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The Kinetic Parameters of *Hafnia alvei* Aspartase from pH Studies

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Abstract: The pH dependence of kinetic parameters in the amination direction of the aspartase from *Hafnia alvei* has been determined. The V/K for fumarate is bell shaped with pK values of 6.4 and 8.7. The maximum velocity for fumarate is also bell shaped with pK values of 7.2 and 9.1. The pH dependence of $1/K_i$ for potassium (competitive inhibitor of ammonia) decreases at low pH with pK 7.6. Together with data [Yoon and Cook (1994) *Korean J. Biochem.* **27**, 1-5] on the deamination direction of the aspartase, these results are consistent with two enzyme groups which are necessary for catalysis. An enzymatic group that must be deprotonated has been identified. Another enzyme group must be protonated for substrate binding. Both the general base and general acid group are in a protonation state opposite that in which they started when aspartate was bound. A proton is abstracted from C-3 of the monoanionic form of L-aspartate by an enzyme general base with a pK of 6.3~6.6 in the absence and presence of Mg^{2+} Ammonia is then expelled with the assistance of a general acid group giving NH_4^+ as the product.

Key words: Aspartase, pH studies, Kinetic parameter.

The enzyme aspartase (aspartate ammonia-lyase, EC 4.3.1.1) catalyzes the deamination of aspartic acid to yield fumarate and ammonia:

$$^{-}OOCCH(NH_3^+)CH_2COO^{-} \rightleftarrows {}^{-}OOCCH{=}CHCOO^{-} + NH_4^+$$

The enzyme from *Hafnia alvei* is a tetramer composed of four apparently identical subunits of molecular weight 48,000 (Williams and Lartigue, 1967), The reaction is reversible and favors aspartate formation with $\Delta G° = 3.2$ kcal/mol for aspartate deamination. The equilibrium constant for the aspartase reaction, measured directly at 25℃ by Bada and Miller (1968), is 5×10^{-3} M.

Nucleotides such as IMP, AMP, GDP and adenosine activate aspartase, while GTP and UTP inhibit it (Williams and Scott, 1968). The nucleotide triphosphates increase $K_{aspartate}$ while the activators decrease $K_{aspartate}$; V_{max} is unaffected. The enzyme was observed to have an absolute requirement for a divalent metal ion activator at higher pH (Rudolph and Fromm, 1971), with some indication that aspartase may possess activity in the absence of divalent metal ions at low pH (Suzuki *et al.*, 1973). Alkali earth metals such as Mg^{2+} and transitional metals such as Mn^{2+} have been shown to provide some degree of activation (Wilkerson and Williams, 1961). Other metals, such as Be^{2+} and Ba^{2+} do not activate aspartase.

Dougherty *et al.* (1972) suggested as a kinetic mechanism a uni-bi rapid equilibrium random mechanism, neglecting any possible role of a metal ion. Nuiry *et al.* (1984) carried out the most complete kinetic mechanism study including a divalent metal ion as a pseudoreactant. They suggested the rapid equilibrium ordered addition of Mg^{2+} prior to aspartate but that there was a completely random release of Mg^{2+}, NH_4^+ or fumarate.

Yoon and Cook (1994) have studied the pH dependence of the kinetic parameters in the deamination direction. The V/K for aspartate is bell shaped with estimated pK values of 6.6 and 7.2. The maximum velocity for aspartate is also bell shaped giving pK values almost identical to those obtained for $V/K_{aspartate}$. They have concluded that two enzyme groups with pK values of 6.6 and 7.2 are necessary for the binding of the substrate and/or catalysis.

Gawron and Fondy (1959), using the data of Englard (1958) and Krasna (1958), showed that ammonia is added trans across the double bond of fumarate. Primary deuterium and ^{15}N, and secondary deuterium isotope effect data are consistent with the formation of a carbanion intermediate following the abstraction of

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the C-3R proton, and this is followed by a rate-determining C-N bond cleavage (Nuiry *et al.*, 1984). In the latter study, the transition state for the C-N bond cleavage has been suggested to be intermediate between the carbanion and fumarate.

In these studies, we present data on the pH dependence of the kinetic parameters in the amination direction of aspartase from *Hafnia alvei* in the presence and absence of Mg^{2+} and discuss complete acid-base-chemistry.

Materials and Methods

Fumaric acid and $(NH_4)_2SO_4$ were from Sigma. $MgSO_4$ was from Fisher Scientific. Matrex gel red A was from Amicon Corp. All other buffers were obtained from commercially available sources and were of the highest quality available.

Enzyme purification

Aspartase obtained from Sigma commercially is contaminated with fumarase which eliminates as a viable assay the monitoring of A_{240} unless the aspartase can be purified. Aspartase was purified by using the dye-ligand affinity chromatography method (Karsten *et al.*, 1985).

The enzyme obtained was approximately 90% pure or apparently homogenous as judged by sodium dodecyl sulfate(SDS)-polyacrylamide gel electrophoresis.

Enzyme assays

Aspartase activity in the amination direction was determined spectrophotometrically at 25℃ by measuring the disappearance of fumarate at 240 nm. Due to the strong absorbance of fumarate at 240 nm, the amination reaction was followed by measuring the disappearance of fumarate on the high-wavelength shoulder (260~290 nm) of the absorbance peak (Karsten *et al.*, 1986). All data were collected by using a Gilford 2600 spectrophotometer. The temperature was maintained at 25℃ by means of a circulating water bath with the capacity to heat and cool the thermospacers in the cell compartment. Assay temperatures were routinely checked using a YSI tele-thermometer with a Teflon probe carried out in a 1-ml cuvette with a 1-cm light path. All cuvettes were incubated for at least 10 min. in the water bath and 5 min. in the cell initiated by the addition of aspartase (1.0~2.0 μg/assay). Aspartase concentration was corrected for complexation with divalent metal by using the following dissociation constant obtained at 0.1 M ionic strength: Mg-fumarate, 10 mM (Dawson *et al.*, 1971). Standard assay mixtures contained 100 mM Hepes buffer, varying amounts of fumaric acid and ammonium sufate, and Mg^{2+} based on metal chelate correction.

pH Studies

Buffers at 100 mM final concentration were used over the following pH ranges: Mes, pH 5.5~6.5 (Pipes replaced Mes for determination of the potassium inhibition constant); Hepes, pH 6.5~8.0; Ches, pH 8.3~9.8. All buffers were titrated to pH with KOH. The pH was measured before and after the reactions. Aspartate and ammonia stock solutions were titrated to the desired pH to prevent significant differences from the pH of the buffer used. In all cases, the assays were repeated several times at a given pH with one of the other buffers to be sure no inhibition or activation was obtained. The concentrations of Mg^{2+} at the pH extremes were doubled in separate assays to be sure they were still saturating: no significant rate change was detected.

Inhibition data were obtained for K^+, competitive inhibitors versus aspartate. The substrate concentration was varied, while the Mg^{2+} concentration was maintained at saturation. Full inhibition patterns for K^+ were obtained at pH 6.8, 8.0, and 9.1 with the substrate varied at several different levels of the inhibitor, including zero. Once the competitive nature of the inhibition was determined to be pH independent, Dixon experiments were performed in which the variable substrate was fixed at a concentration equal to its K_m and the inhibitor concentration was varied over a range from zero to those that result in inhibition. The measured K_i was then divided by 2 to obtain the true K_i.

Data analysis

All data were fitted using the computer program of Cleland (1979), converted to BASIC, and adapted for use on a microcomputer. Reciprocal initial velocities were plotted as a function of reciprocal substrate concentrations. All plots were linear except the initial velocity patterns obtained when one substrate was varied at several fixed levels of the other. Data from Dixon plot analysis were fitted by using Eq. (1). Individual saturation curves were fitted to Eq. (2). Data for the initial velocity patterns in the direction of fumarate amination were fitted line by line to Eq. (3). Competitive inhibition data at each pH were fitted to Eq. (4). Data for the pH profile that decreased with a slope of 1 at low pH were fitted by using Eq. (5). Data for pH profiles that decreased with a slope of 1 at low pH and a slope of -1 at high pH were fitted by using Eq. (6). Data for V pH profiles in the presence of Mg^{2+} where V decreases from a constant value at high pH to a constant value at low pH were fitted to Eq. (7).

$$y=Ax+B \quad (1)$$

$$\upsilon=\frac{VA}{K+A} \quad (2)$$

$$\upsilon=\frac{VA^2}{a+2bA+A^2} \quad (3)$$

$$\upsilon=\frac{VA}{K(1+I/K_{is})+A} \quad (4)$$

$$\log y=\log\left(\frac{C}{1+[H^+]/K_1}\right) \quad (5)$$

$$\log y=\log\left(\frac{C}{1+[H^+]/K_1+K_2/[H^+]}\right) \quad (6)$$

$$\log y=\log\left(\frac{Y_L+Y_H[H^+/K_3]}{[1+[H^+]/K_3]}\right) \quad (7)$$

In Eq. (1), y is 1/υ, x is the inhibitor concentration, A is the slope, and B is the ordinate intercept. Values for K_i are obtained by determining the abscissa intercept (B/A) divided by 2. In Eq. (2) and (3), A is the reactant concentration. V is the maximum velocity, and K is the Michaelis constant for the varied substrate. In Eq. (4), I is the inhibition concentration and K_{is} is the inhibition constant for the slope. In Eq. (5) and (6), K_1 and K_2 represent dissociation constants for enzyme groups, y is V/K, and C is the pH-independent value of y. In Eq. (7), Y_L and Y_H are the constant values of V at low and high pH, respectively, y is the value of V at any pH, H is the hydrogen ion concentration, and K_3 is the acid dissociation constant of the group responsible for the pH dependent activation.

Results

Initial velocity studies in the absence of product and dead-end inhibitors

The stability studies in the deamination direction for aspartase have already shown that a significant decrease in activity did not occur at the extremes, pH 5.0 and 10.0 (Yoon and Cook, 1994). In the direction of fumarate amination, time courses obtained at varied fumarate concentrations and saturating concentrations of NH_4^+ and Mg^{2+} were complicated by the appearance of a lag in the time course followed by a linear steady state rate. The lag in the time course was alleviated in all cases by the addition of 1 mM aspartate to the reaction mixture.

At 1 mM concentration, aspartate has no inhibitory effect on the reaction. The latter behavior is similar to that reported for the enzyme from *Escherichia coli* (Ida and Tokushige, 1985; Karsten *et al.*, 1986).

Nuiry *et al.* (1984) have shown a rapid equilibrium ordered addition of Mg^{2+} prior to aspartate but a completely random release of Mg^{2+}, NH_4^+, or fumarate. An initial velocity pattern was obtained at pH 5.5 and pH 9.0 by varying fumarate at a fixed level of NH_4^+, saturating Mg^{2+}, and 1 mM aspartate intersect to the left of the ordinate. The results at pH 7.0 are shown in Figure 1. Initial velocity patterns qualitatively identical to that observed at pH 7.0 were also obtained at pH 5.5 and pH 9.0 indicating that the kinetic mechanism in the direction of fumarate amination is pH independent.

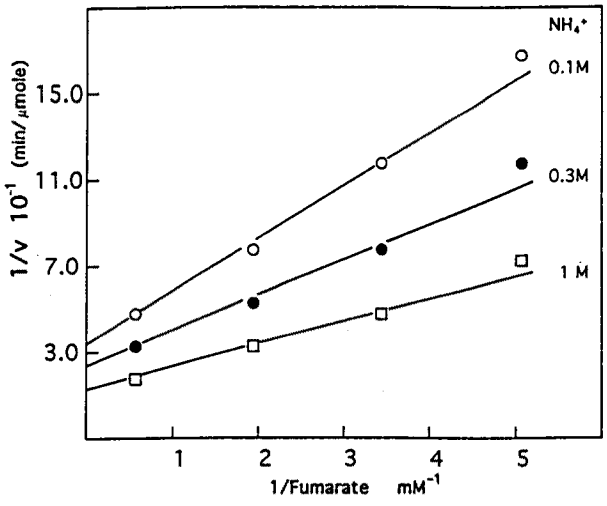


Fig. 1. Initial velocity pattern in amination direction of aspartase at pH 7.0. The ammonium concentration was used as follows: 0.1 M (○); 0.3 M (●); 1 M (□). All substrate concentrations corrected for the amount of metal chelate complex formation as described under Material and Methods. Data were fitted line by line to Eq. (3).

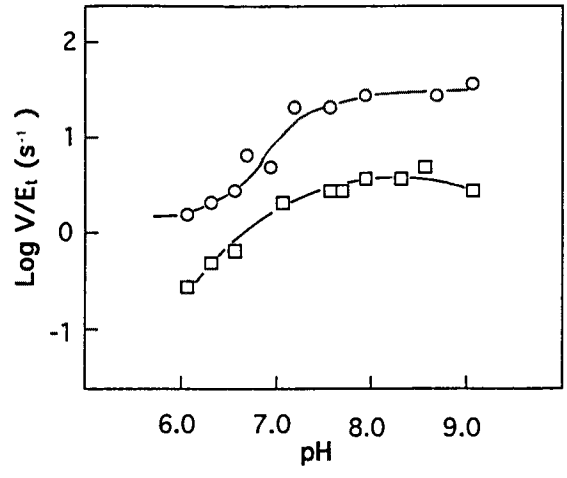


Fig. 2. pH dependence of the maximum velocity of the amination reaction of aspartase. (○)Total enzyme activity in the presence of 20 mM Mg^{2+}. The data were fitted to Eq. (7). (□) Enzyme activity in the absence of added divalent metal ions, where the line is fitted to Eq. (6).

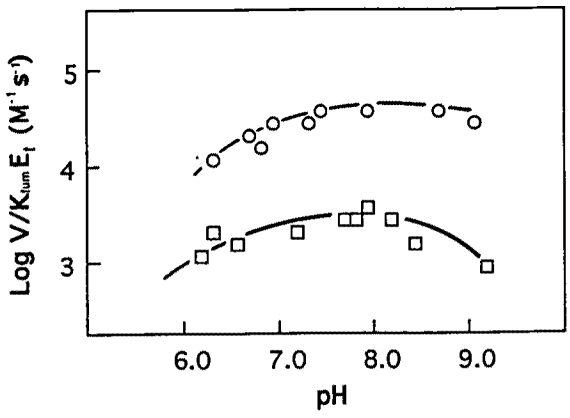


Fig. 3. pH dependence of V/K_{fum} of the amintion reaction of aspartase. (○) Total enzyme activity in the presence of 20 mM Mg^{2+}, which were fitted to Eq. (6). (□) Enzyme activity in the absence of Mg^{2+}, where the line is the fit to Eq. (6).

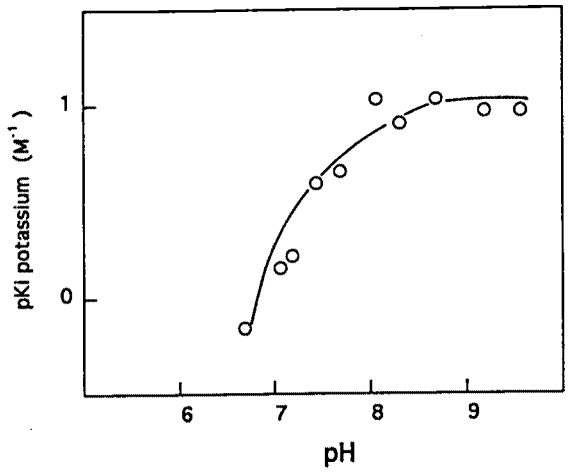


Fig. 4. pH dependence of competitive inhibitor binding to aspartase. All data except pH 6.8, 8.0 and 9.1 were obtained from Dixon plots varying the concentration of potassium in the presence of 10 mM Mg^{2+} and ammonium equal to its K_m. The data were fitted to Eq. (5).

pH Dependence of kinetic parameters

The pH dependence of V for amination was determined in the absence and presence of 20 mM Mg^{2+}. The maximum velocity was pH dependent. The V profile is bell-shaped in the absence of metal, giving pK values of 7.2 and 9.1 and a pH independent value of 9 ± 1 s^{-1}, Fig. 2. The V profile decreases from a constant value (above pH 8) at high pH to another constant value (below pH 5) at low pH in the presence of Mg^{2+}, Fig. 2. The pK values obtained as V becomes constant at low pH and at high pH are 6.4 and 7.5, respectively. The pH independent values of V at high and low pH are 39 ± 6 s^{-1} and 3.5 ± 0.6 s^{-1}, respectively.

The pH dependence of V/K_{fum} was also measured in the absence and presence of 20 mM Mg^{2+}. In both cases, V/K_{fum} is only slightly pH dependent. In the absence of metal, the pK obtained on the acidic side was 6.4, while that on the basic side was 8.7. In the presence of Mg^{2+} only the acidic pK is observed at about 6.5, Fig. 3. The pH independent values of V/K_{fum} in the presence and absence of Mg^{2+} are $(3.6\pm0.4)\times10^4$ $M^{-1}s^{-1}$ and $(2.8\pm0.4)\times10^3$ $M^{-1}s^{-1}$, respectively.

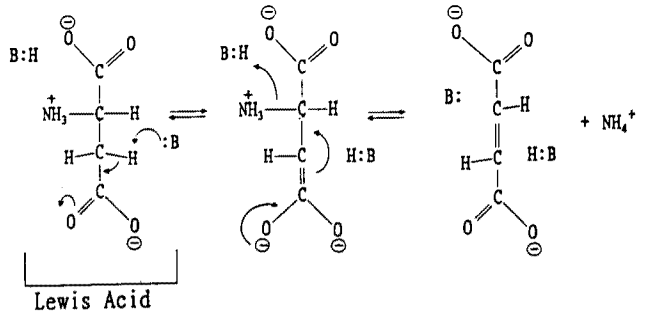
pH Dependence of the potassium dissociation constant

The pH dependence of the dissociation constant for potassium, a competitive inhibitor of ammonia, was determined in order to aid in the assignment of those pK values reflecting groups responsible for substrate binding, and to determine whether intrinsic pK values are observed in the V/K_{fum} pH profile. As a result, true pK values are observed for the pH dependence of K_i values for competitive inhibitors and can be used to check whether or not the observed pK values in the substrate profile are true pK values if the protonation state of these groups affects inhibitor binding, The pK_i potassium profile, Fig. 4, decreases at low pH with limiting slopes of 1, giving pK values of 7.6 ± 0.1. The pH independent value of K_i potassium is 120 ± 10 mM.

Discussion

The pH dependence of the kinetic parameters of the amination direction for the *Hafnia alvei* aspartase enzyme was determined. The V/K for a reactant is the second order rate constant for conversion of free enzyme and free reactant to products. Thus, the pKs observed in the V/K_{fum} pH profiles reflect acid dissociable functional groups in free E in the absence of Mg^{2+} and in free E:Mg in its presence. The V/K_{fum} pH profiles were obtained in the presence of saturating ammonia in the absence or presence of saturating Mg^{2+}, and thus reflect E:ammonia and E:Mg:ammonia, respectively. The V/K_{fum} was bell-shaped in the absence of Mg^{2+} giving pKs of 6.4 and 8.7. A bell-shaped V/K_{fum} profile was observed in the direction of deamination of aspartate and the estimated pK values were 6.6 and 7.2 (Yoon and Cook, 1994). In the presence of Mg^{2+} the acidic pK is about 6.5, while the basic pK is apparently perturbed to a value of >9.5. The V/K for ammonia was reported previously over the pH range 6~9.5 at saturating concentrations of fumarate and Mg^{2+} (Nuiry *et al.*, 1984). The V/K decreases at high pH with a pK of 9.3 indicating the pK for ammonia.

The V/K_{fum} profile decreases on either the acidic or the basic side to a limiting slope of one, indicating that a change in the ionization state of a single group on each side of the profile is involved in the loss of



Scheme 1. Possible chemical mechamism for the aspartase reaction.

activity. Thus, the two groups would have opposite protonation states in the two opposing reaction directions. In addition, potassium binding is only sensitive to the protonation state of general acid in the direction of aspartate deamination. The general acid group must be unprotonated to bind the positively charged monovalent ion, a mimic of the ammonium ion.

The V profiles were pH dependent and have been shown to be bell-shaped with a pK 7.2 at low pH and a pK 9.1 at high pH in the absence of Mg^{2+}. In the presence of Mg^{2+} the V profile shows a decrease from a constant value at high pH to another constant value at low pH, giving a pK of 7.6. The V profiles exhibit similar behavior in both reaction directions (Yoon and Cook, 1994). The two groups observed in the absence of Mg^{2+} likely function in catalysis as discussed above for V/K_{fum}. In the presence of Mg^{2+}, a partial change is observed in the V profiles in both reaction directions. A group with a pK of 7.6 is observed that is required to be unprotonated for optimal activity. The group with a pK of 7.6 is responsible for the pH-dependent activation as it becomes unprotonated. Such activation is also observed in the V/K_{asp} profile as an increase in the V/K between pH 6.5 and 8 (Yoon and Cook, 1994). Neither of the two groups observed in V profiles in forward and reverse reactions (minus divalent metal), the V/K_{asp} profiles (plus and minus divalent metal), and the V/K_{fum} profiles (minus divalent metal) are observed in the V profiles in forward and reverse reactions in the presence of divalent metal. Thus, the two catalytic groups either both interact with aspartate, which is unlikely given the proposed mechanism (Scheme 1), or are environmentally perturbed when aspartate and Mg^{2+} are bound, or a combination of both.

In the direction of fumarate amination, data are qualitatively similar to those obtained in the aspartate deamination reaction direction (Yoon and Cook, 1994). The V decreases at low and high pH as does V/K_{fum} in the absence of Mg^{2+}. Addition of Mg^{2+} causes the basic pK to increase. There are quantitative differences

Table 1. Summary of pK values obtained from the pH dependence of kinetic parameters in the direction of fumarate amination

Parameter	Metal ions	$pK_1 \pm$ SE	$pK_2 \pm$ SE
V/E_t	+	7.6± 0.4	
		6.4± 0.3	
V/E_t	−	7.2± 0.1	9.1± 0.2
$V/K_{fum}Et$	+	6.5± 0.2	
$V/K_{fum}Et$	−	6.4± 0.2	8.7± 0.2
$pKi_{potassium}$	+	7.6± 0.1	

pK_1 indicates that the group must be protonated for enzyme acitivity and pK_2 indicates that the group must be deprotonated. Profiles run in the presence (+) or absence (−) of 20 mM Mg^{2+}.

in the pK values observed in the fumarate direction compared to those in the aspartate direction. The differences likely reflect the complexes that are titrated, that is E:aspartate in one direction and E:fumarate:ammonium in the opposite direction.

Nuiry *et al.* (1984), based on isotope effect studies, and Porter and Bright (1980), based on the high affinity of aspartase for the dianionic form of 3-nitro-L-alanine, have postulated a mechanism in which a stabilized carbanion intermediate is formed. A mechanism taking into account the carbanionic intermediate and the above pH studies is shown in Scheme 1. Aspartate binds to the enzyme as the monoanion with both carboxyl groups and the α-amine ionized. A proton is abstracted from C-3 by an enzyme general base with a pK of 6.3~6.6. The pK for the general base is observed in all of the V profiles in the absence of Mg^{2+}, in all ofthe V/K profiles in the presence and absence of metal ion and in the pK_i succinate profile. The resulting carbanion is stabilized by delocalization of electrons into the β-carboxyl, presumably with the assistance of a one or more positively charged enzyme residues in the vicinity of the β-carboxyl. Nuiry *et al.* (1984) suggested that Mg^{2+} played the role of a Lewis acid coordinated with the β-carboxyl of aspartate, but Mg^{2+} is not absolutely required for the reaction, and the NMR studies of Falzone *et al.* (1988) indicate that this is unlikely. Thus, an enzyme residue must be responsible for the Lewis acid role, but this enzyme group must have a pK higher than 9, since no group that can be ascribed this function has been observed in the pH rate profiles. Ammonia must therfore be expelled with the assistance of a general acid group giving NH_4^+ as the product. The pK for the general acid is about 7.2~7.6 in the absence of Mg^{2+}, but increases to a value of 7.6~8.4 in the aspartate deamination direction in the presence of Mg^{2+} (Yoon and Cook, 1994), and even higher in the fumarate amination direction. The general

acid group is again observed in all of the same pH-rate profiles specified above for the general base, as well as the pK_i potassium profile. The general acid group is likely a neutral acid, since it would be repulsive to the protonated α-amine of aspartate if it were cationic. As a neutral acid there is also a possibility of hydrogen bonding to the positively charged amine. At the end of a catalytic cycle, both the general base and general acid groups are in a protonation state opposite to that in which they started when aspartate was bound. The *Hafnia alvei* aspartase thus has an acid-base catalytic mechanism similar to that suggested for fumarase, which has two catalytic groups, one to accept a proton to form the intermediate and a second to protonate the hydroxyl leaving group (Blanchard and Cleland, 1980). In this regard, *Hafnia alvei* aspartase and fumarase exhibit 37% sequence identity (Sun and Setlow, 1991) as has also been found for the *E. coli* aspartase and class II fumarase.

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