Articles

Activation Changes of *Hafnia alvei* Aspartase by Acetic Anhydride

Im-Joung La, Joungmok Kim, Jeong-Rim Kim,[†]
Ki-Tae Kim,[‡] Jung-Sung Kim,[§] and Moon-Young Yoon[*]

Department of Chemistry, [†]The School of Graduate, Hanyang Univesity, Seoul 133-791, Korea
[‡]Institute of Life Science, Seoulin Scientific Co. Ltd., Seoul 134-030, Korea
[§]Department of Chemical Education, Taegu University, Kyungsan 713-714, Korea
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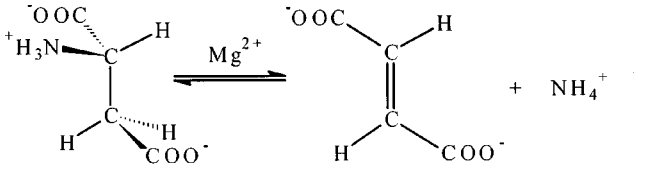
The *Hafnia alvei* aspartase activity with acetic anhydride treatment gradually increased and reached 7.5-fold that of the native one. The activity of the acetylated aspartase was a little higher than that of the native enzyme, indicating that the cooperativity between a substrate and enzyme is increased. The optimum temperature of the native asparatse was 45 °C, and that of the acetylated enzyme shifted to 40 °C. The pH vs. the activity profile of the acetylated asparatse was also different from that of the native enzyme. The initial velocity pattern of the acetylated aspartase intersects to the left of the ordinate, indicating the sequential kinetic mechanism other than a rapid equilibrium ordered one. The reciprocal plots for aspartate of the native aspartase were curved, but those of the acetylated aspartase were linear, indicating the Michaelis-Menten kinetics. The helical content of the acetylated aspartase was rather decreased to 9% than that (63%) of the native one.

Key words : Aspartase, *Hafnia alvei*, Chemical modification

Introduction

Aspartase (L-aspartate ammonia-lyase, EC4.3.1.1) catalyzes the reversible conversion of L-aspasteate to fumarate and an ammonium ion in the following reversible reaction.[1]



$\varepsilon_{240} = 2{,}255 M^{-1}cm^{-1}$

The aspartase is a tetramer composed of four apparently identical subunits of molecular weight 48,000. The enzyme was observed to have an absolute requirement for a divalent metal ion activator at higher pH, with some indication that aspartase may possess some activity in the absence of the divalent metal ion at low pH.[2] Nuiry, *et al.* (1984) carried out a complete kinetic mechanism study, which included a divalent metal ion as a pseudo – reactant utilizing an initial velocity study, and the primary and secondary kinetic isotope effects for aspartase.[3] Initial velocity data for *Hafnia alvei* aspartase are consistent with a rapid equilibrium ordered kinetic mechanism, caused by the ordered addition of Mg^{2+} prior to aspartate, followed by the completely random release of Mg^{2+}, NH^{4+} or fumarate.

[*]To whom correspondence should be addressed. Tel: +82-2-2290-0946; Fax: +82-2-2299-0762; E-mail: myyoon@hanyang.ac.kr

Acetylation on *E. coli* aspartase with acetic anhydride and N-hydroxysuccinimide esters resulted in the alteration of its catalytic and regulatory properties.[4] The two-fold activation at pH 7.0 was observed for 30 min, whereas the activity of the acetylated enzyme at pH 8.5 was lower than that of the native enzyme throughout the range of substrate concentrations tested, while maintaining the V_{max} unchanged. The requirement of divalent metal ion for the enzyme activity increased at both pH 7.0 and 8.5. In the metal-activity relationship before and after acetylation, the ratio of activity in the presence of Mg^{2+} to that in its absence increased upon acetylation from 1.4 to 2.1 at pH 7.0 and from 4.8 to 84.5 at pH 8.5. The acetylated enzyme at pH 8.5 has very little activity in the absence of Mg^{2+}. Acetylation of the enzyme with other acid anhydrides such as *n*-butyric and propionic anhydrides also increased the activity at pH 7.0, albeit to a lesser extent.

As industrial use of aspartase is increasing, some researchers have focused on the structure-function relationship for the activation of asparatse.[5-11] In an effort to elucidate the nature of a more structure-function relationship of the acetylation in the aspartase reaction, we have tried to investigate the activation with limited treatment of the acetic anhydride on aspartase from *Hafnia alvei*. In this paper, the effects of acetylation on the structure and the function properties of *Hafnia alvei* aspartase, as well as some properties of the acetic anhydride-activated aspartase are described.

Material and Methods

Chemicals. *Hafnia alvei* (ATCC 9760) was purchased

from ATCC (American Type Culture Collection, Rockville. USA). 2-Hydroxyethylmercaptan (β-Mercaptoethanol), Potassium phosphate, Tris (hydroxymethyl) aminomethane, L-Aspartic acid and acetic anhydride were purchased from Sigma Chemical Co. (St. Louis, USA). Magnesium chloride was obtained from Yakuri Chemical Co. (Osaka, Japan). All other chemicals were obtained from commercially available sources and were pure or extra-pure for the available analytical grade.

Enzyme Preparations. Aspartase was purified from *Hafnia alvei* as described by Yoon, *et al*. (1998).[12] Briefly, aspartase was obtained from a combination of DEAE-cellulose, Red A-agarose, and Sepharose 6B chromatography. The purified enzyme was divided into aliquots and stored at -70 °C until used. The activity of the enzyme remained stable for at least one month at 4 °C without appreciable loss of enzymatic activity. Protein concentration was determined by Bradfords method. The enzyme preparations used in this investigation were homogeneous as judged by ultracentrifugation and polyacrylamide gel electrophoresis.

Enzyme Assays. The activity of aspartase was determined using a Hewlett-Packard 8452 Diode-Array spectrophotometer equipped with the constant-temperature (25 °C) cell housing. All reactions were carried out in 1 ml cuvettes with a 1 cm light path length, which were incubated for at least 10 minutes in the cell compartment prior to the initiation of a reaction by the addition of aspartase. The progress of the reactions was determined spectrophotometrically and monitored continuously by measuring the formation of fumarate by following the increase in absorbance at 240 nm (ε_{240} = 2,255 $M^{-1}cm^{-1}$) at 40 °C. The standard assay mixture contained, in a total volume of 1.0 mL cuvette, 100 mM Tris-HCl buffer, 10 mM aspartate and 4 mM Mg^{2+}, pH 7.85 and was incubated for 10 minutes in a compartment. The aspartate and Mg^{2+} concentrations were corrected for complexation with a divalent metal using the following dissociation constant obtained at 0.1 mM ionic strength: Mg-aspartate, 4 mM.[10] All other chemicals were also corrected for the metal-chelating effect. The reactions were started by adding appropriate volumes of the acetylated enzyme solution. The increase of absorbance at 240 nm that corresponded to fumarate formation was measured. The pH was recorded before and after the initial velocity data were recorded.

Acetylation Treatment. Aspartase was incubated with acetic anhydride at 0 °C. In 1.5 mL cuvette, 100 mM phosphate buffer, 5 mM β-mercaptoethanol, and various concentrations of acetic anhydride were prepared in a compartment. The reaction mixture contained 17.3 μg of aspartase and incubated for a designated time at pH 7.3.

Initial Velocity Study. The conditions of the initial velocity patterns for *Hafnia alvei* aspartase in the direction of the aspartate deamination reaction at pH 7.8 were the same as those of the enzyme assay condition, described previously, with various concentrations of aspartate and Mg^{2+}. The various concentrations of substrates around K_m were corrected for the Mg-aspartate complex. The kinetic parameters for the enzymes were obtained using Cleland's Method of Net Rate constants (1979).[14]

Circular Dichroism. Circular dichroism (CD) spectra were obtained on a Jovin-Yvan CD6 CD-ORD spectropolarimeter. Samples were contained in a quartz cuvette able to hold a volume of as much as 50 μL. All far-UV spectra were scanned from 250 to 190 nm with a protein concentration of 0.5 mg/mL at intervals of 1 nm. The sample buffer was 10 mM phosphate buffer, pH 7.3. Each spectrum was composed of an average of three scans. The sample spectra were corrected by subtracting the appropriate buffer blanks. The unit of the molecular ellipsoid, [θ], is degree cm^2 per dmol. Values in degree were converted to rad, in order to compare then with the poly-L-lysine reference data in yielding the percentage of α-helix, β-sheet and random coil.

Other Determinations. In order to determine whether the optimum pH and temperature had been changed by the limited acetic anhydride treatment, pH and temperature vs. the activity profiles were compared before and after the acetic anhydride treatment. The pH and temperature dependence of the activity of acetylated aspartase were carried out according to the method of the enzyme assay as described previously (*vide ante*). The pH was maintained using the following buffers at 100 mM concentrations: Hepes, 6.5-8.5; Ches, 8.5-9.0.

Data Processing. Reciprocal values of the initial velocities were plotted as a function of the reciprocal of the substrate concentrations. All data were fitted using the computer program designed by Cleland (1979), converted to BASIC, and adapted for use on a microcomputer.[14] The modified enzyme plots were linear, however, the native one showing the curvature which has positive cooperativity. The initial velocity patterns were obtained by a least square method to the double reciprocal transformation, and data from the Dixon plot analysis were fitted using, Eq. (1).

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

Data for the initial velocity patterns in the direction of aspartate deamination were fitted line by line to Eq. (2).

$$v = VA^2/(a + 2bA + A^2) \quad (2)$$

In Eqs. (1) and (2), A is the reactant concentrations, V is the maximum velocity, and K is the Michaelis constant for the varied substrate.

Results and Discussion

Effect of Acetic Anhydride Treatment on Aspartase Activity. When aspartase was incubated with acetic anhydride at pH 7.3 and 0 °C, the enzyme activity increased to the maximum and decreased gradually after transient, and reached rapidly to complete inactivation (data not shown). With 10 mM acetic anhydride, the activity of acetylated aspartase gradually increased and reached 7.5-fold that of the native aspartase after 10 min of incubation time (Figure 1). When the molar ratio of the acetic anhydride to amino groups of the enzyme was 10^4, the degree of activation was the highest under the experimental condition. The required time to reach the

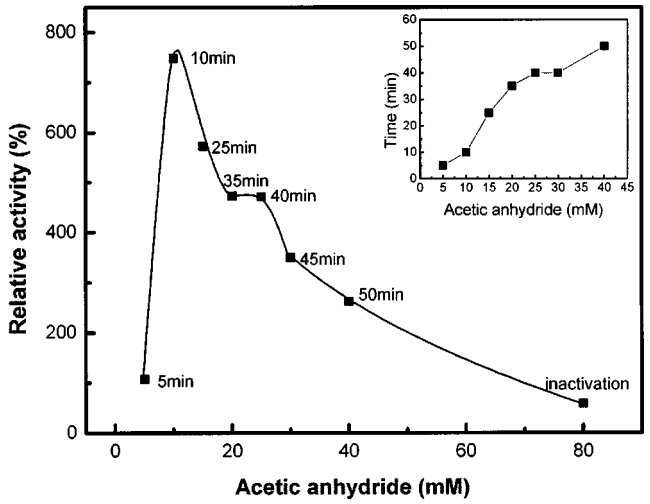


Figure 1. Effect of acetic anhydride treatment on aspartase activity. The reaction mixture contained 100 mM potassium phosphate buffer, pH 7.3, 5 mM β-mercatoethanol, 17.3 μg of aspartase and 20 mM acetic anhydride in a total volume of 0.5 mL. At designed time intervals, aliquots of the reaction mixture were removed and analyzed for enzyme activity. The inset shows the time change as a function of acetic anhydride concentration.

maximum level of activity of acetylated aspartase was increased along with the increasing concentration of acetic anhydride tested (Figure 1, inset). The activation of the enzyme decreased to complete inactivation after increasing to the maximum. The low concentrations of acetic anhydride, from 0 to 4 mM, showed no effects of activation. The L-aspartate saturation profiles of aspartase before and after the acetic anhydride treatment were performed (data not shown). The activity of the acetylated aspartase was a little higher than that of the native enzyme, indicating that the cooperativity between a substrate and enzyme is increased.

Optimum Temperature and pH of Acetylated Aspartase. In order to determine whether or not the optimum temperature and pH of the acetylated aspartase had changed, the relative activities of the native and the acetylated aspartase were determined. Temperature dependence of the relative activity of the acetylated aspartase was bell-shaped, in the range of 20 °C and 60 °C, similar to the native enzyme (Figure 2A). The native aspartase had an optimum temperature of 45 °C. However, the acetylated aspartase had a lower optimum temperature of 40 °C and was less stable at higher temperature (≥ 45 °C). The maximum relative activities of the acetylated and the native aspartase were almost the same. Optimum pH levels of the acetylated and the native aspartase were 7.85 and 8.5, respectively (Figure 2B). The relative activity difference between the native and the acetylated aspartase at the optimum temperature and pH was 20% and 30%, respectively.

Initial Velocity Pattern of Acetylated Aspartase. In order to examine whether or not the functional alteration induced by acetylation involves any kinetic influence, the initial velocity patterns of the native and the acetylated aspartase were determined. The initial velocity pattern of the

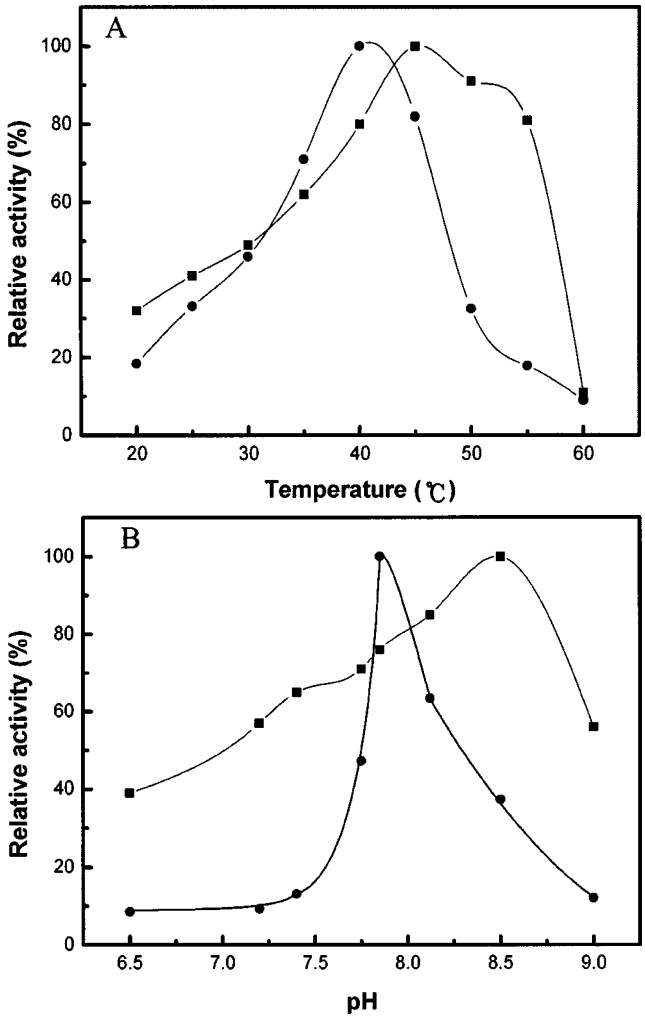


Figure 2. Optimum temperature (A) and pH (B) of the native and the acetylated aspartase. The reaction mixture at 40 °C contained 10 mM aspartate, 100 mM potassium phosphate buffer at pH 7.85, acetylated (●) or native (■) enzyme (1.2 μg), and 4 mM $MgCl_2$ in the total volume of 1.0 mL. All substrate concentrations were corrected for the amount of metal chelate complex formation as described under Materials and Methods.

acetylated aspartase was different from that of the native enzyme. When uncomplexed aspartate was varied at different fixed levels of uncomplexed Mg^{2+}, the initial velocity patterns were obtained. The initial velocity patterns of the native enzyme intersected on the ordinate, indicating the mechanism is the rapid equilibrium ordered addition of Mg^{2+} prior to aspartate (Figure 3A). However, the initial velocity pattern of the acetylated aspartase intersected to the left of the ordinate, indicating that the mechanism is a sequential kinetic mechanism, not including a rapid equilibrium order mechanism. The K_m and V_{max} values of the acetylated aspartase were significantly different from those of the native enzyme. In the values of the kinetic parameters for the acetylated aspartase obtained in the direction of aspartate deamination, the K_m of the aspartate and V_{max} values were 1.07 mM and 7.24×10^{-2} μmol/min, respectively. The K_m value of aspartate of the acetylated aspartase was smaller than that of the native

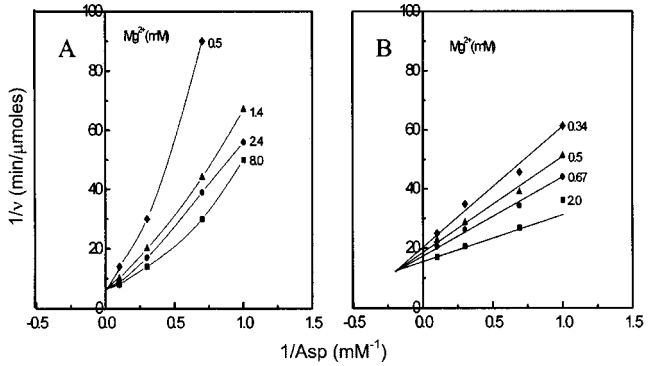


Figure 3. Initial velocity patterns for the native (A) and the acetylated (B) aspartase at pH 7.85. All substrate concentrations were corrected for the amount of metal chelate complex formation as described under Materials and Methods. The data were fitted to Eq. (2).

aspartase (2.5 mM) and the magnitude of V_{max} of the acetylated enzyme was larger than that of the native enzyme (2.5×10^{-3} μmol/min). The K_m (2.1 mM) of Mg^{2+} of the acetylated aspartase was larger than that of the native enzyme (1.05 mM). These results indicate that the acetylated aspartase has higher catalytic efficiencies, a measure of substrate affinity and product releasing for substrates.

Circular Dichroism Spectra. In order to examine whether or not the acetylation of aspartase involves any conformational alteration, the conformational changes of the native and the acetylated aspartase were determined using a CD-ORD spectra (Figure 4). The secondary structures between the native and the acetylated aspartase were different in the values of three basic categories; α-helix, β-strand (often associated with so-called "sheets"), and random coil. The acetylated aspartase exhibited negative cotton effects through 210 nm and 218 nm. From these values, the percentage of α-helix structure

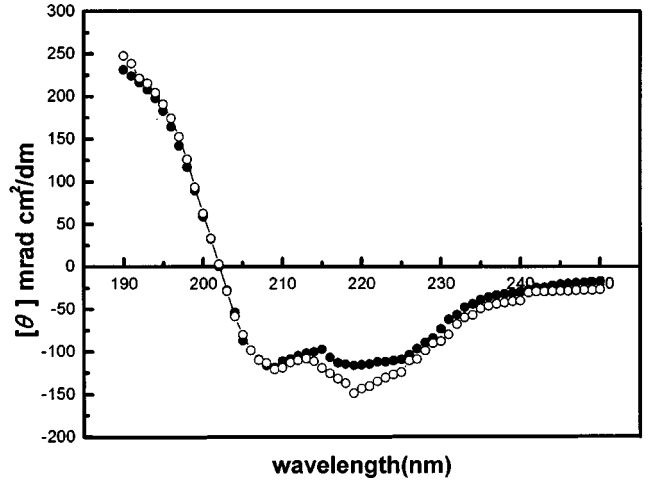


Figure 4. Circular dichroism spectra of the native and the acetylated aspartase. The CD spectra of the native (●) and the acetylated (○) aspartase were measured at 25 °C in a Jovin Yvan CD6 CD-ORD spectropolarimeter with a 0.1 cm light path. The experiments were performed as described under Materials and Methods. The three determinations were obtained using new stock solutions.

of the acetylated aspartase was 54% and that of the native enzyme was 61%, taking that of poly-L-lysine as the standard (100%). The amount of α-helix of native aspartase from *E. coli* was estimated to be 60%.[15] The spectra for the two *Hafnia alvei* aspartase, between the native and the acetylated aspartase, exhibited no significant difference. However, the helical content of acetylated aspartase is about 7% less than that of the native aspartase. In addition, the percentage of its sheet structure was increased from 23% (native) to 29% (acetylated).

Discussion

The effects of various chemical modifications on enzymatic properties have been widely employed as a useful technique for the investigation of the structure-function relationships among various enzymes. The most frequent consequence of chemical modification of aspartase is inactivation.[16,17,18,19] The *Hafnia alvei* aspartase was unexpectedly 7.5-fold activated by the limited treatment of acetic anhydride. However, the low concentrations (≤ 4 mM) of acetic anhydride had no effects of activation, and the activation of aspartase by a certain variation (80 mM) of the molar excess of acetic anhydride was decreased to complete inactivation. The activating effect of acetic anhydride on aspartase may be ascribed to the conformational change brought about by the modification of amino groups (*vide infra*). Several other mono carboxylic acid anhydrides and N-hydroxysuccimide esters have also been used as acylating agents under conditions identical to those for the acetylation of aspartase from *E. Coli*. However, the magnitude of the increases in activity of these acylated aspartase species is less than that of the acetylated aspartase under these experimental conditions.[4]

Upon activation, temperature vs. the activity profile of the acetylated aspartase was essentially changed in comparison with the native aspartase. The optimum temperature of the native aspartase was 45 °C, and that of the acetylated enzyme shifted to 40 °C. The pH vs. the activity profile of the acetylated aspartase was also different from that of the native enzyme. The optimum value of pH of the native aspartase was 8.5 and that of the modified enzyme shifted to 7.85. Concerning all these data, temperature and pH vs. the activity profile of aspartase were affected by acetylation with acetic anhydride, and the acetylated enzyme is a more sensitive molecular species, distinct from the native enzyme. In addition, the activity of the acetylated aspartase was more sensitive to pH values than that of the native aspartase.

The initial velocity pattern of the native aspartase observed when uncomplexed aspartate is varied at several fixed concentrations of uncomplexed Mg^{2+} intersects on the ordinate, indicating a rapid equilibrium ordered addition of Mg^{2+} prior to aspartate. However, the initial velocity pattern of the acetylated aspartase observed under the same conditions intersects to the left of the ordinate, indicating the sequential kinetic mechanism, other then a rapid equilibrium ordered one. These results suggest that the conformational change of aspartase by acetylation induced by the change of the

substrate binding and the central complex (*i.e.* E-Mg-aspartate ↔ E-Mg-fumarate) is not a slow step. The reciprocal plots for aspartate of the native aspartase were curved, indicative of positive cooperativity, but those of the acetylated aspartase were linear, indicative of Michaelis-Menten kinetics.

The helical content of the acetylated and the native aspartase were 54 and 63%, respectively. Yoon, *et al*.(1998) estimated the amount of α-helix of native aspartase from *Hafnia alvei* to be 61%, which is almost the same value as our results.[12] The helical content was rather decreased by the limited modification of acetylation with acetic anhydride. If this change caused the activation of aspartase, the acetylated activation is assumed to mediate a conformational change of the individual subunits. On the basis of these observations, we would suggest that the activating effect of acetic anhydride on *Hafnia alvie* aspartase is ascribed to a conformational change of the individual subunits because of a stoichiometric relationship between the corporation of acetic anhydride to the enzyme and the decrease of the percentage of α-helix of the enzyme upon acetylation.

Among the attempts made in our laboratory, a covalent type activation of the enzyme was attained by a limited proteolysis with trypsin.[20] The polypeptide chain of the native aspartase was cleaved by trypsin at the carboxyl terminal region concomitantly with the activation releasing an oligopeptide. The characteristics of the acetylated aspartase in the present work appear to be somewhat similar to those of the proteinase activated enzyme. However, an attempt to produce further activation by exposing the proteinase activated aspartase to acetylation and vice versa was not successful. Probably, some kind of indirect conformational change by the modification of amino acid(s) appears to be involved in the alteration. The molecular basis of the functional alteration is being investigated in order to clarify the relation between the two enzyme types.

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