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Aspartase (EC 4.3.1.1) catalyzes the reversible deamination of L-aspartate to yield fumarate and ammonia (16) according to Eq. [1].

\[ \text{Aspartate} \rightleftharpoons \text{Fumarate} + \text{NH}_3 \]  

The enzyme is specific for aspartate and fumarate, but \( \text{NH}_2\text{O}H \) can substitute for ammonia as a substrate (5, 12). A variety of divalent metal ions are activators of the reaction including \( \text{Mg}^{2+} \) and \( \text{Mn}^{2+} \) (18), and initial velocity studies obtained for the enzyme from *Hafnia alvei* are consistent with a rapid equilibrium kinetic mechanism in which \( \text{Mg}^{2+} \) binds prior to aspartate, but with a random release of \( \text{Mg}^{2+}, \text{NH}_3, \) or fumarate (14).

The pH dependence of kinetic parameters of the L-aspartase-catalyzed reaction from *Escherichia coli* has been examined in both the amination and deamination directions (12). These authors suggest that aspartase exists in a pH-dependent equilibrium between a high pH form that has an absolute requirement for a divalent metal ion and substrate activation and a low pH form that does not require activation by either substrate or metal ion. Data were consistent with a group with a pK of about 6.5 that acts as a general base in the direction of aspartate deamination and a general acid in the opposite reaction direction.

Gawron and Fondy (8), using the data of Englard (6) and Krasna (13), showed that ammonia is added trans across the double bond of fumarate. Primary deuterium and \( ^{15}\text{N} \) and secondary deuterium isotope effect data are consistent with the formation of an intermediate.

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carbanion following the abstraction of the C-3R proton, and this is followed by a rate-determining C-N bond cleavage (14). In the latter study, the transition state for C-N bond cleavage has been suggested to be intermediate between the carbanion and fumarate.

In this paper the pH dependence of kinetic parameters for the H. alvei aspartase are examined in both reaction directions in the presence and absence of Mg2+ to allow comparison to the enzyme from E. coli (12). In addition, the washin of deuterium from D2O to aspartase during turnover has been measured. Data are discussed in terms of an overall mechanism for aspartase.

**METHODS AND MATERIALS**

**Chemicals and enzyme.** H. alvei aspartase (1 unit/mg) was obtained as a crude preparation from Sigma. The enzyme was purified using a Red A agarose column as suggested by Karsten et al. (11). The purified enzyme had a specific activity of 30 assayed with 20 mM MgSO4 and 10 mM aspartate at pH 8, 100 mM Hepes and was estimated to be 67% pure by SDS-PAGE. L-Aspartate, fumarate, MgCl2, NH4Cl, and Hepes were from Sigma, while ChEs were from Ultron. All other reagents were obtained from commercial sources and were of the highest purity available.

**Initial velocity studies.** All data were collected using either a Gilford 2600 spectrophotometer connected to a Hewlett-Packard flat bed plotter to plot the time course and its derivative or a Gilford 2600 spectrophotometer and a strip chart recorder. The temperature was maintained at 25°C with a circulating water bath with the capacity to heat and cool the thermostators in the cell compartment. All reactions were carried out in 1-ml cuvettes with a 1-cm light path, which were incubated for at least 10 min in a water bath prior to initiation of reaction by the addition of aspartase. The progress of the reaction was monitored using the appearance or disappearance of fumarate at 240 nm (ε240 = 2550 M1 cm−1). Fumarate and aspartate concentrations were corrected for complexation with divalent metal ion using the following dissociation constants obtained at 0.1 M ionic strength: Mg-aspartate, 4 mM; Mg-fumarate, 10 mM (19).

**pH studies.** To be certain that the kinetic mechanism in the direction of fumarate amiation is pH independent and to obtain estimates of the Ka values for fumarate and ammonia, initial velocity patterns in the absence of products were obtained at pH 5.5, 8, and 9 by measuring the initial rate at different levels of fumarate and several fixed levels of ammonium ion and at a single saturating concentration of Mg2+. Once the measured kinetic patterns were shown to be pH independent, saturation curves for aspartate (in the aspartate deamination direction) or fumarate (in the fumarate amiation direction) were obtained at a fixed saturating concentration of the metal ion (and ammonia in the fumarate amiation direction) as a function of pH. The pH was maintained using the following buffers at 100 mM concentrations: Mes, 5.5-6.5; Pipes replaced Mes for determination of the potassium inhibition constant); Hepes, 6.5-8.5; ChEs, 8.5-9.5. Aspartate and ammonia stock solutions were titrated to the desired pH to prevent significant differences from the pH of the buffer used and the final solution pH. The pH was recorded before and after initial velocity data were recorded; no significant differences were detected.

Inhibition data were obtained for succinate and K+, competitive inhibitors versus aspartate. The substrate concentration was varied, while the Mg2+ concentration was maintained saturating. Full inhibition patterns for succinate were obtained at pH 5.5, 8, and 9 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 Ks and the inhibitor concentration was varied from zero over a range that gave inhibition. The measured Ks was then divided by 2 to obtain the true Ks. Full inhibition patterns were obtained for K+ at all pH values except 7, 8.97, 9.15, and 9.34, where Dixon experiments were carried out as above.

**Deuterium washin.** The rate of deuterium incorporation into C-3R of L-aspartate was measured under conditions of turnover by obtaining 1H NMR spectra as a function of time. Spectra were obtained using 2-ml samples in 10 mM Hepes, pH 7.5, at 25°C with 5 mM L-aspartate, 20 mM MgSO4, and ca. 0.01 units/ml aspartase. Control samples were identical, but excluded aspartase. Spectra were measured on a Bruker AM 300 SWB superconducting spectrometer at 300 MHz for 1H, collecting 16K data points with a sweep width of 6024 Hz and a pulse angle of 10°. A total of 12 scans were accumulated per time point, and all spectra were run at 25°C. Data were also collected in the absence of Mg2+. The C-3 protons of aspartate resonate as an octet near 7.27 ppm, while the C-2 proton resonates as a quartet at 3.9 ppm, and the product fumarate resonates at 6.5 ppm. No change in the relative intensities of the quartet or octet was observed in the minus enzyme control, and no appearance of fumarate was observed. Incorporation of 3% deuterium into aspartate could have been detected.

**Data processing.** Reciprocal initial velocities were plotted against reciprocal substrate concentrations and all plots and replots were linear. Data were fitted using the appropriate rate equations and computer programs developed by Cleland (3). Data from Dixon plot analyses were fitted using Eq. (2). Data obtained for substrate saturation curves obtained for the pH dependence of the kinetic parameters were fitted using Eq. (3), while data for pH profiles giving limiting slopes of 1 and -1 were fitted using Eq. (4). Data for competitive inhibition were fitted using Eq. (5).

\[
y = A x + B
\]

\[
\frac{\text{Initial rate}}{[\text{S}] = \frac{Y}{[\text{S}]} = \frac{A}{[\text{S}] + B} = \frac{A}{\text{S} + K_s}
\]

\[
u = \frac{V_{\text{max}}}{[\text{S}] + K_s}
\]

\[
util = \frac{V_{\text{max}}}{K_s}
\]

\[
Y = Y_0 + \frac{V_{\text{max}}}{K_s} \ln \left(1 + \frac{[\text{S}]}{K_s}ight)
\]

In Eq. (2), y is 1/[S] when x is inhibitor concentration, A is the slope, and B is the ordinate intercept. Values for K are obtained by determining the abscissa intercept (B/A) divided by 2. Standard errors were determined using the computer-generated values and Eq. (5) above. In Eqs. (3-5), vi is the initial velocity, V1 is the maximum velocity, A, B and I are reactant and inhibitor concentrations, K, and kb are the Michaelis constants for A and B, Kc is the dissociation constant for A and Kc, and K± are slope and intercept inhibition constants, respectively.

Data for pH profiles giving limiting slopes of 1 were fitted using Eq. (7), while data for pH profiles giving limiting slopes of 1 and -1 were fitted using Eq. (8). Data for V and pH profiles in the presence of Mg2+ where V decreases from a constant value at high pH to a constant value at low pH were fitted to Eq. (9). The acid side of the sink at pH profile including the intermediary plateau was fitted using Eq. (10).

\[
\log y = \log V_{\text{cat}} + \beta \log[\text{S}]
\]
In Eqs. (7) and (8), \( v \) is the observed value of the parameter of interest, e.g., \( V_{IK} \) in the absence of \( Mg^{2+} \) at a given pH. \( C \) is the pH-independent value of \( v \). \( H \) is the hydrogen ion concentration, and \( K_1 \) and \( K_2 \) are acid dissociation constants for enzyme, substrate, or inhibitor functional groups. In Eq. (9), \( Y_e \) and \( Y_w \) are the constant values of \( V \) at low and high pH, respectively, \( y \) is the value of \( Vat \) any pH, \( H \) is the hydrogen ion concentration, and \( K_a \) is the acid dissociation constant of the group responsible for the pH-dependent activation. In Eq. (10), \( y \) is the observed value of \( V_{IK} \) in the absence of \( Mg^{2+} \) at any pH, \( C \) is the pH-independent value of \( y \). \( H \) is the hydrogen ion concentration, and \( K_1 \), \( K_2 \), and \( K_3 \) reflect the three pH-dependent terms for the initial decrease around pH 7.5, leveling off to an intermediary plateau at about pH 7, and the decrease to a limiting slope of 1 at low pH.

RESULTS

Initial velocity studies in the absence of product and dead-end inhibitors. In the direction of aspartate deamination, all time courses were linear for at least 5 min. In the direction of fumarate amination, time courses obtained at varied fumarate concentration 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 \( E. coli \) (9, 10).

Initial velocity patterns obtained by varying the concentration of aspartate at several fixed concentrations of \( Mg^{2+} \), intersect on the ordinate at pH 8, indicating that the kinetic mechanism is equilibrium ordered with \( Mg^{2+} \) binding prior to aspartate as previously suggested by Nuiy et al. (14). Patterns qualitatively identical to that observed at pH 8 were also observed at pH 5.5 and 9.2, indicating that the kinetic mechanism in the direction of aspartate deamination is pH independent. Initial velocity patterns obtained for fumarate amination in the presence of saturating \( Mg^{2+} \) and 1 mM aspartate intersect to the left of the ordinate at pH 5.5, 8, and 9.2, again suggesting that the kinetic mechanism is pH independent.

pH dependence of kinetic parameters in the direction of aspartate deamination. It has been reported by Karsten and Viola (12) that the metal activation of the \( E. coli \) enzyme is dependent on the reaction pH. At pH values below 7, divalent metal ions apparently do not activate, while above this pH divalent metal ion and substrate are essential activators. In view of the similarity in the \( H. alvei \) and \( E. coli \) enzymes with respect to substrate activation, it is important to determine the pH dependence of kinetic parameter plus and minus \( Mg^{2+} \).

Kinetic parameters were obtained in the absence and presence of a saturating noninhibitory concentration of \( Mg^{2+} \) (20 mM). The kinetic parameters obtained under these two conditions are quite different. The maximum velocity in the presence of \( Mg^{2+} \) is constant at pH values above 8 and below 5 (Fig. 1A). In the absence of \( Mg^{2+} \), \( Vma \) decreases at low and high pH with limiting slopes of 1 and -1 (Fig. 1A). The pH-independent values of \( VIE \), in the presence of \( Mg^{2+} \) at high and low pH are 76 ± 4 and 6.1 ± 0.3 s⁻¹, respectively, while that in the absence of \( Mg^{2+} \) is 8.0 ± 0.1 s⁻¹.

The pH dependence of \( V_{IK} \), was likewise determined in the absence and presence of 20 mM \( Mg^{2+} \) (Fig. 1B). In the absence of \( Mg^{2+} \), the \( V_{IK} \) profile decreases to a slope of 1 at low pH with a pK value of 6.3 and a slope of -1 at high pH with a pK of 8.4. An intermediary plateau is observed between pH 6.5 and 7. In the absence of \( Mg^{2+} \), the \( V_{IK} \) profile is also bell-shaped with limiting slopes of 1 and -1, respectively. A pK of 6.6 is observed on the acidic side, identical (within error) to that obtained in the presence of \( Mg^{2+} \), but a lower pK value of 7.2 is obtained on the basic side of the profile. The pH-independent values of \( V_{IK} \), in the absence and presence of \( Mg^{2+} \), are (14 ±...
TABLE I
Summary of pK Values Obtained for the Aspartase-Catalyzed Reaction from *Hafnia alvei*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>20 mM Mg2⁺</th>
<th>Acidic pK</th>
<th>Basic pK</th>
<th>Activation*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7.5 ± 0.2</td>
<td>6.4 ± 0.1</td>
<td>7.4 ± 0.2</td>
<td>7.0 ± 0.2</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Parameter</th>
<th>7.0 ± 0.2</th>
<th>7.4 ± 0.2</th>
<th>7.6 ± 0.1</th>
<th>8.4 ± 0.1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>± 0.1</td>
<td>± 0.1</td>
<td>± 0.1</td>
<td>± 0.1</td>
</tr>
</tbody>
</table>

Aspartate deamination

<table>
<thead>
<tr>
<th>Parameter</th>
<th>6.6 ± 0.2</th>
<th>7.2 ± 0.1</th>
<th>7.6 ± 0.1</th>
<th>8.7 ± 0.3</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>± 0.2</td>
<td>± 0.1</td>
<td>± 0.1</td>
<td>± 0.2</td>
</tr>
</tbody>
</table>

Fumarate amination

<table>
<thead>
<tr>
<th>Parameter</th>
<th>7.2 ± 0.1</th>
<th>9.1 ± 0.2</th>
<th>6.5 ± 0.2</th>
<th>87 ± 0.2</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>± 0.1</td>
<td>± 0.2</td>
<td>± 0.2</td>
<td>± 0.2</td>
</tr>
</tbody>
</table>

*These pK values reflect the pH-dependent activation. The first pK value reflects the initial decrease in activity as the pH is decreased below 8, while the second reflects V leveling off at a lower constant value below pH 6.*

0.1) x 10⁴ and (8 ± 1) x 10⁴ M⁻¹ s⁻¹, respectively. All pK values are summarized in Table I.

**pH dependence of kinetic parameters in the direction of fumarate amination.** The pH dependence of V for amination was determined in the absence and presence of 20 mM Mg²⁺. In the absence of metal, the V profile is bell-shaped, giving a pH-independent value of 9 ± 1 s⁻¹ (Fig. 2A). In the presence of the metal ion, V is pH independent above pH 8 and decreases to a pH-independent value below pH 5 (Fig. 2A). The pH-independent values of V at high and low pH are 39 ± 6 and 3.5 ± 0.6 s⁻¹, respectively.

The pH dependence of V/K⁰a was also measured in the absence and presence of 20 mM Mg²⁺. In both cases, V/K⁰a is only slightly pH dependent. In the absence of Mg²⁺, there is an indication that the parameter decreases below and above the respective pK values, while in the presence of Mg²⁺ only the acidic pK is observed (Fig. 2B). The pH-independent values of V/K⁰a in the presence and absence of Mg²⁺ are (3.6 ± 0.4) x 10⁴ and (2.8 ± 0.4) x 10³ M⁻¹ s⁻¹, respectively.

**pH dependence of the succinate dissociation constant.** The pH dependence of the dissociation constant for succinate, a competitive inhibitor of aspartate, 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/K⁰a pH profile. The pK⁰_ succinate profile (Fig. 3A) decreases at low and high pH with limiting slopes of 1 and -1, respectively. The pK⁰_ potassium profile was also obtained (Fig. 3B) and exhibits a decrease on the acid side. The pH-independent values of K⁰_ succinate and K⁰_ potassium are 11 ± 1 and 120 ± 10 mM, respectively.

**Deuterium washin.** To determine whether the catalytic base is accessible to solvent once it has abstracted...
FIG. 3. pH dependence of \( K_i \) for succinate (A) and potassium (B). Data were obtained at 25°C as discussed under Methods and Materials. Curves are theoretical from fits to Eqs. [8] (A) and [7] (B), while points are experimental values.

the C-3R proton from L-aspartate, the aspartase reaction was carried out in D$_2$O, and the rate of deuterium washin to the C-3R position was monitored by $^1$H NMR. The three regions of importance in the proton NMR spectrum of the aspartate deamination reaction mixture are shown in Fig. 4. The regions of interest are expanded to facilitate visualization. L-Aspartate exhibits an ABX pattern since C-3R and C-3S are magnetically nonequivalent. The resonances near 2.7 ppm reflect the C-3 protons. As stated above, the protons are nonequivalent and as a result C-3R splits C-3S into a doublet and this doublet is further split by C-2 into a quartet. The C-3S and C-2 protons also split C-3R into a quartet, and the C-3R and C-3S quartets produce the observed octet. The resonances centered at 3.9 ppm reflect the C-2 proton split by C-3R and C-3S, giving a quartet, while the resonance near 6.5 ppm reflects the two equivalent protons of fumarate. Thus, the relative intensities of the fumarate to aspartate protons can be used as a monitor of the reaction progress. The spectrum shown in Fig. 4A was taken at 15 min after initiation of the reaction by the addition of aspartase, while that shown in Fig. 4B was taken 435 min after initiation of the reaction; the reaction is more than 50% complete. In addition to the spectra shown, spectra were collected at 0, 30, 45, 60, 90, 120, and 180 min. In no case is there evidence of exchange into the C-3R position. The lack of exchange can most easily be seen from the lack of change in the intensities of the resonances at C-2 relative to one another and to those at C-3. Data collected in the absence of Mg$^{2+}$ are identical (not shown).

**DISCUSSION**

Interpretation of the $V/K_{asp}$ and $V/K_{fum}$ pH profiles. 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 $V/K_{asp}$ pH profiles reflect acid-dissociable functional groups in free E in the absence of Mg$^{2+}$ and in free E:Mg in its presence. In the direction of ammonia addition to fumarate, $V/K_{fum}$ 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.

In the absence of Mg$^{2+}$, a bell-shaped pH profile is obtained for $V/K_{fum}$. Both of the observed pK values must reflect enzyme groups since aspartate exhibits no pKs in the pH range 5-9.5. The presence of Mg$^{2+}$ causes an increase in the $V/K$ from pH 6.5 to about 8 and an apparent perturbation in the value of the basic pK from 7.2 to 8.4. No significant change in the value of the acidic pK is observed. A qualitatively similar behavior is observed in the direction of addition of ammonia to fumarate. The $V/K_{fum}$ is bell-shaped in the absence of Mg$^{2+}$, giving pKs on both the acid and basic sides. In the presence of Mg$^{2+}$ the acidic pK is unchanged, while the basic pK is apparently perturbed to a value of >9.5. Finally, the pH dependence of the $V/I$
K for ammonia has been reported previously over the pH range 6-9.5 (14) at saturating concentrations of fumarate and Mg$_2^+$. The $V/K$ decreases at high pH with a pK of 9.3, consistent with it being the pK for ammonia.

The bell-shaped nature of the pH rate profiles obtained in the absence of Mg$_2^+$, for aspartate and fumarate reflecting the forward and reverse reactions, suggests that the two enzyme groups responsible for the observed pKs function in catalysis. Thus, the two groups would have opposite protonation states in the two reaction directions. These groups are also required in their correct protonation state for the binding of sucinate since its dissociation constant increases above and below the same pKs observed in the $V/K_{asp}$ pH profile. The presence of Mg$_2^+$ causes a pH-dependent activation of aspartase superimposed on the bell-shaped $V/K$ pH profile. The active enzyme becomes less active below a pK of about 7.5 as observed (uide infra) in the $V$, $V/K_{asp}$, and $V/K_{fum}$ pH profiles. In addition, potassium binding is only sensitive to the protonation state of the 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 ammonium ion.

**Deuterium washin.** No washin of deuterium from solvent to aspartate is observed as the reaction progresses to more than 50% completion in either the absence or presence of Mg$_2^+$. A reaction mechanism in which a proton is eliminated from the C-3R position of aspartate prior to the elimination of ammonia has been proposed for aspartase (14, 15). The slow step along the reaction pathway is the elimination of ammonia as shown by the observed $^15$N isotope effect of 24% (1, 14). Thus, if the active site were accessible to solvent during the catalytic cycle, washin of deuterium to aspartate would be expected since the kinetic mechanism is rapid equilibrium (14). A lack of washin of deuterium suggests that the site closes for the catalytic cycle and is not accessible to solvent. The latter has also been shown for fumarase (2) which catalyzes a very similar reaction.

**Interpretation of Vntuprofiles.** The $V$ profiles exhibit similar behavior in both reaction directions. In the direction of the elimination of ammonia from aspartate in the absence of Mg$_2^+$ a bell-shaped pH profile is observed with pK values very similar to those observed in the $V/K_{fum}$ profile in the absence of Mg$_2^+$. The two groups likely function in catalysis as discussed above for $V/K_{asp}$. 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 7.5 is observed that is required unprotonated for optimal activity. The group with a pK of 7.5 is responsible for a pH-dependent activation as it becomes unprotonated. The activation is also observed in the $V/K_{asp}$ profile as an increase in the $V/K$ between pH 6.5 and 8.

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 $V/K_{fum}$ profile (minus divalent metal) is observed in the $V$ profiles in forward and reverse reactions in the presence of divalent metal. Thus, the two catalytic groups both interact with aspartate which is unlikely given the proposed mechanism (uide infra), are environmentally perturbed when aspartate and Mg$_2^+$ are bound, or a combination of both. Consider first the group that must be unprotonated in the direction of aspartate deamination. Causing the environment surrounding the group to become more hydrophobic would be consistent with the deuterium washin experiments which suggest that the active site is closed and not exposed to solvent under these conditions.

The group that must be protonated for activity in the direction of aspartate deamination is also not observed in the $V$ profile measured in the presence of Mg$_2^+$. The pK values obtained from the $V$ and $V/K_{asp}$ pH profiles in the absence of Mg$_2^+$ are the same within error, while in the presence of metal ion the $V/K_{asp}$ pKs are not observed in the $V$ pH profile. Thus, there is apparently a distinctly different conformation of aspartase in the absence and presence of Mg$_2^+$. The latter would be consistent with the suggestion based on the NMR relaxation studies of Falzone et al. (7) that Mn$_2^{2+}$ binds at an allosteric site. The binding of the divalent metal ion to the allosteric site might be expected to produce a different conformation at the active site. The group with a pK observed on the basic side of the $V/K_{asp}$ profiles may interact with aspartate and have its pK perturbed (uide infra). Thus, the perturbation of the basic pK observed in the presence of Mg$_2^+$ is likely a combination of environmental effects and of direct interaction with aspartate.

As a result, in the absence of Mg$_2^+$, aspartase will bind reactant(s) with apparent equal affinity whether or not the enzyme functional groups reflected in the $V$ and $V/K$ profiles are in their correct protonation state. In the presence of Mg$_2^+$, reactants apparently bind only to the correctly protonated form of aspartase.

In the direction of fumarate amination, data are qualitatively similar to those obtained in the aspartate deamination reaction direction. In the absence of Mg$_2^+$, the $V$ decreases at low and high pH as does $V/K_{fum}$. Additions of Mg$_2^+$ causes the basic pK to increase. There are quantitative differences 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.

Taking into account the above data, the mechanism...
in Scheme 1 best describes the pH dependence of kinetic parameters. In Scheme 1 $E_H$ is the least active form of the enzyme that predominates in the absence of Mg$^{2+}$, while $E^*$ is the most active form which predominates in the presence of Mg$^{2+}$ at high pH, and $E_H$:Mg is an intermediate form that predominates at low pH in the presence of Mg$^{2+}$. $H_x$ and $H_y$ represent the protons on the general base and acid, respectively, when they are protonated. The symbols $A$, $P$, $Q$, and Mg represent aspartate, NH$_t$, fumarate, and Mg$^{2+}$, respectively. The constants $k_r$ and $k$, represent the macroscopic rate constants for the catalytic pathway, where the superscripts Mg and * reflect the low pH and high pH Mg-activated forms, respectively.

**Acid-base mechanism.** A mechanism in which a stabilized carbanion intermediate is formed has been postulated by Nuiry *et al.* (14) based on isotope effect studies and the observation by Porter and Bright (15) of the high affinity of aspartase for the dianionic form of 3-nitro-L-alanine. A mechanism taking into account the carbanionic intermediate and the above pH studies is shown in Scheme 2. Aspartate binds to enzyme as the monoanion with both carboxyl groups and the $\alpha$-amine ionized.\(^5\) 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$^{2+}$, all of the $VIK$ profiles in the presence and absence of metal ion, and the $pK$ succinate profile. The resulting carbanion is stabilized by delocalization of electrons into the $\beta$-carboxyl presumably with the assistance of a one or more positively charged enzyme residues in the vicinity of the $\beta$-carboxyl. Nuiry *et al.* (14) suggested that Mg$^{2+}$ played the role of the Lewis acid coordinated to the $\beta$-carboxyl of aspartate, but Mg$^{2+}$ is not absolutely required for the reaction and the NMR studies of Falzone *et al.* (7) indicate that this is unlikely. Thus, an enzyme residue must be responsible for the electrostatic role, but this enzyme group must have a $pK$ higher than 9, since no group that can be ascribed to this function is observed in the $p$H rate profiles. Ammonia is then expelled with the assistance of a general acid group giving NH$_t$ as the product. The general acid is not predicted to protonate the leaving NH$_3$ in the transition state for elimination of ammonia, but rather to trap the expelled NH$_3$ by protonating it once it has been eliminated. The $pK$ for the general acid is about 7.2-7.6 in the absence of Mg$^{2+}$, but is increased to a value of 7.6-8.4 in the aspartate deamination direction in the presence of Mg$^2+$ and even higher in the opposite direction. The general acid group is again observed in all of the same $p$H rate profiles specified above, for the general base, as well as the $pK$ succinate profile. Since the same $pK$ values are observed in the $pK$ succinate and $VIK$ $p$H profile, both enzyme groups must be in their optimal protonation state for efficient binding of reactant in the presence of Mg$^{2+}$.\(^5\)

\(^5\) A $pK$ reflecting the $\alpha$-amine of aspartate is not observed in the $VIK$, ... $p$H rate profile since data were not collected to $p$H 10, the $pK$ value (4). Similar results were presented for the *E. coli* enzyme (12).

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**SCHEME 1.** Kinetic mechanism of aspartase as a function of pH.

**SCHEME 2.** Proposed acid-base chemical mechanism of aspartase.
The general acid group is likely a neutral acid, since it would be repulsive to the protonated a-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 \( H. alvei \) 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 (2). In this regard, Bacillus subtilis aspartase and fumarase exhibit 37% sequence identity (17) as has also been found for the \( E. coli \) aspartase and class II fumarase. 

Similar perturbation of the basic \( pK \) is observed for the \( E. coli \) aspartase (12). These authors interpreted the data based on a pH-dependent conformational activation triggered by Mg\(^{2+} \); that is, enzyme with the protonated a-amine of aspartate was bound. The \( V/K_{\text{act}} \) profile for the \( E. coli \) enzyme decreases at high pH and reflects the \( pK \) of ammonia, while that for the \( H. alvei \) enzyme decreases at high pH and reflects the \( pK \) of ammonia. In addition, the \( V/K_{\text{act}} \) profile for the \( E. coli \) enzyme decreases at high pH with a limiting slope of -2 and reflects the \( pK \)s of two enzyme groups, while that for the \( H. alvei \) enzyme is bell shaped and reflects the same \( pK \)s as those observed in the \( V/K_{\text{act}} \) profile. Thus, although the enzymes are very similar in their behavior, the differences discussed above suggest real differences in the acid-base chemical mechanisms of the two enzymes, particularly with respect to the protonation state of ammonia (ammonium ion) as a reactant.

**Comparison to the \( E. coli \) aspartase.** The pH dependence of kinetic parameters in the direction of elimination of ammonium ion from aspartate is qualitatively very similar for the \( H. alvei \) (this study) and \( E. coli \) (12) enzymes. The \( V/K_{\text{act}} \) profiles for both enzymes exhibit an increase in rate in the presence of Mg\(^{2+} \) at high pH. However, very little metal-dependent increase in activity is observed at low pH for the \( E. coli \) enzyme, while significant activation is observed over the entire pH range for the \( H. alvei \) enzyme. As a result, a difference pH profile is informative in the case of the former, but not in the latter. The \( V \) profiles are also quite similar, with the exception that a \( pK \) of 9.5 is observed in the presence of saturating Mg\(^{2+} \) for the \( E. coli \) enzyme, and data were not collected to high enough pH to define such a \( pK \) for the \( H. alvei \) enzyme. The biggest differences between the two enzymes appear to be in the fumarate amination direction. The \( V \) profiles are similar in that more activation is observed in the fumarate amination direction than in the opposite reaction direction, but almost three orders of magnitude activation is observed for the \( E. coli \) enzyme, compared to a little over an order of magnitude in the case of the \( H. alvei \) enzyme. Even more striking are the differences in the \( V/K_{\text{act}} \) profiles for ammonia and fumarate. The \( V/K_{\text{ammonia}} \) profile for the \( E. coli \) enzyme decreases at low pH and reflects the \( pK \) of ammonia, while that for the \( H. alvei \) enzyme decreases at high pH and reflects the \( pK \) of ammonia. In addition, the \( V/K_{\text{fumarate}} \) profile for the \( E. coli \) enzyme decreases at high pH with a limiting slope of -2 and reflects the \( pK \)s of two enzyme groups, while that for the \( H. alvei \) enzyme is bell shaped and reflects the same \( pK \)s as those observed in the \( V/K_{\text{fumarate}} \) profile. 

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