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Diethylpyrocarbonate Inactivation of Aspartase from *Hafnia Alvei*

Jae-Hee Shim, Hyo-Joon Kim† and Moon-Young Yoon*

Department of Chemistry, Hanyang University, Seoul 133-791, Korea

†Department of Biochemistry and Molecular Biology, Hanyang University, Ansan 425-791, Korea

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An aspartase purified from *Hafnia alvei* was inactivated by diethylpyrocarbonate (DEP) in a pseudo-first-order inactivation. The first-order plot was biphasic. The inactivation process was not saturable and the second order rate constant was 1.3 $M^{-1}s^{-1}$. The inactivated aspartase was reactivated with NH_2OH. The difference absorption spectrum of DEP-inactivated vs native enzyme preparations revealed a marked peak around 242 nm. The pH dependence of the inactivation rate suggests that an amino acid residue having a pK value of 7.2 was involved in the inactivation. L-aspartate, fumarate (substrates), and chloride ion (inhibitor) protected the enzyme against inactivation, indicating that histidine residues for the enzyme activity are located at the active site of this aspartase. Inspection of the spectral change at 240 nm along with inactivation in the presence and absence of Cl^- ion demonstrated that the number of essential histidine residues is less than two. Thus, one or two histidines are in or near the aspartate binding site and participate in an essential step of the catalytic reaction.

Keywords: Aspartase, Chemical modification, Diethylpyrocarbonate.

Introduction

Aspartase [L-aspartate ammonia lyase, EC 4.3.1.1] catalyzes the reversible deamination of L-aspartate to yield fumarate and NH_4^+. It is a tetramer composed of four apparently identical subunits of molecular weight 48,000 (Williams and Lartigue, 1967). The enzyme was observed to have an absolute requirement for a divalent metal ion activator at higher pH (Rudolph and Fromm, 1971), but may possess activity in the absence of divalent metal ions at low pH (Suzuki *et al.*, 1973). Yoon and Cook (1994) have studied the pH dependence of the kinetic parameters in the deamination direction. The *V/K* for aspartate was bell-shaped with estimated pK values of 6.6 and 7.2. The maximum velocity for aspartate was also bell-shaped, giving pK values almost identical to those obtained for $V/K_{aspartate}$. It was 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.

Kim *et al.* (1995) have determined the pH dependence of kinetic parameters in the amination direction. The *V/K* for fumarate was bell-shaped with pK values of 6.4 and 8.7. The maximum velocity for fumarate was also bell-shaped with pK values of 7.2 and 9.1. Taken together, these results are consistent with two enzyme groups which are necessary for catalysis (Yoon and Cook, 1994). One residue that must be deprotonated has been identified, and another residue must be protonated for substrate binding. Both the general base and general acid group are in a protonation state, opposite to that of when aspartate is bound. A proton is abstracted from C-3 of the monoanionic form of L-aspartate by a 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 (Yoon *et al.*, 1995).

A study using an organic solvent perturbation method has recently shown that a possible candidate for the acidic residue in the active site is a histidine, and another possible candidate for the basic residue in the active site is a cysteine (Yoon and Cho, 1998). 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 (Shim *et al.*, 1997). In an attempt to determine whether additional residues are necessary, we carried out inactivation studies using diethylpyrocarbonate (DEP). In these studies, we report that histidine residue(s) located at or near the active site may be required for the catalytic activity of aspartase.

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

Materials and Methods

Chemicals and enzyme Diethylpyrocarbonate (ethoxyformic anhydride), ammonium sulfate, 3-(N-morpholino) propanesulfonic acid (Mops), 2-(N-morpholino) ethanesulfonic acid (Mes), L-aspartic acid, fumaric acid, and D-aspartic acid were obtained from Sigma. Acetonitrile was obtained from Merck. Red-A agarose, sodium dodecyl sulfate, N,N-methylene-bis-acrylamide, acrylamide, and N,N,N′,N′-tetramethylenediamine (TEMED) were obtained from Bio-Rad (Hercules, USA). Nutrient broth and nutrient agar were purchased from Difco Laboratories. All other chemicals were of the highest purity available from commercial sources. Aspartase from *Hafnia alvei* was purified according to the method described by Yoon *et al.* (1998). The enzyme preparations used in this investigation were homogeneous as judged by polyacrylamide gel electrophoresis.

Enzyme assay Aspartase activity was determined spectrophotometrically by measuring the formation of fumarate by following the increase in absorbance at 240 nm and 25°C. All assays were carried out using an HP spectrophotometer. Temperature was maintained at 25°C using a circulating water bath to heat the thermospacers of the cell compartment. The standard assay used in these studies was carried out in a 1 ml solution containing 100 mM TAPS buffer (pH8.5), 2 mM $MgCl_2$, 20 mM L-aspartate, and the enzyme. Stock solutions of diethylpyrocarbonate were prepared in acetonitrile and the molar concentration was determined by reaction with 10 mM imidazole in 0.1 M potassium phosphate buffer, pH 6.0. The increase in absorbance at 240 nm was measured and the total concentration was estimated using an absorptivity of $3.0 \times 103\ M^{-1}cm^{-1}$ (Ovadi *et al.*, 1969). The concentration includes a correction for the Mg-aspartate complex using a dissociation constant of 4 mM (Dawson *et al.*, 1971).

Enzyme modification with diethylpyrocarbonate DEP was freshly diluted in acetonitrile just prior to each experiment. Inactivation by DEP was carried out at 30°C in a reaction mixture containing 100 mM potassium phosphate buffer (pH6.0), enzyme (0.23 mg/ml), and different concentrations of the reagent, as indicated in the figure legends. The reaction was started by the addition of DEP, and 50 μl aliquots of the modified enzyme were assayed for remaining activity at the indicated time intervals. The concentration of acetonitrile was kept below 5% (v/v) throughout the experiment. The number of histidine residues modified by DEP was calculated from the increase in absorbance at 240 nm using an absorptivity of $3.2 \times 10^3\ M^{-1}cm^{-1}$ (Ovadi *et al.*, 1969). Protection experiments were carried out as above with compounds of interest present at the concentrations indicated in Table II.

pH studies The pH dependence of the inactivation rate was obtained with the procedure above using 100 mM potassium phosphate, MOPS (pH 6.0–7.5) and TAPS (pH 7.0–8.5). In all cases, the stability of the enzyme was determined by incubating the enzyme at the pH of interest and assaying aliquots at several intervals at pH 8.5. Since DEP is known to be unstable at higher pH values, it was necessary to determine the half-life of the reagent as a function of pH. The half-life of DEP under various conditions employed for the modification reaction was determined by the method of Berger (1975). In all cases, the degradation of DEP was not a significant problem over the course of the reaction.

Determination of inactivation rate Rates of inactivation were obtained from the initial linear portion of the first-order plots using linear regression analysis. Data obtained for the dependence of K_{inact} on the concentration of DEP were fitted to the equation for a straight line. Data obtained for the pH dependence of k_{inact} were fitted to the following equation (Cleland, 1979).

$$\log y = \log\left(\frac{Y_L + Y_H H/K_1}{1 + H/K_1}\right)$$

where y represents the observed value for k_{inact}; Y_L and Y_H represent the pH independent values of k_{inact} at low and high pH, respectively; K_I is the dissociation constant for the modified histidines, and H is the hydrogen ion concentration.

Results

Diethylpyrocarbonate inactivation Aspartase was inactivated in a time-dependent manner. As shown in Fig. 1, the first-order plot was not linear. There appears to be two phases to the reaction; a fast one followed by a slower one. There are several possible explanations for the biphasic curves in the first-order plots. First, since the reagent itself is unstable, the biphasicity could have resulted from a decrease in reagent concentration with time. Although this may be a factor in long kinetic studies, it is not a major problem in this case. Rates of the fast and slow phases of the reaction are roughly so and 20 times faster than that of reagent loss. Second, there are two or more different histidines with different rate constants for modification which affect activity to differing extents (some directly, others through conformational changes). Third, the modification of histidines which do not affect activity directly may induce protein structural changes influencing histidine(s) that do affect activity.

The second and third explanations differ by their direct or indirect action on activity or binding. In the third case, protein rearrangement upon DEP modification should occur at a rate approximating the slow phase, while this is not necessarily true for the second case. A plot of the pseudo-first-order rate constant extracted from the initial linear region of Fig. 1 indicates that the reaction between the enzyme and the reagent is a simple bimolecular reaction. The second-order rate constant for the reaction of DEP and the enzyme is $1.3\ M^{-1}s^{-1}$. The enzyme is stable over the time course of inactivation as shown in Fig. 1.

Identification of modified residues Although DEP is a fairly specific acylating agent, it has been shown to modify

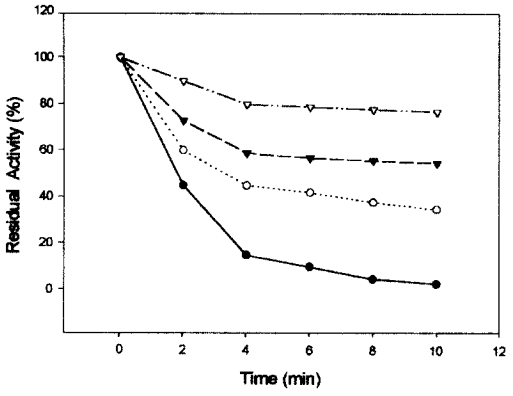

Fig. 1. Inactivation of aspartase by DEP. The enzyme (0.5 mg) was incubated in a solution of 0.1 M KH_2PO_4 buffer (pH 6.0). The reaction was started by addition of indicated concentrations of DEP in acetonitrile, and aliquots of modified enzyme were assayed for remaining activity at the indicated time intervals. The concentrations of DEP were as follows: ▽, 0.75 mM; ▼, 1.5 mM; ○, 3.0 mM; ●, 4.5 mM.

amino acids other than histidines. However, reversal of the inactivation by DEP using hydroxylamine will occur only when the imidazole side chain of histidine or the phenolic side chain of tyrosine is modified (Melchior and Farney, 1970). As shown in Table 1, treatment of the enzyme with 3 mM DEP results in 81% inactivation after 40 min compared to the control activity. Addition of 80 mM hydroxylamine results in restoration of 76% of the original activity. However, an addition of hydroxylamine to untreated enzyme decreases the original activity by 7%. When this is taken into account, the percentage of the initial activity is identical in both experiment and control after 80 min. The difference in absorption spectrum between the inactivated diethylpyrocarbonate and native enzyme preparations was recorded (data not shown). The peak around 240 nm is characteristic of histidine modification (Ovadi *et al.*, 1969). When the tyrosine residue is modified, absorbance at 280 nm is expected to decrease (Burstein *et al.*, 1974). In the case of aspartase, however, there was no absorbance change above 270 nm, suggesting that no tyrosine residue reacted with diethylpyrocarbonate.

pH dependence of K_{inact} A pK value of the modified residue can be estimated from the pH dependence of the inactivation rate. The rate of inactivation of aspartase by DEP was determined using 3 mM DEP as a function of pH. The dependence of the inactivation rate on the concentration of DEP was obtained at pH 5.5 and 8.5. At both pH values, the process is first order with respect to DEP as is true at pH 7.0. Thus, a 3 mM concentration of DEP was used at all pH values. The dependence of the inactivation rate on pH is shown in Fig. 2. The inactivation rate decreases below a pK of about 7.2, suggesting that the inactivation was due to a histidine modification.

Table 1. Reversal of DEP inactivation by hydroxylamine[a].

Additions[b]	Time (min)	Percent activity (experimental)	Percent activity (control)
3mM DEP[c]	0	100	100
	5	50	98
	40	14	95
80mM NH_2OH[c]	50	40	95
	80	90	93

[a] Aspartase activity was assayed as described in Materials and Methods using a 10 μl aliquot at time zero, 5 min, and 40 min, since an equal volume of hydroxylamine was added to the enzyme inactivation mixture at 40 min. All the remaining assays were performed with 20 μl aliquots.

[b] The additions listed were made only to the experimental takes in the case of NH_2OH.

[c] DEP was added at time zero, while NH_2OH was added at 40 min. The concentrations listed are final concentrations in the incubation mixture.

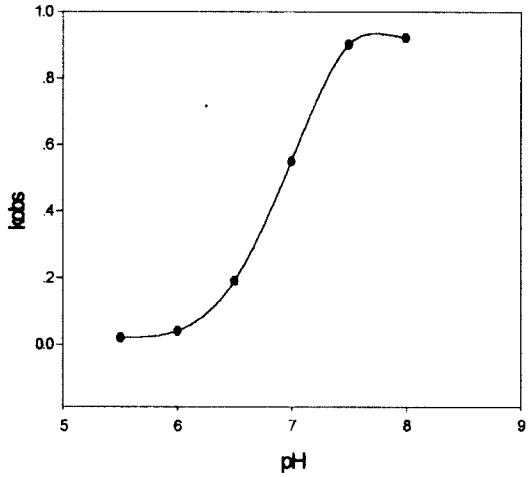

Fig. 2. pH dependence of the inactivation rate.

Protective effects of various compounds Protective effects of various compounds were tested in order to examine whether or not the modified histidine residues were located at the active site. As shown in Table 2, L-aspartate and fumarate at 5 mM, which are substrates of the enzyme, exhibited about 70% protection of the enzyme against DEP inactivation. In contrast, glutamate, succinate, and some other related compounds, which do not serve as substrates, provided less than 45% protection at the same concentrations, possibly due to a low affinity of the compounds towards the active site. KCl showed a marked protective effect of about 86%. We further examined the effect of Cl^- ion on the enzyme reaction and found that it provided 78% protection of the enzyme reaction at pH 6.0 (Fig. 3).

Table 2. Protective effects of various compounds against DEP-inactivation of aspartase.

Addition (mM)	Residual activity (%)
3 mM DEP (none)	38
5 mM L-Aspartate	69
5 mM Fumarate	69
5 mM KCl	86
5 mM Succinate	46
5 mM Glutamate	43
5 mM $(NH_4)_2SO_4$	15

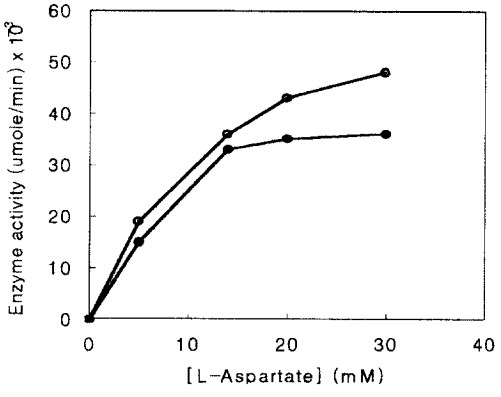

Fig. 3. Substrate saturation profiles in the presence (o) and absence (●) of Cl^-.

Estimation of the number of essential histidine residues Carbethoxylation of the imidazole side chain of histidine by DEP results in an increase in absorbance at 242 nm as a result of formation of carbethoxyhistidine (Miles, 1977). By this method, we examined the relationship between the number of modified histidine residues and the residual activity. As shown in Fig. 4, modification of six to seven histidine residues per subunit resulted in a complete inactivation of aspartase (based on linear extrapolation), indicating that most of the whole histidine residues reacted with diethylpyrocarbonate. However, in the presence of Cl^- ion, a protective agent against inactivation, only 20% activity was lost concomitant with the modification of five residues per subunit. This result suggests that five out of six to seven residues modified in the presence of Cl^- ion are located apart from the active site and were modified without causing inactivation, and so there are one or two essential histidine residues per subunit.

Discussion

Diethylpyrocarbonate reacts not only with imidazole, but also with other nucleophilic groups such as cysteine,

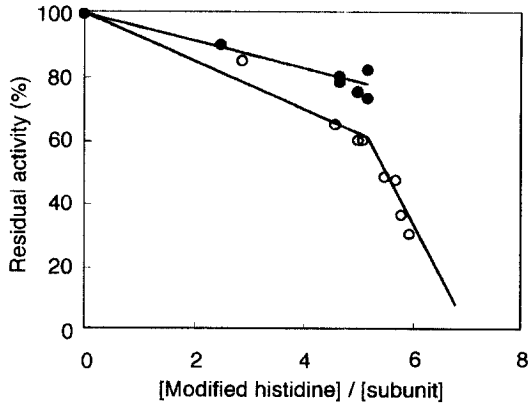

Fig. 4. Correlation between enzyme activity and the number of modified histidine residues. Assays were performed as described under Materials and Methods in the presence (●) or absence (○) of 10 mM KCl. Small aliquots (20 μl) were withdrawn and the enzyme activity was measured. The number of modified histidine residues was calculated as described in Materials and Methods.

tyrosine, and lysine residues (Melchior and Fahrney 1970; Wells 1973; Burstein *et al.*, 1974). However, the present experimental evidence strongly supports the view that the inactivation of aspartase was due to the modification of histidine residues based on the following observations: (1) Enzyme activity was completely restored by NH_2OH treatment. (2) The difference absorption spectrum between modified and native enzyme preparations exhibited a peak of around 240 nm without an appreciable change at around 280 nm. (3) The pH dependence of the inactivation rate indicated the involvement of a residue having a pK value of about 7.2.

The biphasicity as discussed in the Results section is most likely a result of two or more different histidines which react at different rates with DEP. The biphasic nature is not due to a loss of reagent since the rates of both phases are significantly faster than the rate of reagent loss. The second-order rate constant (1.3 $M^{-1}s^{-1}$) obtained for the reaction of DEP with aspartase was somewhat smaller than the value reported for the reaction with free histidine, imidazole, and N-acetylhistidine, which are 54 $M^{-1}s^{-1}$ (Melchior and Fahrney, 1970) and 24 $M^{-1}s^{-1}$ (Holbrook and Ingram, 1973), respectively. Parallel modification of as many as seven residues per subunit in the case of aspartase may reflect this low reactivity of the essential histidine residue. The pH dependence of the DEP inactivation rate gives a pK of 7.2 for the histidine(s) modified concomitant with activity loss. The ratio of the rate of modification at high and low pH is about 10.

The pH dependence of the kinetic parameters for aspartase has recently been determined (Yoon *et al.*, 1995). There are two acid/base catalytic groups required for reaction, one with a low pK of 6.6 which must be unprotonated for activity, and the other with a pK of about

7.2 in the *V/K* for aspartate. This profile represents the titration of a residue on the enzyme in the E:Mg^{2+} complexes. The pK of 7.2 obtained in these studies is most likely the same residue with a pK of 6.6. A proton is abstracted from C-3 by an enzyme general base with a pK of 6.6. This group is most likely responsible for the protonation of the carbanion intermediate of aspartate. The Cl^- ion protected the enzyme against inactivation and also inhibited the enzyme reaction. The inhibitory effect of Cl^- ion was not conspicuous until a 5 mM aspartate concentration was reached, suggesting that the Cl^- ion protects the enzyme not binding to the active site, but rather probably by reducing the reactivity of histidine at the active site through binding to a distant site. The number of histidines modified by DEP concomitant with activity loss is dependent on the concentration of the reagent and/or pH.

Over the last several years, a number of papers have reported the reaction mechanism of aspartase (Porter and Bright, 1980; Nuiry *et al.*, 1984; Kim *et al.*, 1994; Yoon *et al.*, 1995). Aspartate binds to the enzyme as a 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 6.6. The resulting carbanion is stabilized by delocalization of electrons into the β-carboxyl, presumably with the assistance of one or more positively-charged enzyme residues in the vicinity of the β-carboxyl. In consideration of this hypothesis, it is possible that a histidine residue(s) is involved in the proton abstraction. Further investigation is needed for elucidation of the role of histidine as well as other amino acid residues.

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