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INTERACTION BETWEEN L-ASPARTIC ACID AND L-ASPARAGINASE FROM *ESCHERICHIA COLI*: BINDING AND INHIBITION STUDIES

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Experiments using equilibrium dialysis and fluorescence quenching provided direct evidence that approximately four moles of L-aspartic acid were bound per mole of tetrameric L-asparaginase from *Escherichia coli*, with a dissociation constant on the order of 60–160 μM. In addition, a set of weaker binding sites with a dissociation constant in the millimolar range were detected. Kinetic studies also revealed that L-aspartic acid inhibited L-asparaginase competitively, with an inhibition constant of 80 μM at micromolar concentrations of L-asparagine; at millimolar concentrations of the amide, an increase in maximal velocity but a decrease in affinity for L-asparagine were observed. L-Aspartic acid at millimolar levels again displayed competitive inhibition. These and other observations suggest that L-aspartic acid binds not only to the active site but also a second site with lower intrinsic affinity for it. The observed "substrate activation" is most likely attributable to the binding of a second molecule of L-asparagine rather than negative cooperativity among the tight sites of the subunits of this tetrameric enzyme. Further support for L-aspartic acid binding to the active site comes from experiments in which the enzyme, when exposed to various group-specific reagents suffered parallel loss of catalytic activity and in its ability to bind L-aspartic acid.

Different commercial preparations of *Escherichia coli* L-asparaginase were found to contain ~ 2–4 moles of L-aspartic acid; these were incompletely removed by dialysis, but could be removed by transamination or decarboxylation. Efficiency of dialysis increased with increasing pH. Taken together, this set of results is consistent with the existence of a covalent β-aspartyl enzyme intermediate.

KEY WORDS: L-Aspartic acid, L-asparaginase, L-asparagine, inhibition, binding, DONV.

INTRODUCTION

L-Asparaginase [EC 3.5.1.1] from *Escherichia coli*, (EC-2), a tetrameric enzyme consisting of identical subunits, has been extensively studied because of its therapeutic value as an antineoplastic agent. The interaction of this enzyme with its product L-aspartic acid, has however not been characterized adequately: Campbell and Mashburn[1] reported that L-aspartic acid failed to inhibit the amidohydrolysis of L-asparagine; conversely, Ehrman *et al.*[2] observed a progressive incorporation of [^{18}O] into L-aspartic acid, when the amino acid was incubated with L-asparaginase in $H_2[^{18}O]$. Furthermore, Citri *et al.*[3] demonstrated that L-aspartate protected L-asparaginase against iodination, proteolysis, and thermal inactivation. In view of these and other contradictory observations, as well as our finding that each mole of commercial crystalline preparations

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of *E. coli* L-asparaginase invariably contained ~2–4 moles of "free" L-aspartic acid, we undertook a study of the interaction between this amino acid and the enzyme.

MATERIALS

Radiochemicals

L-[U-^{14}C]Asparagine (specific radioactivity 133 mCi/mmole), L-[U-^{14}C]aspartic acid (specific radioactivity 226 mCi/mmole) and L-[4-^{14}C] aspartic acid (specific radioactivity 17.6 mCi/mmole) were products of Amersham Searle Corp., Arlington Heights, IL. Phenylglyoxal and diethylpyrocarbonate were obtained from Sigma Chemical Co. All other chemicals were of analytical reagent grade.

Enzymes

L-aspartate aminotransferase (GOT) and L-malate dehydrogenase (MDH), were products of Boehringer Mannheim Corp., Indianapolis, IN. Polyphenol oxidase (Tyrosinase) from mushrooms was obtained from Worthington Biochemical Corp., Freehold, NJ.

L-Asparaginase treated with 5-diazo-4-oxo-L-norvaline, 99% inactivated (DONV-asparaginase[4]) and the enzyme treated with tetranitromethane, 90% inactivated (nitro-asparaginase),[5] were generous gifts from Dr. R. E. Handschmacher, Yale Univ., New Haven, Connecticut.

Additional L-asparaginases from various sources were obtained from the Drug Development Branch, National Cancer Institute, Bethesda, MD.

Various batches of L-asparaginase form *E. coli* were generously provided by Dr. Harold Peck, Merck Sharp and Dohme Research Lab., Rahway, NJ: no dialysis step was used during purification and neither L-asparagine nor L-aspartic acid were used as protectors.[6]

METHODS

L-Asparaginase activity was measured by an enzyme-coupled spectrophotometric technique[7,8] or, when L-aspartic acid was examined as an inhibitor of the reaction, by radiometric methodology.[9] The product was analyzed by high voltage electrophoresis on Whatman 3M paper applying a current of 2500 V and 100 mA for 1 hr at 4° C.

L-Aspartic acid was measured by a coupled assay[8] or by automated column chromatography on the JEOL amino acid analyzer, model JLC-5AH, using the lithium citrate system of Benson.[10]

Equilibrium dialysis experiments were performed in lucite chambers (Gateway Immunosera, Chahokia, IL) at atmospheric pressure, using constant sample and bath volumes of 50 μl. Aliquots from the two sides of the dialysis chamber were taken after equilibration at 4° C for 18 h and the radioactivity measured by scintillation spectrometry. Binding constants were analyzed by means of the Scatchard equation:[11]

$$v/A = n/K_d - v/K_d,$$

where v is the molar ratio of bound L-aspartic acid to enzyme, A is the concentration of free L-aspartic acid; K_d is the overall association constant for the ligand, and n is the number of binding sites present on L-asparaginase.

Fluorescence quenching studies were carried out at 20° C with a Hitachi MPF-2A fluorescence spectrophotometer equipped with temperature control. The sample contained 10 nmoles of L-asparaginase in 2 ml of 0.05 M Tris buffer, pH 8.5. Titration was done by the addition of 2–5 μl aliquots of appropriate concentrations of L-aspartic acid, pH 8.5. The excitation and emission wavelengths used were 290 nm and 324 nm, respectively. Data were analyzed by the rearranged Klotz equation:[12]

$$\frac{1}{1 - \bar{x}} = \frac{1}{k_d}\left(\frac{A_0}{\bar{x}} - nE_0\right)$$

where $\bar{x}$ equals the extent of saturation and is equivalent to % total quenching, k_d is the dissociation constant of the ligand, A_0 is the total ligand concentration; n is the number of ligand binding sites; and E_0 is the total enzyme concentration, in terms of L-asparaginase tetramers.

Preparation of "L-aspartate-free" L-asparaginase

E. coli L-asparaginase was dissolved in 0.01 M Tris buffer, pH 7.0 (30 mg/ml) and dialyzed against several changes of the same buffer for 48 h at 4° C. The resulting enzyme was termed "native enzyme." One ml aliquots of the native enzyme were: (a) treated with 0.19 mg NADH, 1.0 mg of neutralized α-ketoglutaric acid (α-KG), 2 μl GOT and 0.25 μl of MDH in 0.01 M Tris-HCl buffer, pH 7.0 for 72 h at 4° C; or (b) treated at pH 5.0, in 0.01 M sodium acetate buffer with 0.01 units of L-aspartate-β-decarboxylase purified from *E. coli* by the method of Shukuya and Schwert[13] for 72 h at 4° C and then dialyzed against 0.01 M Tris-HCl buffer, pH 7.0 for 48 h at 4° C. No L-aspartic acid could be detected in these preparations, hereafter referred to as "L-aspartate-free" enzymes.

Chemical Modification of Amino Acid Residues of L-Asparaginase

Six hundred μl aliquots (containing 19 mg protein) each of the preparations of dialyzed L-asparaginase and dialyzed L-aspartate-free L-asparaginase were: (a) treated with 3 μl of acetic anhydride for 1 h at 4° C and then dialyzed against 4 L of 0.01 M Tris-HCl buffer, pH 7.0 for 48 h; (b) dialyzed for 24 h against 50 ml of 0.2 M sodium nitrite in 0.05 M sodium acetate buffer, pH 4.8 and later redialyzed against 4 L of 0.01 M Tris-HCl buffer, pH 7.0 for another 24 h; (c) dialyzed for 24 h against 50 ml of 0.3 M 2-mercaptoethanol in 0.01 M Tris-HCl buffer, pH 7.0 at 4° C; (d) dialyzed against 50 ml of 0.3 M phenylglyoxal for 1 h at 25° C and then redialyzed against 4 L of 0.01 M Tris-HCl buffer, pH 7.0; (e) treated with 1.8 mg L-tyrosinase (polyphenoloxidase) for 1 h at 4° C and then dialyzed against 0.01 M Tris-HCl buffer, pH 7.0 for 48 h; (f) treated for 30 min with a freshly prepared ethanol solution of diethylpyrocarbonate to achieve a final concentration of 0.03 M at pH 7.0 on ice and then dialyzed for 24 h against 4 L of 0.01 M Tris-HCl buffer, pH 7.0 at 4° C for 24 h.

DONV-asparaginase and nitro-asparaginase preparations were dissolved in 0.01 M Tris-HCl buffer, pH 7.0 and then dialyzed against the same buffer at 4° C for 48 h prior to use.

RESULTS

L-Aspartate Associated with L-Asparaginase Preparations

In the course of experiments entailing the enzymatic digestion of chemically modified L-asparaginase with pronase, even control preparations not exposed to this protease

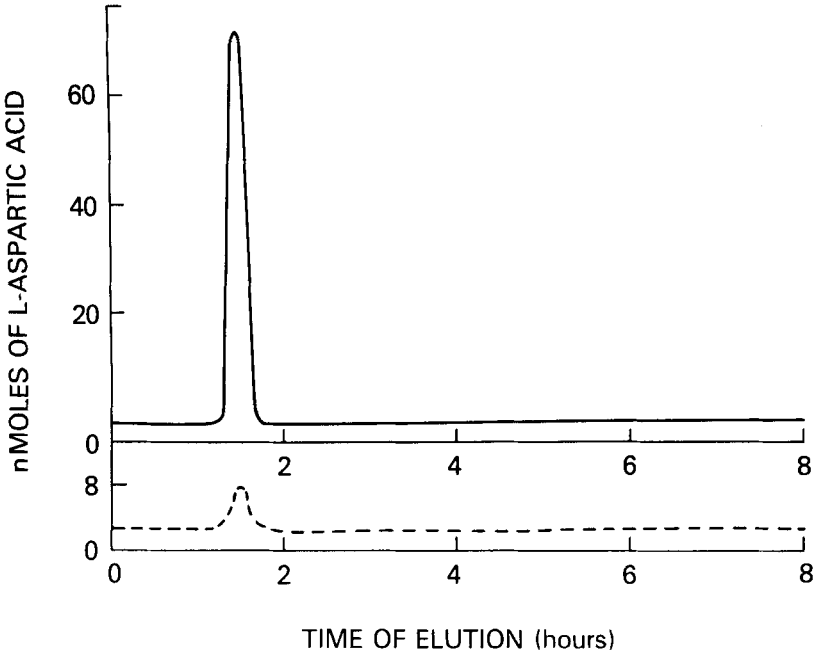

FIGURE 1 The elution pattern of amino acids on the long column of the amino acid analyzer using lithium citrate buffers. Analyses were carried out according to the procedures outlines in the Methods Section. 2.5 mg of *E. coli* L-asparaginase was analyzed; a. (———) without any treatment; and b. (– –) after exhaustively dialyzing against 4 L of 0.05 M Tris-buffer, pH 8.0 at 4° C for 48 h.

showed the presence of apparently free L-aspartic acid. No detectable quantity of any other amino acid was associated with the enzyme as adjudged by automated column chromatography on the amino acid analyzer (Figure 1). This prompted us to examine the source of the L-aspartic acid.

Ten batches of crystalline *E. coli* L-asparaginase from Merck Laboratories were found to contain L-aspartic acid in amounts ranging from 1.50 to 3.63 moles per mole of the enzyme (Table I). Considerable amounts of this amino acid resisted removal by dialysis at pH 7.5 (Table II). *E. coli* L-asparaginase preparations from Bayer and Squibb also contained L-aspartic acid, although not to the same extent as the preparations from Merck (Table II). These variations might be due to the use of different isolation procedures by these manufacturers. L-Asparaginases from other bacterial sources did not contain detectable amounts of free L-aspartic acid (Table II).

Effect of pH on Removal of L-aspartate

In order to examine the influence of pH on the affiliation of L-aspartic acid with crystalline preparations of L-asparaginase from *E. coli*, the enzyme was dialyzed against water or 0.01 M Tris-HCl buffer at pH's ranging from 5.0 to 9.0 (Figure 2). Dialysis of the enzyme preparations at alkaline pH effectively removed most of the free L-aspartic acid; under acidic conditions, only minimal amounts of L-aspartic acid were dialyzable.

To determine whether removal of L-aspartic acid might be associated with denaturation of the enzyme, the catalytic activity of L-asparaginase was studied after

TABLE I
Removal by dialysis of L-aspartate from various batches of *E. coli* L-asparaginase

Batch No.	L-Aspartate (nmoles/mole tetramer) before dialysis	After dialysis
CE-123	3.63	1.39
CE-124	3.23	0.65
CD-333	2.40	0.38
CD-371	1.50	0.36
CD-480	2.55	0.25
CD-554	2.80	0.49
CD-715	1.60	0.38
CD-726	2.20	0.34
CD-903	2.50	0.20
CD-925	3.20	0.25

To vials containing 10,000 U (30 mg protein)/vial, 1 ml of 0.01 M Tris-HCl buffer pH 7.5 was added; 0.5 ml of this solution was dialyzed against 4 L of the same buffer for 48 h at 4° C. Measurements of L-aspartate were done on dialyzed and undialyzed enzyme preparations by using the coupled spectrophotometric assay detailed in the Methods Section.

TABLE II
Estimation of free L-aspartate in L-asparaginases for various sources

Source	Moles of L-aspartate/mole of L-asparaginase	
	Before dialysis	After dialysis
L-Asparaginase from *E. coli*		
Merck, Sharp and Dohme (CD 925)	3.20	0.25
Bayer	1.25	0.98
Squibb	0.21	0.21
L-Asparaginase from *Erwinia carotovora*	0.00	0.00
L-Asparaginase from *Vibrio succinogenes*	0.00	0.00
L-Glutaminase–L-asparaginase from *Achromobacter sp.*	0.00	0.00

L-Aspartic acid and protein determinations of various preparations were done before dialysis and after dialysis against 0.05 M Tris HCl, pH 7.0 at 4° C, according to the methodology detailed in the Methods Section.

dialysis at various pH. Dialysis at alkaline pH produced a significant loss of the catalytic activity with L-asparagine as substrate; however, no such loss was seen when dialysis was carried out at pH 5.0 (Figure 2).

To examine the influence of various pH's on the binding of L-aspartic acid, equilibrium dialysis studies were carried out with enzyme preparations at pH 5, 7 and 9; and compared with analogous preparations previously subjected to exhaustive dialysis against buffers of the same pH. Studies with preparations dialyzed at pH 5.0 and 7.0 indicated a binding of about 4 moles of L-aspartate per mole of enzyme. As was mentioned, all of the L-aspartate was lost from preparations of L-asparaginase dialyzed at pH 9.0; this was accompanied by a 50% reduction in enzyme activity.

Binding Studies by Equilibrium Dialysis

The binding of L-aspartate to L-asparaginase was next examined by subjecting four preparations of L-asparaginase from *E. coli*, to equilibrium dialysis versus L-[4-^{14}C]

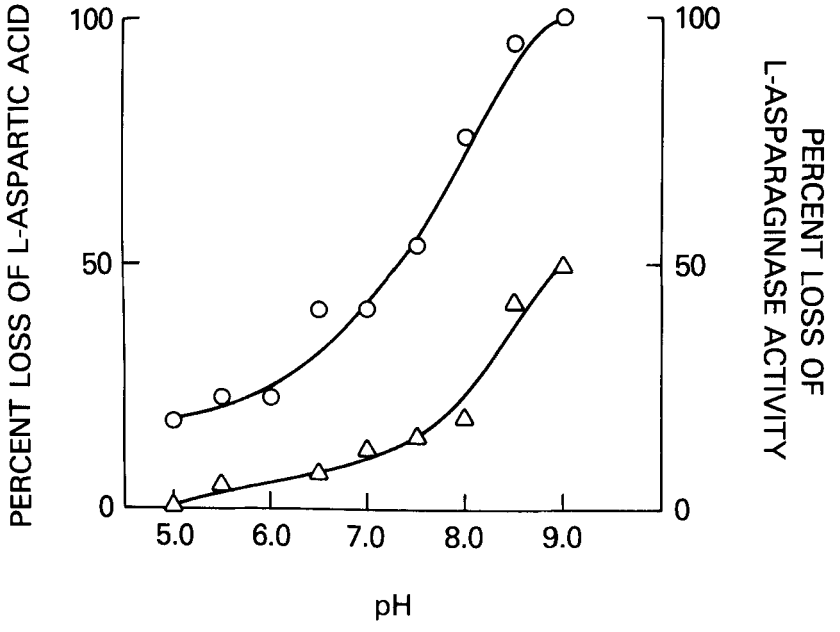

FIGURE 2 Influence of dialysis of *E. coli* L-asparaginase at various pH's on the content of L-aspartic acid and L-asparaginase activity. A 30 mg/ml solution of *E. col* L-asparaginase was prepared in 0.05 M Tris-buffer or appropriate pH and dialyzed against 4 L of the same buffer at 4° C for 48 h. The enzyme preparation was then analyzed for L-aspartic acid and L-asparaginase activity as detailed in the Methods Section. Symbols: (○) refers to % loss of L-aspartic acid; and (△) refers to the % loss of L-asparaginase activity.

aspartic acid at pH 7.0 and 4° C for 18 h: (1) crystalline enzyme dialyzed exhaustively against 0.01 M Tris-HCl buffer, pH 7.0 (native enzyme); (2) L-aspartate-free enzyme; (3) DONV-asparaginase;[4] and (4) nitro-asparaginase.[5] Samples (1) and (2) exhibited full catalytic activity, (3) was inactive, and (4) was 90% inactive with L-asparagine as substrate. An example of a Scatchard plot of the binding studies is presented in Figure 3; it can be seen that there are 4 binding sites for L-aspartic acid on the native tetrameric enzyme. The dissociation constant was determined to be $\sim 160\,\mu$M. The L-aspartate free enzyme demonstrated a similar binding constant (180 μM) for L-aspartic acid. Conversely, DONV-asparaginase and nitro-asparaginase bound no L-aspartic acid.

Fluorescence Quenching Studies

The L-tryptophanyl groups of L-asparaginase displayed an emission peak at 324 nm when excited at 290 nm (Figure 4A, solid line). This fluoresence was quenched upon addition of L-aspartic acid. At saturation, the total quenching by L-aspartate amounted to 18% (Figure 4A, dashed line) of the initial fluorescence amplitude. An example of the titration data is shown in Figure 4B as the rearranged Klotz plot.[12] From several titrations, it was determined that there were four binding sites for L-aspartate, with a dissociation constant of $\sim 60\,\mu$M. No discernible cooperative phenomenon was observed (cf. Ref. 3). The L-aspartate-free enzyme preparations gave similar results.

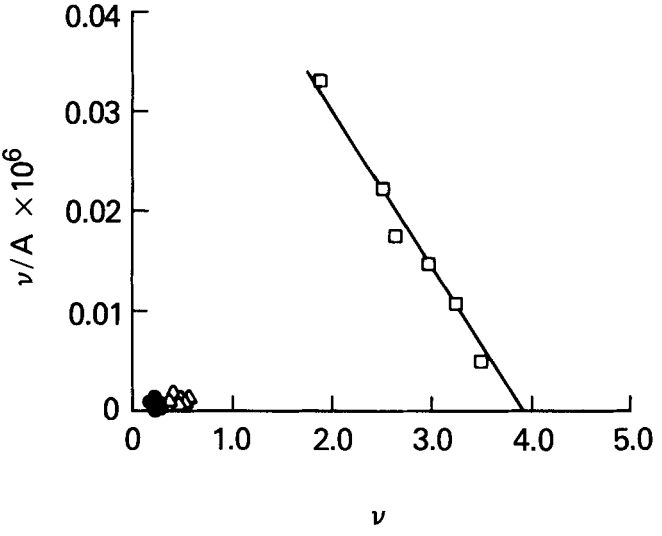

FIGURE 3 Binding of L-aspartic acid by L-asparaginase. Equilibrium binding studies were performed as detailed in the Methods Section. Fifty μl of the "native" enzyme preparation (500 μg protein) was dialyzed against various concentrations of L-[4-^{14}C]aspartic acid (0.125–2.5 μCi; 4.5–90 nmole) in a 50 μl volume for 18 h at 4° C and then the radioactivity in aliquots from the two sides of the dialysis chamber were counted by scintillation spectrometry. Symbol [□] refers to native L-asparaginase, (△) refers to DONVasparaginase, and (·) refers to nitro-asparginase.

The fluorescence titration curves reached an apparent plateau when the amount of L-aspartate added was about 0.5–1.0 mM. Further addition of millimolar levels of L-aspartic acid, however, resulted in further fluorescence quenching, indicating the presence of weaker binding sites. "Filter effect" was not involved in this additional quenching since spectrophotometric examination of the L-aspartate solution revealed no ultraviolet absorption due to any contaminant in the 290 nm range.

Inhibition Studies

Initial velocity studies when plotted as double reciprocal plots demonstrated that L-aspartate was a competitive inhibitor of L-asparaginase with L-asparagine as the substrate in the 6–50 μM range. The inhibition constant, 80 μM, agreed quite well with the dissociation constant for L-aspartate calculated from fluorescence titrations. Similar studies performed over the 4.4–44 mM range of L-asparagine showed that the maximal velocity actually increased by 300-fold; L-aspartate remained competitive. Judged from the weak binding sites uncovered in fluorescence quenching experiments, the observed "substrate activation" conceivably was the result of binding of a second molecule of L-asparagine to the enzyme rather than negative cooperativity among the tight binding sites.

Chemical Modification Studies

The results presented thus far support the concept that L-aspartate binds to the catalytic center of L-asparaginase. For this reason, it became of interest to study the

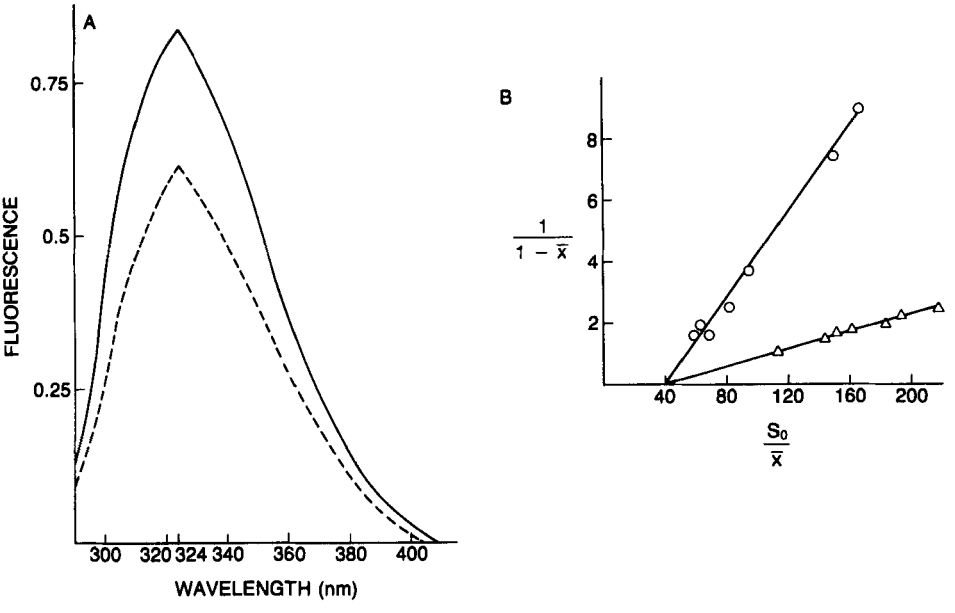

FIGURE 4 Emission spectrum of L-asparaginase. Florescence spectrophotometric studies were carried out as detailed in the Methods Section. Experiments shown here were performed with native L-asparaginase and the procedure is detailed in the Methods Section. *Panel A*. Emission spectrum of native L-asparaginase before addition of L-aspartic acid (———) and after saturating amount (1 mM) of L-aspartic acid was added (— —). *Panel B* Klotz plot drawn from the fluorescence titration data (cf. Methods Section).

influence of modifications of some of the amino acid residues of L-asparaginase on its catalytic activity and also on the binding of L-aspartate to the enzyme. For this study, both the native and L-aspartate-free enzymes (cf Methods Section) were treated with group-specific reagents, and the catalytic activity of the proteins so modified, as well as the number of binding sites for L-aspartic acid were simultaneously determined (Table III). Acetylation of either of the two enzyme preparations with acetic anhydride under mild conditions, did not influence the binding of L-aspartic acid. Similarly, preparations of native and L-aspartate-free L-asparaginases exposed to sodium nitrite did not show significant alterations either in binding characteristics or in catalytic activity. Generally, the major sites of modification in proteins as a result of reaction with these two reagents are α and ε amino groups. Treatment of native and L-aspartate-free enzymes with 2-mercaptoethanol, a reagent known to reduce disulfide bonds, did not alter either their catalytic activity or binding properties; this negative result is consonant with the absence of disulfide bonds in L-asparaginase from *E. coli*. Treatment with L-tyrosinase, which is known to modify the accessible L-tyrosine residues of proteins, produced a pronounced decrease in catalytic enzyme activity with a concomitant decrease in the capacity of the enzymes to bind L-aspartate. As pointed out earlier, DONV-asparaginase, wherein DONV-binds irreversibly to the active site[14] and nitro-asparaginase wherein 25% of the L-tyrosine residues are converted to nitrotyrosine,[5] did not show any binding of L-aspartic acid. Native and L-aspartate-free enzymes treated with diethylpyrocarbonate, a reagent known to modify L-histidine residues,[15,16] exhibited a loss of enzyme acitivity and a

TABLE III
Influence of modifiers on the binding of L-aspartate to L-asparaginase from *E. coli* (Merck)

			Type of L-Asparaginase			
			Native enzyme		L-Aspartate-free enzyme	
Modifier	Group modifier	Reference	Binding of L-aspartate (%)	Enzyme activity (%)	Bind of L-aspartate (%)	Enzyme activity (%)
None			100	100	100	100
Acetic anydride	Amino groups	20	83	100	100	100
Sodium nitrite	L-Lysine	21	81	100	92	100
2-Mercaptoethanol	Disulfide bridges		91	100	100	100
L-Tyrosinase	L-Tyrosine	14	42	17	13	18
Diethylpyrocarbonate	L-Histidine	16	46	10	29	4
Phenylglyoxal	L-Arginine	17	38	14	6	13
L-DONV	L-Serine	4	0	1	–	–
Tetranitromethane	L-Tyrosine	5	0	10	–	–

L-Asparaginase preparations were treated with modifiers, assayed for enzyme activity and L-aspartate binding, as detailed in the Methods Section. Binding of L-aspartic acid to the enzyme in the presence of modifiers is expressed as % binding in relation to that of enzyme without any treatment. Four moles of L-aspartic acid were bound to native and L-aspartate-free enzymes, in the absence of modifiers.

marked reduction in the capacity of the enzymes to bind L-aspartate. When enzyme preparations were treated with phenylglyoxal, a reagent known to modify L-arginine residues in proteins,[17–19] for 1 h at 25° C a marked reduction in the catalytic activity of the enzyme and a loss in binding activity ensued.

DISCUSSION

The studies presented here indicate that there are on the average, ~4 tight binding sites for L-aspartate in L-asparaginase. The competitive nature of inhibition by L-aspartate and the concomitant loss of both catalytic activity and binding affinity for the amino acid are consistent with the notion that L-aspartate binds to the active site of the enzyme. Equilibrium dialysis and fluorescence studies also provide direct evidence that there is one tight binding site per L-asparaginase monomer. Evidence for low affinity binding sites comes from the additional fluorescence quenching and the observed "substrated activation" at high L-asparagine concentrations. Citri *et al.*[3] have previously shown that L-aspartate is competitive with L-asparagine. The inhibition constant reported by these authors, 250 μM, is higher than the 80 μM determined by us. However, in view of the fact that there are separate binding sites for L-aspartate, it is likely that a higher inhibition constant may be obtained, depending on the range of L-asparagine concentrations employed in the kinetic studies.

No cooperativity was discernible for the tight binding sites. This is again in agreement with the finding of Citri *et al.*[3] The fact that four equivalent tight binding sites were found by two different techniques eliminates the possibility of "substrate activation" as the consequence of negative cooperativity. Although interactions between the high and low affinity binding sites cannot be ruled out, the second set of binding sites is undoubtedly involved in the observed substrate activation.

The persistence of L-aspartate even after dialysis can be attributed to a β-aspartyl enzyme intermediate. The data of Ehrman *et al.*[2] strongly suggested the existence of such an intermediate, which was also supported by the labeled peptide found when L-asparaginase was reacted with [^{14}C]DONV – a reagent that acts both as an irreversible inhibitor and an alternative substrate. The β-aspartyl group can be hydrolyzed and reformed in the presence of L-aspartate:

$$\text{E-Asp} \rightarrow [\text{E Asp}] \rightarrow \text{E} + \text{Asp}.$$

Conceivably, during dialysis at acid pH, the rate of recombination of L-aspartate with the enzyme is greater than the rate of diffusion across the dialysis tubing; moreover, the enzyme concentration is high (15 mg/ml), so that a substantial amount of L-aspartate will remain associated with the enzyme. The addition of GOT or β-decarboxylase, however, destroys the free L-aspartate, interrupts the cyclic process of E-Asp formation, and thereby effectively removes all the L-aspartate.

DONV is reported to bind irreversibly to the catalytic site of L-asparaginase causing complete inactivation of the enzyme.[1,4] Liu and Handschumacher have shown that, in nitro-asparaginase, up to 60% of the monomers of this tetrameric enzyme are covalently cross-linked as a consequence of tetranitromethane treatment.[5] Although extensive inactivation took place, the binding of DONV to the active site of the nitrosylated enzyme was increased two-fold. This augumentation points to the extensive rearrangement of the catalytic center of the enzyme. In our hand, no binding of L-aspartate to nitro-asparaginase was observed.

It is tempting to speculate on the possible physiologic significance of L-aspartic acid affiliated to the enzyme. As was mentioned, L-aspartic acid protects the catalytic activity of the enzyme from inactivation by pronase, heat or iodination.[21,22] Thus, it is reasonable to suggest that L-aspartate plays a role in stabilizing the native enzyme.

It is also relevant to point out that the affiliation of free L-aspartic acid with L-asparaginase gives rise to significant error in studies on the amino acid composition of the enzyme. As an example, Davidson *et al.* have reported that there are 202.8 L-aspartyl residues/mole of *E. coli* L-asparaginase.[22] If the binding of L-aspartic acid to the enzyme is taken into consideration, then this value must be revised to 198.8 residues of L-aspartic acid/mole of enzyme. In fact, it has been demonstrated by enzymatic means that L-asparaginyl residues account for about 50% of the so called L-aspartyl residues (more appropriately called L-asx residues), in L-asparaginase from *E. coli*.[23] If account is taken of this percentage, then the molar error introduced by free L-aspartic acid in the computation of the number of actual L-aspartyl residues is proportionately increased.

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