ratio, with $K_{eq}$ being taken as the point where the resulting curve crosses the Dfumarate axis. The $K_{eq}$ value ([fumarate][NH$_4^+$]/[aspartate]) obtained from these methods at pH 7.0 and 25 °C is 6.0 ± 0.2 × 10$^{-3}$ M. The equilibrium constant for the reversible reaction can be measured using the Haldane relationship. It is necessary for the initial velocity data to adhere to the Haldane relationship.$^{19}$ The Haldane relationship for the aspartase mechanism in the absence of metal is as follows:

$$K_{eq} = (V_f/K_{aspartate})K_{fumarate}/[V_r/K_{NH4}].$$
A value of $1.18 \times 10^{-3}$ M at pH 7.0 is calculated for $K_{eq}$. This is in excellent agreement with the value of $5 \times 10^{-3}$ M, which was determined by Bada and Miller.\(^2\)

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**References**