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STUDIES ON ASPARTASE

II. ROLE OF SULFHYDRYL GROUPS IN ASPARTASE FROM *ESCHERICHIA COLI*

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Summary

Aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1) of *Escherichia coli* W contains 38 half-cystine residues per tetrameric enzyme molecule. Two sulfhydryl groups were modified with *N*-ethylmaleimide or 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) per subunit, while 8.3 sulfhydryl groups were titrated with *p*-mercuribenzoic acid. In the presence of 4 M guanidine · HCl, 8.6 sulfhydryl groups reacted with DTNB per subunit.

Aspartase was inactivated by various sulfhydryl reagents following pseudo-first-order kinetics. Upon modification of one sulfhydryl group per subunit with *N*-ethylmaleimide, 85% of the original activity was lost; a complete inactivation was attained concomitant with the modification of two sulfhydryl groups. These results indicate that one or two sulfhydryl groups are essential for enzyme activity. L-Aspartate and DL-*erythro*-β-hydroxyaspartate markedly protected the enzyme against *N*-ethylmaleimide-inactivation. Only the compounds having an amino group at the α-position exhibited protection, indicating that the amino group of the substrate contributes to the protection of sulfhydryl groups of the enzyme. Examination of enzymatic properties after *N*-ethylmaleimide modification revealed that 5-fold increase in the K_m value for L-aspartate and a shift of the optimum pH for the activity towards acidic pH were brought about by the modification, while neither dissociation into subunits nor aggregation occurred. These results indicate that the influence of the sulfhydryl group modification is restricted to the active site or its vicinity of the enzyme.

Abbreviations: DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); MES, 2-(*N*-morpholino)-ethanesulfonic acid; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; TAPS, tris-(hydroxymethyl)-methylaminopropanesulfonic acid; CAPS, cyclohexylaminopropanesulfonic acid.

Introduction

Aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1) which catalyzes the reversible conversion of L-aspartate to fumarate and NH_4^+ has recently been purified to homogeneity from *Escherichia coli* [1,2]. Molecular weight, subunit structure, amino acid composition, and enzymatic properties of the enzyme from *E. coli* W were described in a previous report [1]. The enzyme has a molecular weight of 193 000 and is composed of seemingly identical four subunits. The enzyme contains 38 half-cystine residues per tetrameric enzyme molecule as determined by amino acid analysis. In addition to our observation [1], several other investigators showed that *p*-mercuribenzoate inactivated aspartase of various microorganisms [3—5], suggesting that sulfhydryl group(s) of the enzyme protein plays an important role in the catalytic activity. However, detailed information is not available about the nature and the function of the sulfhydryl groups in connection with the structure and the reaction mechanism of the enzyme. The present investigation was undertaken to obtain a clue for elucidation of these problems mainly by means of chemical modification of the sulfhydryl groups.

Materials and Methods

Chemicals

p-Mercuribenzoate, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB), *N*-ethylmaleimide, iodoacetamide, D-aspartic acid, DL-α-methylaspartic acid, DL-β-methylaspartic acid, dithiothreitol, Tris, 2-(*N*-morpholino)-ethanesulfonic acid (MES), *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES), tris-(hydroxymethyl)-methylaminopropanesulfonic acid (TAPS) and cyclohexylaminopropanesulfonic acid (CAPS) were from Sigma. *p*-Mercuribenzoate was purified by the method of Boyer [6] using repeated dissolution in aqueous NaOH and precipitation with HCl. DL-*erythro*-β-Hydroxyaspartic acid was from Calbiochem. *N*-ethyl-[1-^{14}C]maleimide (11 Ci/mol) was from New England Nuclear. Sephadex G-25 was from Pharmacia. All other chemicals were of analytical grade.

Enzyme preparation

Aspartase was purified from *E. coli* W cells as described previously [1]. The enzyme preparations used in this investigation were homogeneous as judged by ultracentrifugation and polyacrylamide gel disc electrophoresis.

Enzyme assay

The activity of aspartase was routinely determined spectrophotometrically by measuring the formation of fumarate following the increase in absorption at 240 nm at 30° C. The standard assay system contained, in a total volume of 1.0 ml, 0.1 M sodium L-aspartate (pH 7.4), 2 mM $MgCl_2$, 0.1 M Tris · HCl buffer (pH 7.4), and the enzyme. The reaction was initiated by the addition of the enzyme.

Sulfhydryl group determination

Prior to chemical modification, the enzyme was freed from excess thiol by

gel filtration on a small column of Sephadex G-25 (1 cm × 15 cm) equilibrated with 20 mM potassium phosphate buffer, pH 6.6.

Titration of enzyme with *p*-mercuribenzoate was carried out according to the method of Boyer [6]. Determination of sulfhydryl groups with DTNB was carried out according to the method of Ellman [7]. The number of sulfhydryl groups reacted per subunit was calculated with a molar extinction coefficient of $1.36 \cdot 10^4$ at 412 nm for free 5-thio-2-nitrobenzoic acid.

Number of sulfhydryl groups modified with *N*-ethylmaleimide was determined as follows. The enzyme was incubated with ^{14}C-labeled *N*-ethylmaleimide in the presence of Tris · HCl buffer, pH 7.4, in a total volume of 1.5 ml at 0° C. At various time intervals, 0.1 ml portions of the incubated mixture were transferred into test tubes containing 0.9 ml each of 10 mM 2-mercaptoethanol for the termination of the reaction. After removal of an aliquot for assay of the residual activity, 0.1 ml of 1% bovine serum albumin was added as carrier, and the protein in the solution was precipitated by adding 2 ml of 10% trichloroacetic acid. The precipitate formed was washed three times with 10% trichloroacetic acid on a glass fiber filter (Whatman, 2.5 cm GF/C), washed with ether, airdried, and was transferred into a vial together with the filter. The protein fraction was dissolved in 0.5 ml of 90% formic acid and the radioactivity was determined in a Beckman Model LS-230 liquid scintillation spectrometer with the use of the toluene-ethanol solvent system [8] as scintillator. The number of sulfhydryl groups was calculated from the amount of radioactive *N*-ethylmaleimide incorporated into protein.

Other determinations

Polyacrylamide gel disc electrophoresis was carried out according to the method of Davis [9]. Protein concentration was determined by the method of Lowry et al. [10] using bovine serum albumin as standard. All spectrophotometric determinations were carried out with a Hitachi 124 recording spectrophotometer equipped with a constant temperature cell housing.

Results

Sulfhydryl group determination

Determination of the reactive sulfhydryl group in aspartase was carried out by using *N*-ethylmaleimide, DTNB and *p*-mercuribenzoate. In the native enzyme, approximately 2 out of 9.5 sulfhydryl groups reacted with *N*-ethylmaleimide and DTNB per subunit of the enzyme as shown in Table I. When the enzyme was denatured in 4 M guanidine · HCl, 8.6 sulfhydryl groups reacted with DTNB per subunit. When determination of the sulfhydryl groups was carried out in the presence of as low as 0.3 M guanidine · HCl, 8 to 9 sulfhydryl groups per subunit were already susceptible to the reaction with DTNB.

The results of titration of the enzyme with *p*-mercuribenzoate are shown in Fig. 1. In contrast to the reactivity of *N*-ethylmaleimide and DTNB, 8.3 mol of *p*-mercuribenzoate reacted per mol of subunit.

Inactivation of aspartase by sulfhydryl reagents

When the enzyme was incubated with various sulfhydryl reagents, the

TABLE I

SULFHYDRYL GROUPS IN ASPARTASE

The reaction mixture for modification of sulfhydryl groups with DTNB contained 8.8 mM potassium phosphate buffer, pH 7.4, 0.80 mg (without guanidine · HCl) or 0.32 mg (with guanidine · HCl) of enzyme protein, 0.21 mM EDTA, designated concentration of guanidine · HCl, and 63 μM DTNB in a total volume of 2.4 ml. The reaction was started by addition of DTNB and the increase in absorbance was measured at 412 nm at room temperature. Number of sulfhydryl groups modified with *N*-ethylmaleimide and *p*-mercuribenzoate was obtained from the results in Figs 1 and 4.

Reagents	Sulfhydryl groups modified per subunit
^{14}C-labeled *N*-ethylmaleimide	1.8
DTNB	2.0
DTNB in 0.3 M guanidine · HCl	8.5
DTNB in 4 M guanidine · HCl	8.6
p-Mercuribenzoate	8.3

enzyme activity decreased following pseudo-first order kinetics as shown in Fig. 2. The rate of the inactivation decreased in the order, *p*-mercuribenzoate > DTNB > *N*-ethylmaleimide > iodoacetamide. The inactivation by *p*-mercuribenzoate occurred quite rapidly and completed in 30 s. When the enzyme was incubated with DTNB, *N*-ethylmaleimide and iodoacetamide for 10 min, the residual activity was 10, 15 and 90%, respectively.

In Fig. 3 is shown the effect of pH on the rate of inactivation of the enzyme by DTNB. The results indicate that the rate of inactivation by DTNB increases, as pH increases. For example, the rate constants were 0.023 and 0.33 at pH 7.0 and 9.5, respectively. Inactivation of the enzyme by *p*-mercuribenzo-

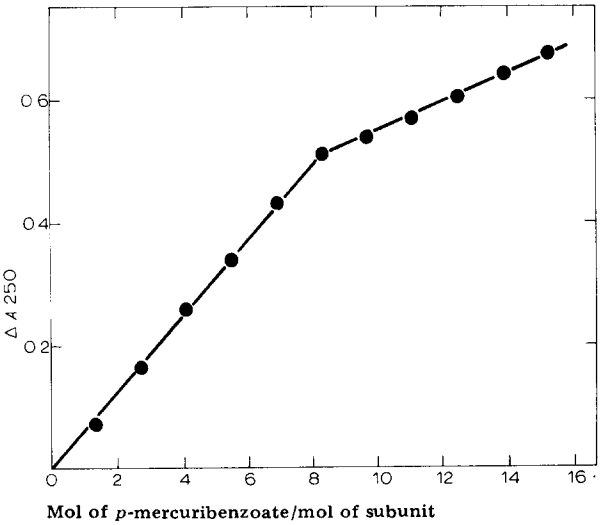

Fig. 1. Titration of aspartase with *p*-mercuribenzoate. Changes in absorbance at 250 nm were measured approximately 10 min after the addition of various concentrations of *p*-mercuribenzoate to a mixture of 0.48 mg of enzyme protein and 0.1 M potassium phosphate buffer, pH 7.0 in a total volume of 2.0 ml at 30° C.

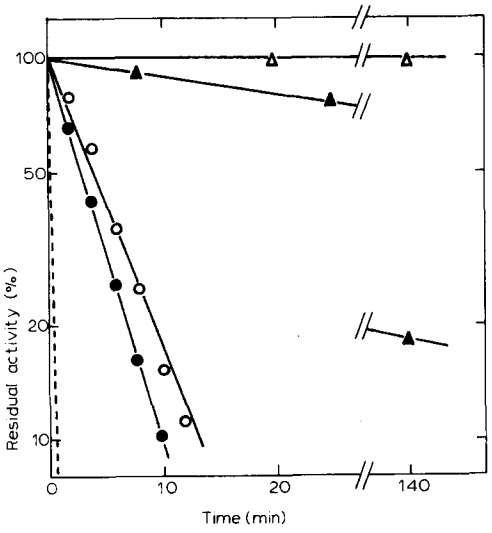

Fig. 2. Inactivation of aspartase by various sulfhydryl reagents. Aspartase (20 μg of protein) in 0.1 M potassium phosphate buffer, pH 7.0, was allowed to react at 0° C with 0.2 mM sulfhydryl reagent in a total volume of 0.25 ml. Aliquots of the reaction mixture were removed and assayed for residual activity at indicated time intervals. △———△, control (in the absence of sulfhydryl reagents); ▲———▲, iodoacetamide; ○———○, *N*-ethylmaleimide; ●———●, DTNB; - - - - - -, *p*-mercuribenzoate.

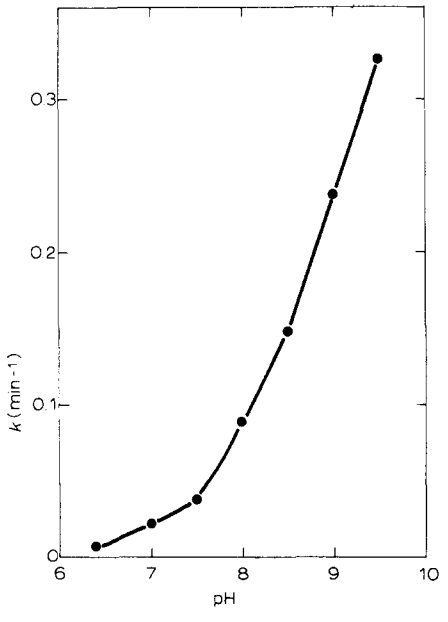

Fig. 3. Effect of pH on the rate of inactivation. The enzyme (27 μg of protein) was incubated at 0° C with 50 μM DTNB and 0.1 M Tris · HCl buffer at indicated pH in a total volume of 0.2 ml. Aliquots were taken at various time intervals for determination of the residual activity. The rate constants (k) were calculated as follows: $k = 2.303 \cdot 1/t$ (log C_O − log C_t), where C_O is original activity (100%) and C_t is relative activity after t min as per cent of the original activity.

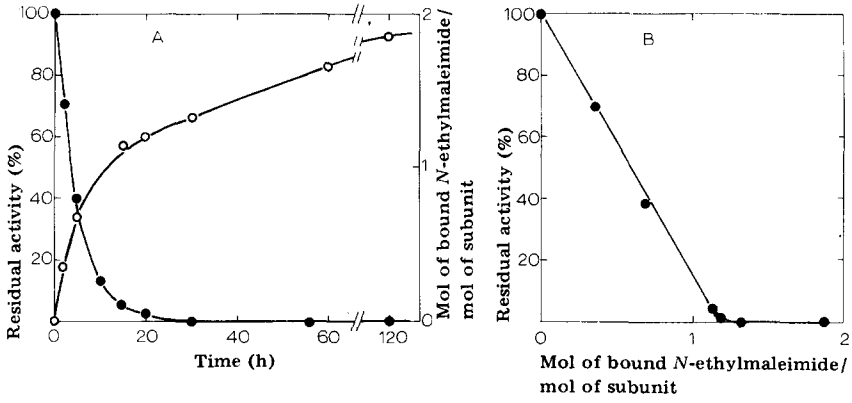

Fig. 4. Relationship between the number of *N*-ethylmaleimide bound to the enzyme and alteration of enzyme activity in the *N*-ethylmaleimide modification. The enzyme (0.82 mg) was incubated with 1.3 mM ^{14}C-labeled *N*-ethylmaleimide (2.91 · 10^6 dpm) in the presence of 33 mM Tris · HCl buffer, pH 7.4 in a total volume of 1.5 ml at 0° C. At various time intervals, residual activity (●——●) and the number of *N*-ethylmaleimide bound to the enzyme (○——○) were determined as described under Materials and Methods. Data in (A) were replotted as shown in (B).

ate or DTNB was reversed by the addition of dithiothreitol. For example, when 4 mM dithiothreitol was added soon after complete inactivation by DTNB, 80% of the original activity was restored in 30 min.

Relationship between enzymatic activity and sulfhydryl group modification

Mode of *N*-ethylmaleimide binding to the enzyme and the effect of the sulfhydryl group modification on the enzyme activity were examined with the use of ^{14}C-labeled *N*-ethylmaleimide (Fig. 4A). After 2 h of the reaction approximately two sulfhydryl groups were modified with ^{14}C-labeled *N*-ethylmaleimide with a concomitant disappearance of the activity. Data in Fig. 4A were replotted to illustrate the relationship between the sulfhydryl group modification and the degree of inactivation (Fig. 4B). Upon modification of one sulfhydryl group per subunit, 85% of the original activity was lost; a complete inactivation was attained concomitant with the modification of two sulfhydryl groups. Extrapolation of the line in Fig. 4B to zero activity indicates that complete inactivation corresponds to modification of an average of 1.2 sulfhydryl groups per subunit. When the enzyme was modified with DTNB, a value of 1.2 was also obtained.

Effect of substrates and related compounds on sulfhydryl group modification

In Fig. 5 is shown the effect of L-aspartate on the reactivity of sulfhydryl groups towards *N*-ethylmaleimide-modification. In the absence of L-aspartate, 1.8 sulfhydryl groups were modified with *N*-ethylmaleimide per subunit of the enzyme after a 2 h incubation. When 0.1 M L-aspartate was present, however, only 0.6 sulfhydryl group was modified. Thus, 1.2 sulfhydryl groups were protected by L-aspartate. By contrast, D-aspartate at the same concentration gave no effect on the modification. When the enzyme was modified with DTNB in the presence and absence of L-aspartate, 1.0 and 2.0 sulfhydryl groups were determined, respectively.

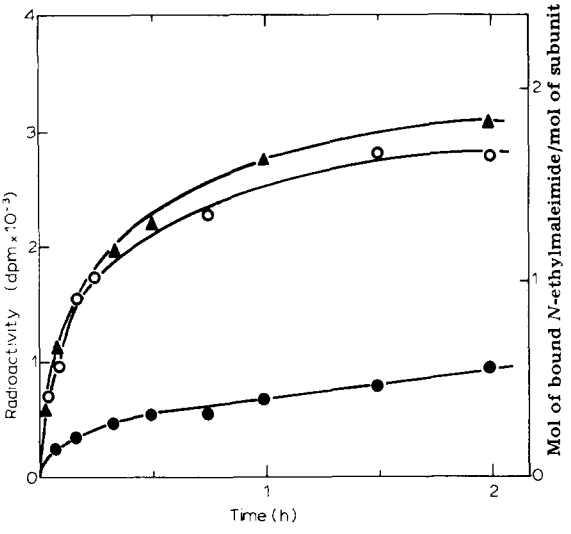

Fig. 5. Effect of L-aspartate on *N*-ethylmaleimide-modification. The number of *N*-ethylmaleimide bound to the enzyme was determined in the presence of 0.1 M L-aspartate or D-aspartate. The methods were the same as those described in Fig. 4, except that L-aspartate or D-aspartate was added. ▲———▲, no addition; ●———●, L-aspartate added; ○———○, D-aspartate added. pH of amino acids was adjusted to 7.4 with NaOH.

In order to examine whether or not the substrates and their structural analogues have a protective effect against the inactivation by sulfhydryl reagents, the enzyme was exposed to *N*-ethylmaleimide in the presence of various compounds. As shown in Table II, L-aspartate markedly protected the enzyme activity and combination of fumarate and NH_4^+ exhibited a protection to a lesser extent. However, when tested individually, fumarate and NH_4^+ gave no protection. In the presence of EDTA, L-aspartate did not give protection at all. $MgCl_2$ exhibited no protection, when added singly.

Among the substrate analogues tested, DL-*erythro*-β-hydroxyaspartate was

TABLE II

PROTECTIVE EFFECT OF REACTION COMPONENTS AGAINST INACTIVATION OF ASPARTASE BY *N*-ETHYLMALEIMIDE

The enzyme (35 μg) was incubated with 38 μM *N*-ethylmaleimide in the presence of 0.1 M Tris · HCl buffer, pH 7.4, and indicated compounds (pH was adjusted to 7.4 with NaOH) in a total volume of 0.25 ml at 0°C. After 45 min, aliquots were removed for the assay of the residual activity. The activity was expressed as relative value to the original enzyme activity.

Additions (mM)	Residual activity (%)
None	39
L-Aspartate (10) + $MgCl_2$ (4)	93
L-Aspartate (10) + EDTA (4)	41
Fumarate (10) + NH_4Cl (100) + $MgCl_2$ (4)	78
Fumarate (10) + $MgCl_2$ (4)	35
NH_4Cl (100) + $MgCl_2$ (4)	32
$MgCl_2$ (4)	37
EDTA (4)	39

TABLE III

EFFECT OF SUBSTRATE ANALOGUES ON INACTIVATION OF ASPARTASE BY *N*-ETHYLMALEIMIDE

The experimental conditions were the same as those described in Table II, except that indicated compounds (pH was adjusted to 7.4 with NaOH) and 4 mM $MgCl_2$ were added.

Additions (0.1 M)	Residual activity (%)
None	38
DL-*erythro*-β-Hydroxyaspartate	96
DL-α-Methylaspartate	58
L-Glutamate	38
DL-β-Methylaspartate	36
D-Aspartate	34
L-Malate	30
Succinate	27

most effective and DL-α-methylaspartate exhibited a protection to a lesser extent as shown in Table III. D-Aspartate and succinate did not give protection, although they are relatively potent competitive inhibitors: inhibition constants are 10 and 26 mM for D-aspartate [1] and succinate, respectively. When DTNB was used, similar results were obtained as to the effect of substrates and related compounds.

Effect of sulfhydryl group modification on enzymatic properties

The apparent K_m value for L-aspartate was determined before and after the sulfhydryl group modification. The K_m value of the native enzyme was 2.5 mM at pH 7.4, while that of the *N*-ethylmaleimide-modified enzyme was 12.5 mM, a five-fold higher value than the K_m of the native enzyme as shown in Fig. 6. The *N*-ethylmaleimide-modified enzyme preparation used in this experi-

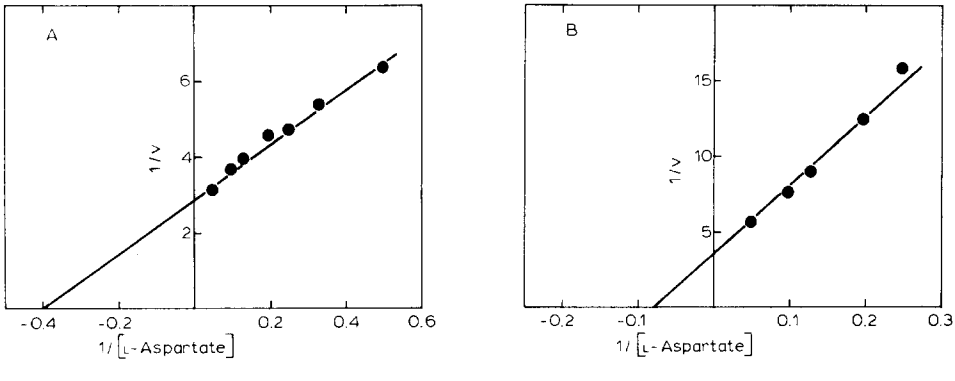

Fig. 6. Effect of *N*-ethylmaleimide-modification on K_m for L-aspartate. The enzyme activity was determined by the standard assay system, except that L-aspartate concentration was varied. Double reciprocal plots were obtained according to the method of Lineweaver and Burk [11]. The enzyme was incubated with 0.6 mM *N*-ethylmaleimide in the presence of 19 mM potassium phosphate buffer, pH 6.6. When the residual activity reached 15% of the original activity, dithiothreitol was added to the reaction mixture to a final concentration of 13 mM for termination of the reaction. The enzyme preparation thus obtained was used as *N*-ethylmaleimide-modified enzyme. (A), native enzyme (2.3 μg of protein/ml); (B), *N*-ethylmaleimide-modified enzyme (22 μg of protein/ml).

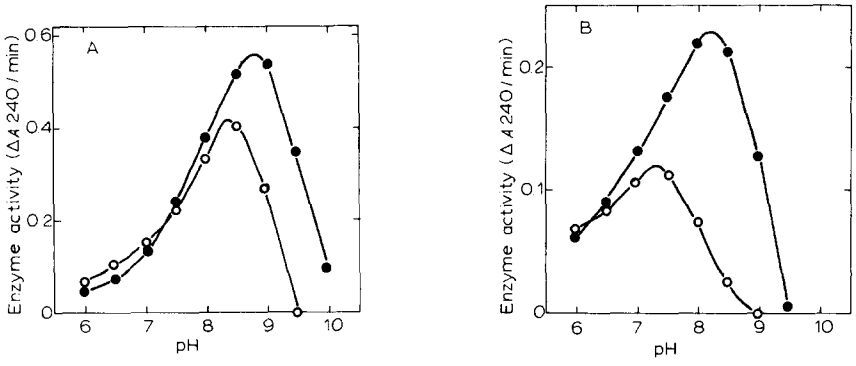

Fig. 7. Effect of *N*-ethylmaleimide-modification on pH-activity profile. The reaction mixture contained 0.1 M L-aspartate (pH was adjusted to indicated value with KOH), designated buffer (0.1 M), the enzyme, and 2 mM $MgCl_2$ (●———●) or in its absence (○———○) in a total volume of 1.0 ml. MES-KOH (pH 6.0—7.0), HEPES-KOH (pH 7.0—8.0), TAPS-KOH (pH 8.0—9.0), CAPS-KOH (pH 9.5—10.0). Temperature was 30°C. (A), native enzyme (1.4 μg/ml); (B), *N*-ethylmaleimide-modified enzyme (9.1 μg/ml). *N*-Ethylmaleimide-modified enzyme (residual activity, 11%) was obtained as described in Fig. 6.

ment retained 15% of the original activity and had an average of one *N*-ethylmaleimide-modified sulfhydryl group per subunit.

Fig. 7 shows the pH-activity profile of the native and the *N*-ethylmaleimide-modified enzyme preparations. In a previous report was shown that the optimum pH for the activity was altered in the presence of added divalent metal ions [1]. The optimum pH for the activity of the native enzyme was 8.8 in the presence of 2 mM Mg^{2+}, while it shifted to 8.2 upon *N*-ethylmaleimide-modification. In the absence of the added metal ions, the optimum pH values for the native and the *N*-ethylmaleimide-modified enzyme preparations were 8.3 and 7.3, respectively. Metal requirement was also varied by *N*-ethylmaleimide-modification. For example, when the native enzyme was employed at pH 9.0 in the absence of the added metal ions, the enzyme activity was a half of that assayed in the presence of the added metal ions. In contrast, when the *N*-ethylmaleimide-modified enzyme was used, there was no enzyme activity detected in the absence of the added metal ions.

As already shown, the substrate saturation profile of aspartase exhibited a cooperative nature at higher pH values [1—3]. When the substrate saturation curve was plotted by using *N*-ethylmaleimide-modified enzyme, a homotropic effect was also observed at pH 8.6. The Hill coefficients of L-aspartate were 1.5 for both the native and the *N*-ethylmaleimide-modified enzyme preparations.

In order to test whether or not the enzyme undergoes a change in its quaternary structure upon sulfhydryl group modification, polyacrylamide gel disc electrophoresis was carried out. The results (not shown) indicated that both the partially and the completely inactivated enzyme preparations have the same mobility as the native enzyme at pH 8.9.

Discussion

The reactivity of the sulfhydryl groups and the mode of inactivation of *E. coli* aspartase varied to a considerable extent depending upon the sulfhydryl

reagents and the interaction with ligands. Two sulfhydryl groups per subunit were modified with DTNB and *N*-ethylmaleimide, while eight reacted with *p*-mercuribenzoate. Almost all of the sulfhydryl groups seem to be located at or near the surface of the enzyme structure, in consideration of the fact that 8 to 9 sulfhydryl groups readily reacted with DTNB in guanidine · HCl as low as 0.3 M, where no large conformational alteration such as unfolding or dissociation into subunits occurred, as examined by ultracentrifugation and circular dichroism analysis (unpublished observation). The fact that modification of an average of 1.2 sulfydryl groups per subunit resulted in complete loss of the enzyme activity indicates that one or two sulfhydryl groups are needed for the activity. The results of the experiments shown in Fig. 5 suggest, however, that one sulfhydryl group is enough for the activity. That is, in the absence of the substrate, two sulfhydryl groups reacted with *N*-ethylmaleimide, while only one reacted in the presence of the substrate. An additional support is provided by the following experiments. Aspartase was not readily inhibited by arsenite and 50% inhibition was attained in the presence of as high as 10 mM arsenite. Arsenite inhibition is usually taken as evidence for the presence of the two sulfhydryl groups in close proximity [12—14]. A combination of 10 μM *o*-phenanthroline and 5 μM Cu^{2+}, which is known to catalyze air-oxidation of vicinal sulfhydryl groups to yield disulfide linkage [15] did not inhibit aspartase, at all.

Inspection of the protective effect of various compounds revealed that only the compounds having an amino group at the α-position were effective. This suggests that the interaction between the sulfhydryl group of the enzyme and the α-amino group of the substrate plays an important role in the deamination reaction catalyzed by aspartase.

Role of divalent metal ions appears to be rather complex. As previously shown [1], the requirement of the enzyme for divalent metal ions is essentially undetectable at neutral pH, whereas an absolute requirement is observed at alkaline pH; attempts to remove intrinsic metal ions, if any, from enzyme preparations and the assay mixture by means of solvent extraction with dithizone did not improve the situation. Nevertheless, addition of EDTA to the assay system resulted in a complete inhibition of the activity [1]. Protective effect of L-aspartate against sulfhydryl group modification was abolished upon addition of EDTA.

Modification of the catalytically essential sulfhydryl group did not result in alteration of the molecular size. In contrast, catalytic properties, such as pH optimum for the activity and the apparent K_m for the substrate were subjected to a considerable modification. These results indicate that the influence of the sulfhydryl group modification is restricted to the active site or its vicinity of the enzyme molecule.

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