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# Allosteric Properties of *Hafnia alvei* Aspartase by Nucleotide Effectors

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**The nucleotide effects of *Hafnia alvei* aspartase were investigated. Purine nucleosides, such as adenosine and guanosine, increased the aspartase activities; whereas, purine nucleotides, such as AMP, ATP, GTP and IMP, caused little change in the aspartase activities. However, pyrimidine derivatives, such as cytidine and CTP, decreased the aspartase activity. The nucleotide and nucleoside effects by the limited trypsin-treated aspartase were similar to those of a native enzyme. These results indicate that the COOH-terminal region and an allosteric site might be located away from each other. The initial velocity study in the presence of adenosine showed that $K_m$ for aspartate was decreased to one-sixth of that in the absence of adenosine, but $V_{max}$ was unchanged. The significance of the distinct allosteric effect for the enzyme-nucleotide interaction is discussed.**

**Keywords:** Aspartase, *Hafnia alvei*, Nucleotides, Trypsin.

## Introduction

Aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1) catalyzes the reversible deamination of L-aspartate to yield fumarate and ammonia (William and Lartigue, 1967). It was observed that the enzyme requires a divalent metal ion activator at higher pH (Rudolph and Fromm, 1971). The enzyme is specific for the amino acid substrate, L-aspartate, and fumarate. However, $NH_2OH$ can substitute for ammonia as a substrate (Karsten and Viola, 1991).

Bacterial aspartase has long been the center of controversy regarding the role of nucleotides and nucleosides in the reaction (William and Lartigue, 1967). On the one hand, the aspartase activity of aged whole cells was increased by the addition of adenosine or AMP to cell suspensions. On the other hand, the same activation was not obtained with cell-free extracts. Nucleotides, such as IMP, AMP, GDP and adenosine, activate aspartase, while GTP and UTP inhibit it. The nucleotide triphosphates such as GTP and UTP increase $K_{aspartate}$, while the activators such as AMP, GDP, IMP decrease $K_{aspartate}$. Aspartase fractions that had been dialyzed for 72 h against EDTA buffers at pH 7.0 retained only 11% of their original activity (Scott, 1959). The 80% of the activity that was recovered, by incubating the preparation for 1 h with 1.3 mM IMP and 100% of the activity, was restored by adding 0.4 mM $Mg^{2+}$ in addition to the IMP. The reaction mechanism of an intermediate ammonia acceptor and an intermediate ammonia source in the aspartase is not clear. In many cases (refer to Results and Discussion) the nucleotides, or nucleosides by the preceding reaction(s), increase enzyme activity (Kim and Weete, 1999; Duggleby and Pang, 2000; Kim *et al.*, 2000). They may also act as allosteric activating agents.

Because of our interest in the mechanism of these aspartate-utilizing enzymes, we have undertaken many studies (Lee *et al.*, 1999; Paik *et al.*, 1999; Shim *et al.*, 1999). In an effort to better elucidate the structure-function relationships and the mechanism in the enzymatic reaction, we present in this study data of the nucleotide and nucleoside effects for aspartase from *Hafnia alvei.*

## Materials and Methods

**Chemicals** *Hafnia alvei* (ATCC 9760) was purchased from ATCC (American Type Culture Collection). L-Aspartic acid, Adenosine, Cysteine, Guanosine, Adenosine 5'-monophosphate, Adenosine 5'-triphosphate, Cytidine 5'-triphosphate, Guanosine 5'-triphosphate, Inocinate 5-triphosphate, 2-Hydroxyethylmercaptan (β-Mercaptoethanol), and 4-(2-hydroxyethyl) piperazine-1-ethane sulfonic acid (HEPES) were purchased from Sigma (St. Louis, USA). Magnesium chloride was obtained from Yakuri (Osaka, Japan). All other buffers were obtained from commercially available sources and were pure or extra pure for the available analytical grade.

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**Enzyme** Aspartase from *Hafnia alvei* was purified according to the method described by Yoon *et al.* (1998). Briefly, aspartase was purified according to a scheme including cell extraction, streptomycin sulfate fractionation, heat treatment, ammonium sulfate fractionation, DEAE-cellulose, Red A-agarose, and Sepharose 6B chromatography. The nucleotide is either so tightly bound or so well shielded from the solvent that efforts to remove it fail in most preparations of aspartase. Aspartase is judged from $A_{280}$: $A_{260}$ ratios in order to obtain a nucleotide sensitive enzyme. The enzyme preparations used in this investigation were homogeneous as judged by polyacrylamide gel electrophoresis.

**Enzyme assay** All data were collected using a Hewlett Packard 8452 Diode-Array spectrophotometer and a Shimadzu UV-2101PC. The temperature was maintained at 25°C using a circulating water bath with the capacity to heat and cool the thermospacers in the cell compartment. All of the reactions were carried out in 1 mL cuvettes with a 1 cm light path length, which were incubated for least 10 min in the cell compartment prior to initiation of the reaction by the addition of aspartase. The progress of the reaction of aspartase was routinely determined spectrophotometrically and monitored continuously by measuring the formation of fumarate by following the increase in absorbance at 240 nm ($\varepsilon_{240}$= 2,255 $M^{-1}cm^{-1}$) at 25°C. The standard assay mixture contained in the total volume of a 1.0 mL cuvette consisted of 100 mM Tri-HCl buffer, 10 mM aspartate and 4 mM $Mg^{2+}$, pH 8.0 and was incubated for 10 minutes in a compartment. The aspartate and $Mg^{2+}$ concentrations were corrected for complexation with $Mg^{2+}$ using the following dissociation constant obtained at 0.1 mM ionic strength (Dawson *et al.*, 1971): Mg-aspartate, 4 mM. All other chemicals were corrected for the metal-chelating effect. The reaction started by adding the appropriate volumes of aspartase. The increase of absorbance at 240 nm that corresponded to fumarate formation was measured.

**Data processing** Reciprocal initial velocities were plotted versus reciprocal substrate concentrations. Data were fitted using the appropriate rate equations and the Fortran programs of Cleland (1979). Initial velocity data conforming to a sequential mechanism were fitted using Eq (1)

$$v = VAB/(K_{ia}K_b + K_aB + K_bA + AB) \quad (1)$$

Where v and V represent initial and maximum velocity; $K_a$ and $K_b$ are $K_m$ values for A and B; and A and B represent reactant concentrations, respectively. $K_{ia}$ is the substrate inhibition constant for A.

## Results and Discussion

The question of the stimulation of aspartase by nucleotides and nucleosides is an old one, to cite the widely published and readily reproducible effects obtainable with aged whole cells. Because the activation is not easily reproduced with cell-free preparations, the enhancement due to these activators has been attributed to secondary effects, such as the stimulation of aspartate transported across cell membranes (William and Scott, 1968).

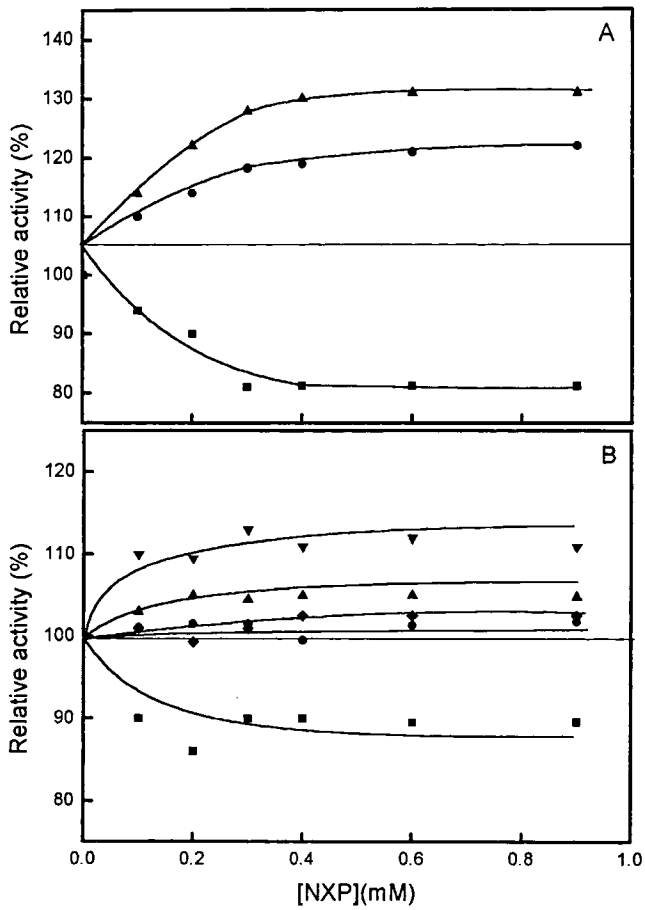


**Fig 1.** Effects of Nucleotides and Nucleosides on the Activity of Aspartase. The enzymatic activity was assayed as described in Materials and Methods. Data were obtained in the presence of varying total nucleotide concentrations ([NXP]) at 25°C. The following NXP were used: (A) adenosine (▲), guanosine (●), cytidine (■); (B) IMP (▼), AMP (▲), GTP (◆), ATP (●), CTP (■).

The influence of increasing concentrations of nucleotides and nucleosides on the rate of the reaction catalyzed by aspartase was determined and compared with the effects. Aspartase reactions were performed at each concentration of nucleotides and nucleosides at 25°C, 100 mM Hepes (pH 8.0). The results obtained are shown in Figure 1. The addition of exogenous nucleotides and nucleosides to the incubation media affected the enzyme activity. Purine nucleosides, such as adenosine and guanosine, increased the aspartase activities to approximately 130% and 120%, respectively. Purine nucleotides, such as AMP, ATP, GTP and IMP, caused little change in enzyme activity, although a small increase was noted for IMP. On the other hand, the pyrimidine nucleotide and nucleoside, such as cytidine and CTP, decreased the aspartase activities to approximately 80% and 87%, respectively. The effects of adenosine, guanosine and cytidine on the aspartase activities were shown to be dependent on the concentration.

At increasing concentrations, adenosine, guanosine, IMP

**Table 1.** Synergistic effect on activity of aspartase[a]

| | AMP | ATP | Guanosine | GTP | Cytidine | CTP | ITP |
|---|---|---|---|---|---|---|---|
| Adenosine | 113 | 125 | 91 | 113 | 108 | 110 | 115 |
| AMP | • | 100 | 92 | 97 | 103 | 95 | 92 |
| ATP | • | • | 96 | 89 | 102 | 85 | 85 |
| Guanosine | • | • | • | 96 | 93 | 88 | 93 |
| GTP | • | • | • | • | 100 | 89 | 89 |
| Cytidine | • | • | • | • | • | 96 | 85 |
| CTP | • | • | • | • | • | • | 87 |

[a]The aspartase activity was assayed at 25°C, pH 8.0 100 mM Hepes buffer and each nucleotide and nucleoside concentration 0.4 mM. All experiments were repeated at least 3 times. The value of data reveals the percentage of activity.

and AMP behaved as an allosteric activator on the rate of the reaction catalyzed by aspartase. However, at increasing concentrations, cytidine and CTP behaved as allosteric inhibitors on the rate of the reaction catalyzed by aspartase. The allosteric effects of the nucleosides are larger than that of the nucleotides. In addition, the concentration of nucleosides must be 2-3 times higher than that of nucleotides in order to promote activation or inhibition of aspartase activity. On the other hand, ATP and GTP have almost no effect on the aspartase activity, even at high concentrations. These results indicate that the nucleosides having the purine ring, and the nucleotides having a less phosphate group (comparing α-phosphate and γ-phosphate), reveal a higher affinity for the regulatory sites.

The influence of the possible combinations of nucleotides and nucleosides on the activities of aspartase is shown in Table 1. Other nucleoside, or nucleotide combinations with adenosine, promoted the synergistic phenomena. The values of the overall effects are approximately 110-120%, except in the case of guanosine, which decreased to 91%. These values were revealed to be less than that of adenosine alone (130%).

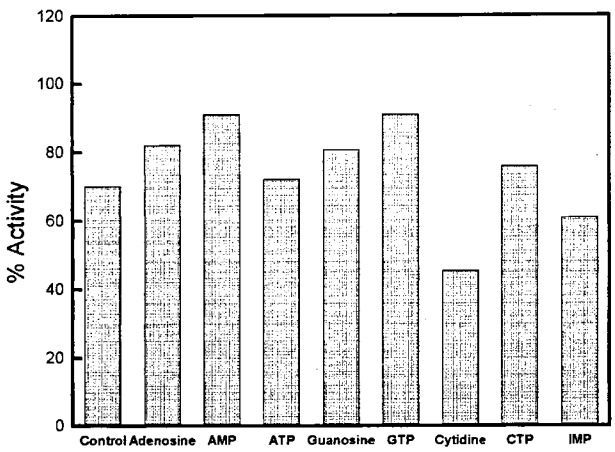


**Fig. 2.** Nucleotides Effect on the Activity of Trypsin treated Aspartase. The enzymatic activity was assayed as described in Materials and Methods. The original activity of aspartase decreased to 70%. The trypsin treated aspartase was allowed to react with each 0.4 mM nucleotides and nucleosides.

In contrast, other combinations of nucleotides and nucleosides used were unable to induce any synergistic phenomena.

In order to understand the correlation of the binding site of the nucleotide (or nucleoside) and the activity of the enzyme, trypsin treated aspartase experiments were performed. After aspartase (0.2 mg) was treated with trypsin (0.01 mg) at pH 7.4 and 30°C for 24 h, the original activity of the aspartase decreased to 70% (Figure 2). When trypsin treated aspartase was incubated with the nucleotides and the nucleosides, most of the nucleotide and nucleoside derivatives increased the activities of the enzymes. Adding AMP and GTP restored the original activity (100%). Ninety percent of the original activity was recovered by adding adenosine and guanosine. However, cytidine and IMP revealed lower activities than the control one.

The aspartase from *E. coli* and *H. alvei* was markedly activated by trypsin treatment without an appreciable alteration of its molecular weight (Yumoto *et al.*, 1980; Lee *et*

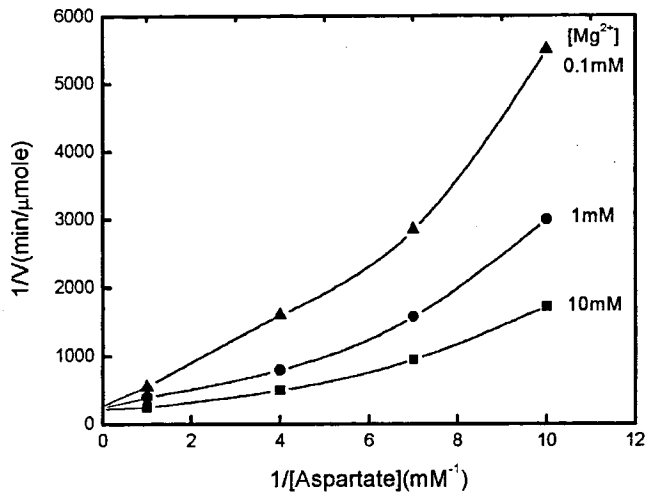


**Fig. 3.** Initial Velocity Pattern of Aspartase in the presence of 0.4 mM Adenosine at pH 8.0. The enzymatic activity was assayed as described in Materials and Methods. Data were obtained by varying the concentration of aspartate as indicated at each of the following $Mg^{2+}$ concentrations: 10 mM (■), 1 mM (●), 0.1 mM (▲). The points are experimental, while the lines are theoretical from a fit of Eq. 1 to the data.

*al.*, 1999). The released peptide from *E. coli* was separated by a high voltage paper electrophoresis, and the amino acid composition and terminal residues were determined. The results showed that one or more residues are related to the COOH-terminal upon activation. This indicates that the COOH-terminal of pro-aspartase is probably related to the aspartase activation that penetrates the plasma membrane. In this study, most of the nucleotides and nucleosides tested increased the activity of the enzyme 130%, except cytidine and IMP (*vide ante*). The nucleotides and nucleosides effects by limited trypsin digestion are similar to those of a native enzyme. These results indicate that the COOH-terminal region may affect an allosteric site.

When aspartate concentration was varied at different fixed levels of uncomplexed $Mg^{2+}$ with 0.4 mM adenosine, the initial velocity pattern that is shown in Figure 3 was obtained. Intersection on the ordinate is indicative of the rapid equilibrium ordered addition of $Mg^{2+}$ prior to aspartate. These results agree with the kinetic mechanism in the absence of adenosine (Nuiry *et al.*, 1984; Yoon *et al.*, 1995; Shim *et al.*, 1997). The $K_m$ for aspartate and $Mg^{2+}$ was 0.37 mM and 0.9 mM in the presence of adenosine, respectively. In the absence of adenosine, the $K_m$ for aspartate and $Mg^{2+}$ was 2.5 mM and 1.05 mM, respectively (Nuiry *et al.*, 1984; Yoon *et al.*, 1995). The $K_m$ for aspartate in the presence of adenosine was decreased to about one-sixth of that in the absence of adenosine. On the other hand, the $K_m$ for $Mg^{2+}$ in the presence of adenosine did not change when compared to that in the absence of adenosine.

The kinetic studies of the aspartase reaction revealed the cooperative effects of substrates, which are especially pronounced at above pH 8.0 (Nuiry *et al.*, 1984; Yoon *et al.*, 1995). The 1/velocity vs. 1/substrate plot is not truly hyperbolic at lower than pH 7.5, but drift upward at the low concentrations. A marked sigmoidicity appeared as the pH rose above 7.5. These results indicated that the system might be subject to nucleotide regulation as are so many other enzymes possessing a quaternary structure. Previous work (Williams and Lartigue, 1967) on *Bacterium cadaveris* showed that the AMP and IMP displayed kinetic effects that are characteristic of an allosteric activator. The kinetic curve shifts from cooperative to Michaelis-Menten, the $V_{max}$ is unchanged and the $K_m$ decreased. The results of binding studies and the kinetic experiments presented here are consistent. Aspartase exhibited a true $K_m$ of 0.37 mM aspartate and a true $K_m$ of 1.05 mM $Mg^{2+}$ in the presence of adenosine. The $K_m$ for aspartate in the presence of adenosine was decreased about six-fold. Adenosine leads to a tight affinity between the substrate, aspartate, and the enzyme, i.e. the aspartase reaction revealed the positive cooperative effects of the substrate. However, the $K_m$ for $Mg^{2+}$ was almost the same in the presence and absence of adenosine.

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