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Chemical Modification of Cysteine Residues in *Hafnia alvei* Aspartase by NEM and DTNB

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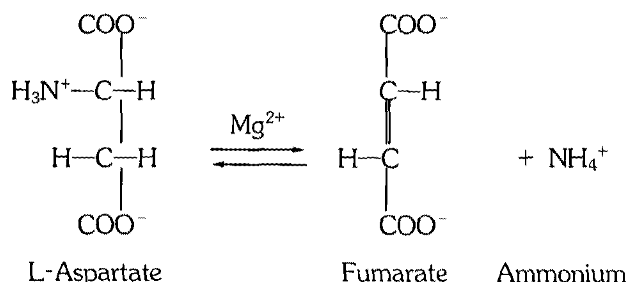
Abstract: Aspartase from *Hafnia alvei* was inactivated by N-ethylmaleimide (NEM) and 5,5′-Dithiobis-(2-nitrobenzoic acid) (DTNB) following pseudo-first order kinetics. Their apparent reaction orders were 0.83 and 0.50 for NEM and DTNB modifications, respectively, indicating that inactivation was due to a sulfhydryl group in the active site of aspartase and participation of the sulfhydryl group in an essential step in the catalytic reaction. When aspartase was modified by DTNB, the enzyme activity was restored by dithiothreitol treatment, indicating that cysteine residue(s) is(are) possibly at or near the active site. The pH-dependence of the inactivation rate by NEM suggested that an amino acid residue having pK value of 8.3 was involved in the inactivation. When aspartase was incubated with NEM and L-aspartate together, L-aspartate markedly protected the enzyme from inactivation by NEM, but the other reagents used did not.
Key words: aspartase, chemical modification, cysteine, enzyme inactivation.

Aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1) catalyzes the reversible deamination of L-aspartate to yield fumarate and ammonia (Quastel and Woolf, 1926):



The aspartase reaction was first demonstrated in bacteria by Harden (1901). Aspartase has been regarded as a catabolic enzyme in both bacteria and plants, but the reaction is reversible and favors aspartate formation with ΔG^0=3.2 kcal/mol for fumarate amination. The equilibrium constant for the aspartase reaction, measured directly at 25℃ by Bada and Miller (1968), was 5×10^{-3} M.

The aspartase 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), with some indication that aspartase may possess activity in the absence of divalent metal ions at lower pH (Suzuki *et al.*, 1973). Other alkali metals such as Be^{2+} and Ba^{2+} do not activate aspartase.

Dougherty *et al.* (1972) suggested a uni-bi rapid equilibrium random process as the kinetic mechanism. Later, Nuiry *et al.* (1984), using a divalent metal ion as a pseudo-reactant, carried out an extensive kinetic mechanism study for aspartase using initial velocity, primary and secondary kinetic isotope effects. They suggested a rapid equilibrium ordered addition of Mg^{2+} prior to aspartate but completely random release of Mg^{2+}, NH^{4+} or fumarate.

Yoon and Cook (1994) have recently studied the pH dependence of the kinetic parameters in the deamination direction reaction. 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/Kaspartate. They have 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 also 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

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and 8.9. Together with the data of Yoon and Cook (1994), these results are consistent with two enzyme groups being necessary for catalysis. An enzymatic group that must be deprotonated has been identified. Another enzyme group must be protonated for substrate binding. Both the general base and general acid groups are in a protonation state opposite that in which they started when aspartate was bound. A proton is abstracted from C-3 of the monoanionic form of L-aspartate by general base with 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.

In an attempt to determine which residues at or near the active site of the aspartase, chemical modification studies using NEM and DTNB were carried out. In these studies, we report the requirement of cysteine residues for the catalytic activity of aspartase. They are located at or near the active site of aspartase.

Materials and Methods

Materials

Aspartase, L-aspartic acid, D-aspartic acid, NEM, DTNB, fumaric acid and dithiothreitol were obtained from Sigma (St. Louis, USA). $(NH_4)_2SO_4$ and $MgSO_4$ were obtained from Fischer Scientific. Matrix gel Red-A was obtained from Amicon Corp. Sodium dodecyl sulfate, N,N-methylene-bis-acrylamide, Acrylamide and N, N,N,N-tetramethylenediamine (TEMED) were obtained from Bio-Rad (Richmond, USA). All other buffers were obtained from commercially available sources and were of the highest quality available.

Enzyme purification

Aspartase obtained from Sigma was contaminated with fumarase which eliminate the monitoring of ε_{240} as a viable assay unless the enzyme can be purified. Aspartase was purified by using the dye-ligand affinity chromatography method (Karsten *et al.*, 1985). The enzyme obtained was approximately 90% pure or apparently homogeneous as judged by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis.

Enzyme assay

All data were collected using the Hewlett Packard 8452 Diode-Array spectrophotometer and the Contron UVKon 860. 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 reactions were carried out in a 1 ml cuvette with a 1 cm light path. All cuvettes were incubated for at least 10 min in a water bath and for 5 min in the cell compartment prior to incubation of reaction.

The reaction was started by the addition of aspartase. The aspartate concentration was corrected for complexation with Mg^{2+} using the following dissociation constant obtained at 0.1 mM ionic strength: Mg-aspatate 4 mM (Dawson *et al.*, 1971). Aspartase activity was assayed continuously using the absorbance of fumarate at 240 nm (ε_{240}=2,255 $M^{-1} \cdot cm^{-1}$) (Cook *et al.*, 1980). Standard assay mixture contained 100 mM Hepes (pH 8.0) buffer, 10m M aspartate, and 20 mM Mg^{2+} based on the metal chelate correction.

Enzyme modification with NEM and DTNB

Chemical modification of aspartase by NEM and DTNB was carried out at 0°C in a reaction mixture containing 10 mM Hepes (pH 7.0) and different concentrations of the reagent as indicated in the figure legends. The reaction was started by the addition of NEM and DTNB, and then aliquots of the modified enzyme were assayed for remaining activity at indicated time intervals.

Data processing

Rate of inactivation was obtained from the initial linear portion of the first order plots using linear regression analysis. Data obtained for the dependence of Kinact on the concentrations of NEM and DTNB were fitted to the equation for a straight line. Data obtained for the pH dependence of Kinact were fitted using

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

Y represents the observed value for K_{inact}; Y_L and Y_H represent the pH independent value of K_{inact} at low and high pH, respectively; K_I is the dissociation constant for the modified cysteine; and H is the hydrogen ion concentration.

Results

Inactivation of aspartase by NEM

Aspartase was inactivated by incubation with various concentrations of NEM in 10 mM Hepes buffer, pH 8.0 (Massey and Lenard, 1987). The time course for inactivation of the enzyme depended on the concentration of the reagents used.

As shown in Fig. 1A, the first-order plots were not linear. The plot of residual activity versus incubation time yielded a biphasic inactivation pattern: initially rapid, and then slowing, indicating that there may exist two types of modifiable sulfhydryl residues. From the slopes of the faster inhibition phase obtained at dif-

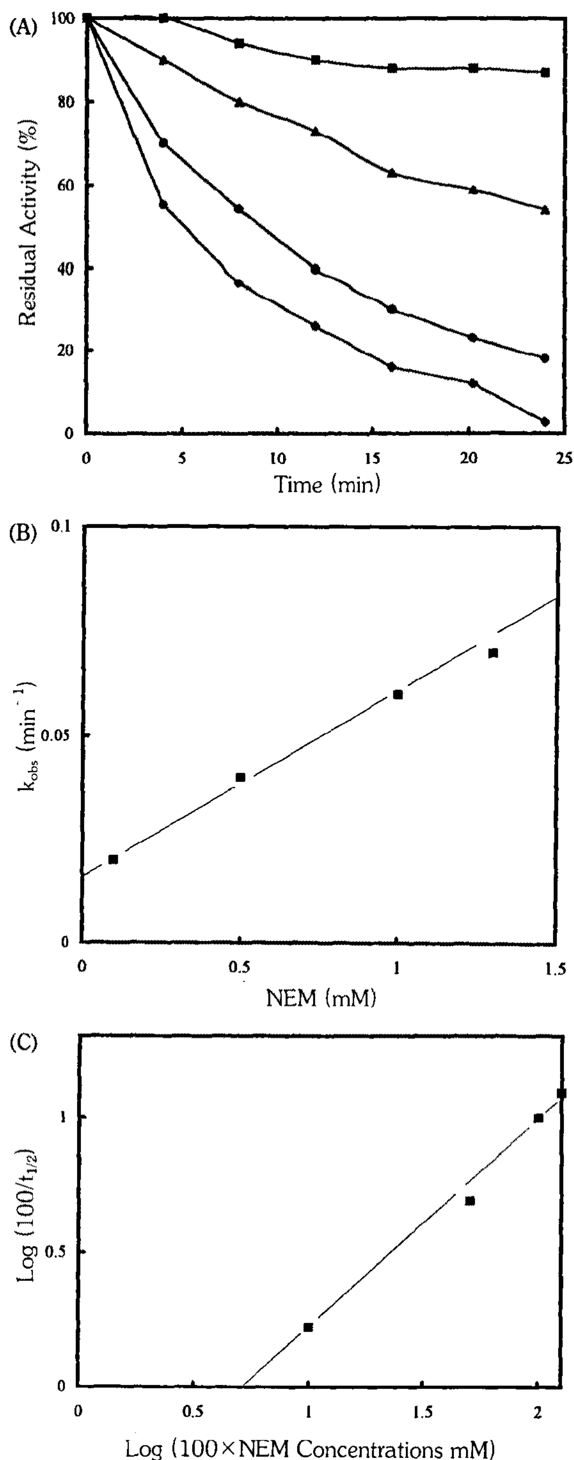

Fig. 1. (A) Inactivation of aspartase by NEM. The enzyme (0.5 mg) was incubated in a solution (0.5 ml) 10 mM Hepes buffer (pH 8.0) at 0°C. The reaction was started by the addition of indicated NEM concentrations and aliquots of modified enzyme were assayed for remaining activity at indicated time intervals. The concentrations of NEM were as follows: ■ : 0.1 mM NEM, ▲ : 0.5 mM NEM, ● : 1.0 mM NEM and ◆ : 1.3 mM NEM. (B) Plot of second order rate constant for the inactivation of aspartase obtained at various concentrations of NEM. (C) Double-logarithmic plot of the half-times of inactivation of aspartase at various concentrations of NEM.

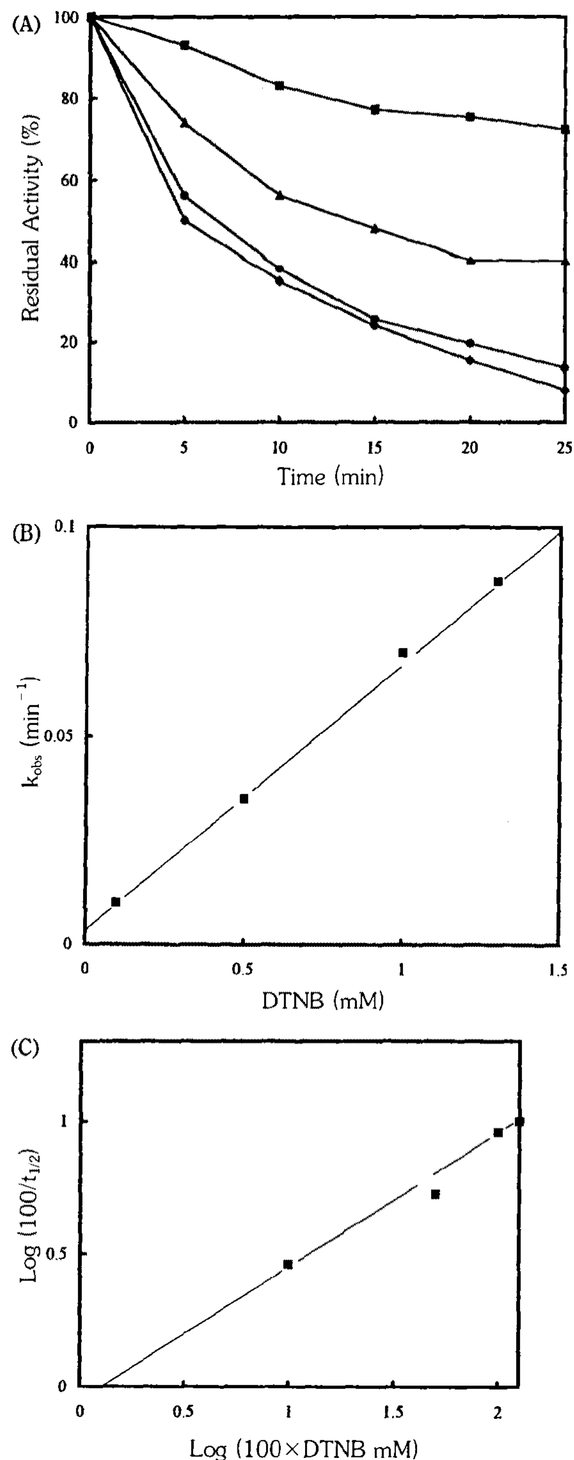

Fig. 2. (A) Inactivation of aspartase by DTNB. The enzyme (0.5 mg) was incubated in a solution (0.5 ml) 10 mM Hepes buffer (pH 8.0) at 0°C. The reaction was started by the addition of indicated DTNB concentrations and aliquots of modified enzyme was assayed for remaining activity at indicated time intervals. The concentrations of DTNB were as follows: ■ : 0.1 mM DTNB, ▲ : 0.5 mM DTNB, ● : 1.0 mM DTNB and ◆ : 1.3 mM DTNB. (B) Plot of second order rate constant for the inactivation of aspartase obtained at various concentrations of DTNB. (C) Double-logarithmic plot of the half-times of inactivation of aspartase at various concentrations of DTNB.

ferent concentrations of NEN, the second order rate constant for inactivation was 65 $M^{-1} \cdot min^{-1}$ (Fig. 1B). A double logarithmic plot of the reciprocal of the half time of inactivation against NEM concentration yielded reaction order of 0.83 with respect to the inhibitor (Fig. 1C), suggesting that there is one essential sulfhydryl residue per active enzyme molecule.

Inactivation of aspartase by DTNB

The effect of DTNB, which is a cysteine modification reagent, on the activity of aspartase was examined in a 10 mM Hepes buffer, pH 8.0 (Fig. 2A). The reaction of aspartase with DTNB caused a complete loss of enzyme activity. A residual activity versus time course showed a biphasic mode: initially rapid, and then slowing, indicating the existence of two types of cysteine residues whose modification affects enzyme activity. From the initial fast inactivation reaction represented in Fig. 2A. The second order rate constant of 42 $M^{-1} \cdot min^{-1}$ was determined from the slope of the linear relationships between the Kobs values and DTNB concentration (Fig. 2B). A double-logarithmic plot of the reciprocal of the half-time of inactivation against DTNB concentration yielded a reaction order of 0.5 with respect to the inhibitor (Fig. 2C), suggesting that there may be one essential cysteine residue per active enzyme molecule.

Inactivation of aspartase by DTNB and reactivation by DTT

The inactivation of aspartase by DTNB was reversed upon the addition of excess DTT (25 mM) (Miles, 1977; Olano *et al.*, 1992). The 90% inactivated enzyme was restored to more than 95% of the native enzyme after 20 min in dithiothreitol (Fig. 3). This inactivation and reactivation study suggested that the cysteine residue(s) is(are) possibly located at or near the active site of aspartase.

pH-dependence of aspartase inactivation by NEM

The pH-dependence of the inactivation constant for aspartase was determined in order to aid in the assignment of pK value with active site amino acid residue(s).

A pK value of the modified residue can be estimated from pH-dependence of the inactivation rate. The inactivation rate of aspartase by NEM was determined by using 0.3 mM NEM as a function of pH. At both extreme pH values, the process is first order in NEM as is true at pH 8.0 (data not shown). Thus, a 0.3 mM concentration of NEM was used at all pH values. The dependence of inactivation rate on the NEM concentration was obtained from pH 6.0 to 10.5. As shown in Fig. 4, the inactivation rate decreases below a pK of about 8.3, suggesting that the apparent pK value was approximately 8.3, and the inactivation was likely due to cysteine residue modification.

Protective effects of various compounds

The inactivation of aspartase activity by chemical modification does not always directly imply that these chemically modifiable residues are present at the active site (Nakanishi *et al.*, 1989). However, protection of an enzyme activity by a substrate against inactivation may suggest whether the amino acid residues that are pro-

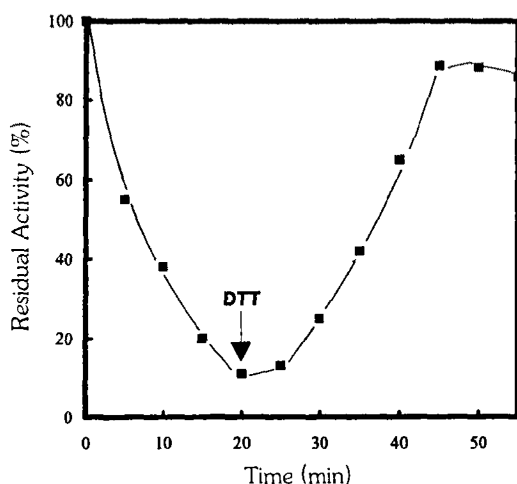

Fig. 3. Reactivation of DTNB-modified aspartase by dithiothreitol. The enzyme (0.5 mg) was allowed to react with DTNB (1 mM) in a solution 0.5 ml containing 10 mM Hepes (pH 8.0) at 0°C. DTNB-inactivated aspartase was reactivated by the addition of dithiolthreitol (25 mM) within 20 min.

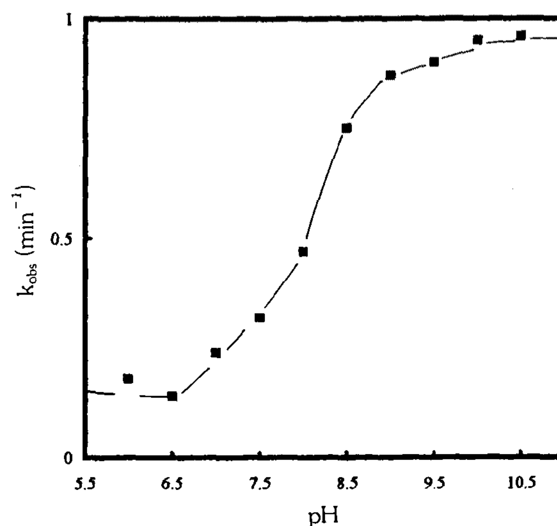

Fig. 4. pH-dependence of inactivation rate. The enzyme (0.06 mg) was incubated at 0°C with 0.3 mM NEM and 10 mM buffer at various pH values. Aliquots were taken at various time intervals for determination of the residual activity.

Table 1. Protective effects on aspartase by various compounds against NEM inactivation

Reagent concentration (mM)	Residual activity (%)
native enzyme	100
aspartate (10)+$MgSO_4$ (4)+NEM (0.1)	96.3
aspartate (10)+NEM (0.1)	99
$MgSO_4$ (4)+NEM (0.1)	51.2
aspartate (10)+EDTA (1)+NEM (0.1)	79.3
fumarate (10)+NH_4Cl (10)+NEM(0.1)	40
fumarate (10)+NEM (0.1) 54.2	54.2
succinate (10)+NEM (0.1) 55.4	55.4
NH4Cl (10)+$MgSO_4$ (4)+NEM (0.1)	60.3
EDTA (1)+NEM (0.1)	56.1
NEM (0.1)	59.8

Aspartase was incubated with 0.1 mM NEM and indicated concentration of each reagent for 30 min.

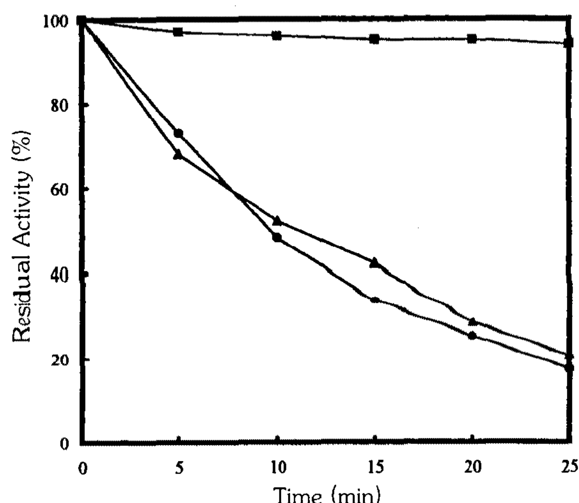

Fig. 5. Effects of substrate and structural analogue on protective effect. The enzyme (0.2 mg) was allowed to react at 0°C with 10 mM D-aspartate and L-aspartate with 0.1 mM NEM in a solution of 0.5 ml containing 10 mM Hepes buffer (pH 8.0). ■ : with L-aspartate, ▲ : with D-aspartate and ● : none.

tected from modification are located at or near the active site. As shown in Table 1, L-aspartate markedly protected the enzyme against NEM inactivation. In contrast, other related substrates and analogues such as fumarate, NH_4OH and succinate, exhibited little protective effects at the same concentration, possibly due to the low affinity of the compounds towards the active site cysteine residue.

In order to examine whether or not the substrate and its structural analogues have protective effects against NEM modification, L-aspartate and D-aspartate were incubated with NEM, respectively. As shown in Fig. 5, L-aspartate completely protected against NEM inactivation, but D-aspartate did not.

Discussion

NEM and DTNB react with sulfhydryl groups such as cysteine residue (Melchior and Fahrney, 1970). The following experimental evidence strongly supports the possibility that the inactivation was due to the modification of cysteine residue(s). (1) the enzyme was completely inactivated by sulfhydryl reagents such as NEM and DTNB. (2) the enzyme activity was almost restored by dithiothreitol treatment. (3) the pH-dependence of the inactivation rate indicated the involvement of a residue having a pK value of about 8.3.

The biphasicity as mentioned under Results is most likely a result of two or more different cysteines which react with different rates with NEM and DTNB. The biphasicity nature is not due to a loss of reagent since the rate of both phases are significantly faster than the rate of reagent loss.

The second order rate constants obtained from the reaction of NEM and DTNB with aspartase (65 $M^{-1} \cdot min^{-1}$ and 42 $M^{-1} \cdot min^{-1}$ at pH 8.0) were similar to the values, which were reported previously for the reaction of the enzyme transamidinase (74 $M^{-1} \cdot min^{-1}$) (Cho and Lee, 1993). The reactivity of the cysteine may be similar in different enzymes, indicating that the reaction order for NEM and DTNB may be similar in different enzymes also. Inactivation by DTNB and reactivation by dithiothreitol of aspartase imply that the inactivation of aspartase can be attributed not just to change in the conformation of the enzyme but to modification of sulfhydryl group, indicating that cysteine residue(s) is(are) possibly located at or near the active site of aspartase.

The pH-dependence of the NEM inactivation rate gives a pK of 8.3 for the modified cysteine modified concomitant with activity loss. 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 catalysis. One with pK 7.2 must be deprotonated for activity and the other with pK of 6.6 must be protonated in the V/K profile for aspartate. The profile represents the titration of a residue on enzyme in the enzyme: Mg^{2+} complexes. The pK of 8.3 obtained in these studies is likely this same residue with the pK 7.2. This group is most likely responsible for ammonia expulsion of carbocation intermediate of aspartate.

Protective effects against NEM inactivation by the addition of L-aspartate, the substrate for aspartase, support the hypothesis that modification occurs at or near the enzyme active site. Previous work has shown that

L-aspartate binds only at the active site and that at higher pH the binding of aspartate requires the presence of an activator and divalent metal ions (Falzone *et al.*, 1988). The role of divalent metal ions appears to be rather complex. The enzyme was observed to have an absolute requirement for a divalent metal ion activator at higher pH (Rudolph and Formm, 1971), with some indication that aspartase may possess activity in the absence of divalent metal ions at low pH. Alkaline earth metals such as Mg^{2+} and transitional metals such as Mn^{2+} have been shown to provide some degree of activation (Wilkerson and William, 1961). In summary, the inactivation of native enzyme by NEM and DTNB in the presence of aspartate suggests the involvement of cysteine in the active site.

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