Articles

Kinetic Studies of Aspartase from *Hafnia alvei* by Temperature Dependence Activity Changes

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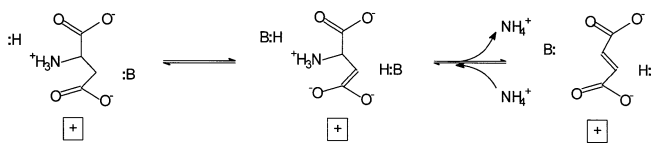
The temperature dependence of the kinetic parameters of the aspartase-catalyzed reaction has been examined in the direction of deamination. The pK_1 values at 37 °C, 25 °C, 16 °C and 7 °C were 6.2 ± 0.1, 6.3 ± 0.1, 6.7 ± 0.3 and 6.9 ± 0.3, respectively. On the other hand, the pK_2 values at 37 °C, 25 °C, 16 °C and 7 °C were 8.1 ± 0.2, 8.3 ± 0.2, 8.2 ± 0.3 and 8.0 ± 0.2, respectively. The enthalpy of ionization, ΔH_{ion}, calculated from the slope of pK_1, are 6.0 ± 0.3 kcal/mol. These results validate the prediction that aspartase requires a histidine residue for a general base, and a cysteine (or having a carboxyl functional group) for a general acid.

Introduction

L-aspartate ammonia-lyase [EC 4.3.1.1] (aspartase) catalyzes the reversible deamination of L-aspartate to fumarate and ammonia.[1] The enzyme is specific for aspartate and fumarate, but NH_2OH can be substituted for ammonia as a substrate.[2] Aspartase has been regarded as a catabolic enzyme in both bacteria and plants, but the reaction is reversible and favors aspartate formation with $\Delta G^o = 3.2$ kcal/mol for fumarate amination. The equilibrium constant for the aspartase reaction, measured directly at 25 °C was 5×10^{-3} M.[3] A variety of divalent metal ions, such as Mg^{2+}, Mn^{2+}, Zn^{2+} and Co^{2+} activate the reaction.[4,10] Initial velocity studies obtained for the enzyme from *Hafnia alvei* are consistent with a rapid equilibrium kinetic mechanism in which Mg^{2+} binds prior to aspartate, but with a random release of Mg^{2+}, NH_4^+, or fumarate.[5]

Yoon *et al*. has proposed an acid-base chemical mechanism for *Hafnia alvei* aspartase using pH studies and deuterium wash-in (Scheme 1).[6] Data are consistent with a proton which is abstracted from C-3 of the monoanionic form of aspartate by an enzyme general base with a pK of 6.3-6.6 in the absence and presence of Mg^{2+}. The resulting carbanion is presumably stabilized by delocalization of electrons into the β-carboxyl with the assistance of a protonated enzyme group in the vicinity of the β-carboxyl. Ammonia is then expelled with the assistance of a general acid group that traps an initially expelled NH_3 as the final NH_4^+ product. The pK for the general acid is about 7 in the absence of Mg^{2+}, but is increased by about a pH unit in the presence of Mg^{2+}. Since



Scheme 1

the same pK values are observed in the $pK_{i\ succinate}$ and V/K pH profile, both enzyme groups must be in their optimum protonation state for efficient binding of reactant in the presence of Mg^{2+}. At the end of a catalytic cycle, both the general base and general acid groups are in a protonation state opposite that in which they started when aspartate was bound. When the aspartase reaction is run in D_2O to greater than 50% completion no deuterium is found in the remaining aspartate, indicating that the site is inaccessible to solvent during the catalytic cycle.

A study using an organic solvent perturbation method has shown that a possible candidate for the acidic residue in the active site is a histidine, and another possible candiate for the basic residue in the active site is a cysteine.[8] A chemical modification study using N-ethylmaleimide and 5,5'-dithiobis-(2-nitrobenzoic acid) which is specific to cysteine has confirmed the requirement of a cysteine residue for catalytic activity.[11] In an attempt to determine whether additional residues are necessary, I carry out a kinetic study using a temperature dependence.

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 (HEPES), 2-(N-morpholino)ethanesulfonic acid (MES), 2-(N-cyclohexylamino)ethanesulfonic acid (CHES), 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.[10] 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 disappearance of fumarate following the decrease in absorbance at 240 nm (ε_{240}=2,255 M^{-1} 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. Assay for temperature dependence was performed at 7 °C, 16 °C, 25 °C and 37 °C. One unit of enzyme activity was defined as the amount of 1 μmol of fumarate consumed per minute under the standard assay conditions.

pH studies. Determination of V and V/K values for aspartate were carried out by varying the levels of aspartate at saturating concentrations of Mg^{2+}. All assays reflected the initial velocity conditions with less than 10% of the limiting reactant used over the time course of the reaction. Buffers used at 100 mM concentration were MES at pH 5.5-6.5, HEPES at pH 6.5-8.5, CHES at pH 8.5-9.5. All buffers were titrated to the appropriated pH level with KOH. The pH was measured before and after the reactions. Several of the assays were repeated at a given pH using different buffers to eliminate the possibility of activation by the buffers. At the pH extremes the concentration of Mg^{2+} was doubled in separate assays to be sure that they were still saturated. No significant rate change was detected.

Data analysis. All data were fitted by the computer program of Cleland, converted to BASIC, and adapted for use on a microcomputer, which assume equal variance for the velocities of the logarithm of the fitted parameter. Data was analyzed according to the appropriate rate equation by using the Fortran programs of Cleland (1979).[20] Reciprocal plots at each pH with the fixed substrate saturating were fit to Eq. (1), where v is the experimentally determined velocity, V is the maximum velocity, A is the variable substrate concentration, and K is the Michaelis constant. All data to determine pK values were fit to the approciate equation listed below where y is equal to V_{max} or V/K. These data are fitted by assuming that the variance of the velocities is proportional to the square of the velocity.[20] Eq. (2) was used to fit pH profiles in which activity decreases were observed in both the acidic and the basic regions, and Eq. (3) for pH profiles in which activity decreases were observed in wave form.

$$v = VA/ K + A \quad (1)$$

$$\log y = \log\{C/(1 + [H^+]/K_1 + K_2/[H^+])\} \quad (2)$$

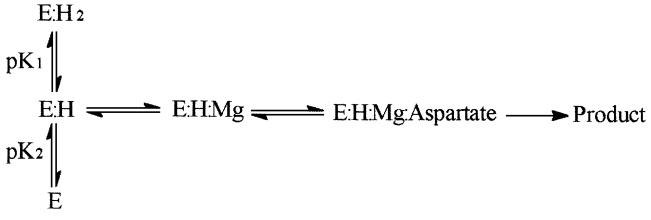
$$\log y = \log\{(Y_L + Y_H[H^+/K_3])/(1 + [H^+]/K_3)\} \quad (3)$$

Results and Discussion

Efforts to identify the amino acid or other residues involved in the catalytic function of an enzyme on the basis of pK values of ionizing groups derived from kinetic measurements are valid, and no conformational changes occur in the enzyme under the conditions of the experiment. If such changes occur, then it can be argued that an ionization, which affects V_{max} or K_m or both, does so by changing the conformation of the enzyme and does not involve any residue that directly participates in the catalytic process. No such conformational changes have been shown to occur in the case of aspartase in the pH range 5.0 to 9.5.[8] Thus, absorption spectra of the enzyme in the 225 nm to 330 nm range under these conditions (data not shown) have been found to be identical and superimposable, indicating a lack of alteration either in the environment of aromatic residue or in the peptide backbone structure of the enzyme. An estimate of the variation of the kinetic mechanism over the pH range to be studied is required in order to ensure that the enzyme complex is being titrated. Initial velocity patterns were obtained at pH 5.0 and pH 9.0 by varying aspartate at a fixed level of Mg^{2+}. These results are in agreement with the kinetic mechanism data (rapid equilibrium ordered addition of Mg^{2+} prior to aspartate) of Nuiry *et al*.[5] Absence of disturbance in the environment of aromatic chromophores in solvent-water and the kinetic mechanism detailed above, indicates the direction of shifts in pK values by temperature at the active site and no denaturation by temperature.

One method for identifying the functional group of residue at the active site of an enzyme is based on the temperature dependent method of pK values.[7] The method of temperature dependence of pK values depends on the different enthalpy of ionization (ΔH_{ion}) values of a functional group of the residues involved in catalysis and /or binding, and as long as the ionizations are not accompanied by conformational changes in the enzyme which have large ΔH_{ion} values that are often 15-25 kcal/mol. The carboxyl group shows almost no temperature dependence. On the other hand, the imidazole group shows 6-7.5 Kcal/mol.[7]

Figure 1A shows the log V_{max}/K_m versus pH plots for the enzyme in 37 °C (top curve), in 25 °C (second curve), in 16 °C (third curve), and in 7 °C (bottom curve). The log V_{max}/K_m plots implies the effects of temperature on the pK values of the ionizing groups on the free enzyme. The pH dependent hydrolyses over the entire temperature range were shown by a kinetic scheme requiring minimally two critical ionization of the free enzyme (EH_2 and E). Consider the following mechanism:



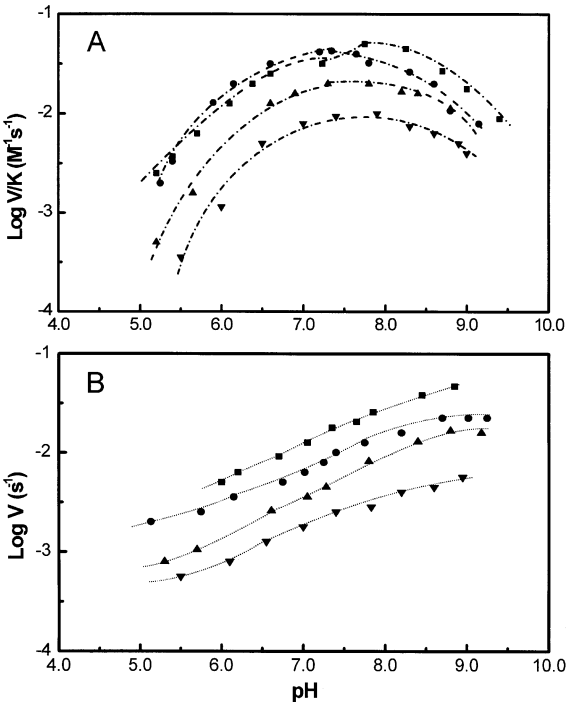


Figure 1. pH dependence of the kinetic parameters for Aspartase on the Temperature effect. (A) pH dependence of V_{max}/K_m. (■) Kinetic parameters at 37 °C. (●) Kinetic parameters at 25 °C. (▲) Kinetic parameters at 16 °C. (▼) Kinetic parameters at 7 °C. The data were fitted to Eq (2). (B) pH dependence of V_{max}. (■) Kinetic parameter at 37 °C. (●) Kinetic parameters at 25 °C. (▲) Kinetic parameters at 16 °C. (▼) Kinetic parameters at 7 °C. The data were fitted to Eq (3). The points shown are experimentally determined values.

The pK_1 values in the acidic side were changed in proportion to the decrease of temperature. The pK_1 values at 37 °C, 25 °C, 16 °C and 7 °C were 6.2 ± 0.1, 6.3 ± 0.1, 6.7 ± 0.3 and 6.9 ± 0.3, respectively in Table 1. On the other hand, the pK_2 values in the basic side were not changed in proportion to the decrease of temperature within error. pK_2 values in 37 °C, 25 °C, 16 °C and 7 °C were 8.1 ± 0.2, 8.4 ± 0.1, 8.2 ± 0.1 and 8.0 ± 0.2, respectively in Table 1. The V/K profile decreases on either acidic or 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 activity. Figure 1B shows the log V_{max} versus pH plots for the enzyme at 37 °C (top curve), at 25 °C (second curve), at 16 °C (third curve), and at 7 °C (bottom curve). The log V_{max} is pH independent above pH 8.5 and decreases to become constant below pH 5.5. The log V_{max} plots implies the effect of these temperatures on the pK values of the ionizing groups on the enzyme-substrate complex responsible complex responsible for its catalytic activity. Two groups were observed in V_{max}/K_m profile (*vide ante*). On the other hand, neither of the two groups is observed in V profiles. These results indicate that the two catalytic groups are environmentally perturbed when aspartate and Mg^{+2} are bound, or a combination of both.[6]

The enthalpy of ionization was obtained from the Van't Hoff equation, $(dlnk)/dT = \Delta H_{ion}/RT^2$. The value of ΔH_{ion} is determined from a plot of pK versus reciprocal absolute temperature, which has a slope of $\Delta H_{ion}/2.303R$. Figure 2 shows pK versus 1/T plots that are from Table 1. These pK values vary linearly with the reciprocal of absolute temperature. The enthalpy of ionization, ΔH_{ion}, calculated from the slope of pK_1 and pK_2, are 6.0 ± 0.3 Kcal/mol and 0.0 ± 0.3 Kcal/mol, respectively. The value for pK_1 closely resembles that of an imidazole group of free histidine (ΔH_{ion}=6.0 Kcal/mol). The enthalpy of the ionization which control a general acid (pK_2) is similar to it for caboxyl residue of free amino acid (ΔH_{ion}=0.0 Kcal/mol).

Table 1. Summary of pK values obtained from the pH dependence of V_{max}/K_m

	V_{max}/K_m	
Temp (°C)	pK_1 ± SE	pK_2 ±SE
37	6.2 ± 0.1	8.1 ± 0.2
25	6.3 ± 0.1	8.3 ± 0.2
16	6.7 ± 0.3	8.2 ± 0.3
7	6.9 ± 0.3	8.0 ± 0.2

pK_1 indicates that the group must be protonated for enzyme activity and pK_2 indicated that the group must be deprotonated.

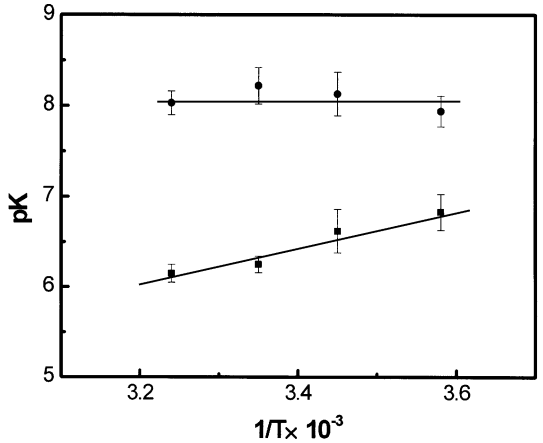


Figure 2. Temperature data from pH profiles for aspartase. (●) pKs of the general acid enzyme group. (■) pKs of the general base enzyme group. Heats of ionization are obtained from Van't Hoff equation, $(dlnk)/dT=\Delta H_{ion}/(RT^2)$.

A chemical modification study using diethylpyrocarbonate which is specific to histidine has speculated about participation in an essential step of the catalytic reaction.[12] Another chemical modification using N-ethylmaleimide and 5,5'-Dithiobis-(2-nitrobenzoic acid) which are specific to cysteine and pH profile studies has speculated about the requirement of a cysteine residue for catalytic activity.[11,13,14] However, site-directed mutagenesis of a highly conserved cysteine, which is present throughout the fumarase-aspartase family, did not confirm the essentiality of this functional group.[15,16] Peptide mapping has identified a cysteinyl and a lysyl residue of *E. coli* aspartase that have been modified by a mechanism based inactivator.[17] A study using an organic solvent perturbation method has recently shown that a possible candidate for the acidic residue of pK_1 in the active site

of aspartase from *Hafnia alvei* is the cationic type, and another possible candidate for the basic residue of pK_2 in the active site is the neutral type.[8] Chemical modification and sequence homology studies have suggested the potential involvement of a number of residues in the activity of aspartase. However, in most of these cases, replacement of these amino acids by site-directed mutagenesis has either eliminated them from consideration or relegated them to an indirect role in binding or catalysis in aspartase.

In summary, the current work supports the previously suggested location of the active site.[6,11,12,18,19] The deamination of aspartate is proposed to involve acid-base catalysis by *Hafnia alvei* aspartase, with base catalyzed removal of the pro-R proton on carbon 3 followed by protonation and expulsion of ammonium ion (Scheme 1). Among the preliminary candidates for the role of the general base catalyst that is responsible for removal of the proton at carbon 3 of the substrate, the most likely candidate enzyme group is a histidine residue. The possible candidate for the role of the general acid catalyst that is responsible for protonation and expulsion of ammonium ion is a cysteine or an amino acid having a carboxyl functional group. In regard to the general acid, the final conclusion cannot be drawn on the basis of the presently available data.

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