Elimination of the Sensitivity of L-Aspartase to Active-Site-Directed Inactivation without Alteration of Catalytic Activity†

Francesco Giorgianni, Šárka Beranová, Chrys Wesdemiotis, and Ronald E. Viola*

Department of Chemistry, The University of Akron, Akron, Ohio 44325-3601

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ABSTRACT: The catalytic activity of the enzyme L-aspartase from *Escherichia coli* has previously been shown to be sensitive to sulfhydryl reagents. The use of group-specific reagents, and a sequence homology comparison study among the fumarase−aspartase family of enzymes, has not, however, lead to the identification of a specific, essential cysteinyl residue. We have recently shown that L-aspartate-β-semialdehyde is an alternative substrate for L-aspartase, producing fumaric acid semialdehyde (FAA) which specifically inactivates the enzyme [Schindler, J. F., & Viola, R. E. (1994) *Biochemistry 33*, 9365]. Proteolytic digests of the resulting inactivated enzyme have now been mapped by HPLC and mass spectrometry. A specific residue (Cys-273) has been determined to be the site of FAA modification. Site-directed mutagenesis of this cysteine in the *E. coli* enzyme has produced altered enzymes which are considerably less sensitive to active-site-directed inactivation, while retaining full catalytic activity. Thus, cysteine-273 has been identified as an active-site nucleophile that, while not directly involved in catalysis in L-aspartase, is poised to attack an activated double bond in an enzyme-bound product analogue.

L-Aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1) catalyzes the reversible deamination of L-aspartic acid to form fumaric acid and ammonia. The enzyme from *Escherichia coli* is tetrameric, with the sequence of the *aspA* gene (Takagi et al., 1985) encoding a subunit composed of 477 amino acids with a molecular weight of 52 224.

L-Aspartase has previously been shown to have absolute specificity for its natural substrate (Ellfolk, 1954; Falzone et al., 1988). Unlike other amino acid deaminating enzymes, L-aspartase does not require cofactors or coenzymes, except that divalent ions have been shown to activate the reaction at alkaline pH (Rudolph & Fromm, 1971; Falzone et al., 1988). The deamination of aspartic acid has been shown to proceed *via* a carbanionic intermediate (Porter & Bright, 1980; Nuiry et al., 1984). However, the detailed reaction mechanism of the enzyme and the structure of its active site have not yet been fully elucidated.

Chemical modification and pH profile studies aimed at the identification of the amino acid residues that are implicated in catalysis have indicated the potential involvement of cysteine (Mizuta & Tokushige, 1975), histidine (Ida & Tokushige, 1984; Karsten & Viola, 1991), and lysine residues (Karsten & Viola, 1991) in the catalytic activity of L-aspartase. In an attempt to characterize the specific cysteine residue the enzyme had been treated with *N*-[7-(dimethylamino)-4-methyl-5-coumarinyl]maleimide (DACM),[1] a fluorescent reagent which is specific for sulfhydryl groups. Out of a total of 11 cysteines per enzyme subunit, 2 cysteines (identified by tryptic mapping as cysteines-140 and -430) were found to be modified by this reagent (Ida & Tokushige, 1985). However, subsequent mutagenesis of cysteine-430 did not support the essentiality of this group for catalysis (Murase et al., 1991). Site-directed mutagenesis of a highly conserved cysteine (cysteine-389), which is found throughout the fumarase−aspartase family of enzymes, also had no effect on the activity of L-aspartase (Saribas et al., 1994).

Recently, we have shown that the substrate analogue L-aspartate β-semialdehyde (L-ASA) is a mechanism-based inactivator of the enzyme, and that total protection from inactivation can be achieved by the addition of the product fumarate in the presence of magnesium ions (Schindler & Viola, 1994). The proposed mechanism of inactivation involves the catalytic formation of the product analogue fumaric acid semialdehyde (FAA), followed by nucleophilic attack on the double bond by an enzyme residue with consequent covalent modification. The enzyme from *E. coli* is highly sensitive to FAA inactivation, but studies with L-aspartase from *Pseudomonas fluorescens* have reported that this enzyme, which is 77% identical in amino acid sequence to the *E. coli* enzyme, is completely insensitive to L-ASA treatment (Takagi et al., 1984).

L-ASA has been used to specifically inactivate L-aspartase from *E. coli*. The single peptide bearing a modified amino acid residue has been mapped and characterized by reverse-phase HPLC and mass spectrometry of proteolytic digests. A specific amino acid (Cys-273) has been found as the site of FAA modification. The role of this residue has been examined by site-directed mutagenesis.

MATERIALS AND METHODS

Materials. L-ASA was synthesized and purified according to the procedure of Black and Wright (1955). Enzyme-grade buffer salts and urea were purchased from U. S. Biochemical. Acetonitrile (HPLC grade) was from Fisher, sequencing-grade trifluoroacetic acid (TFA) was from Pierce, α-chymotrypsin and trypsin [HPLC-purified before use (Titani et

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[1] Abbreviations: L-ASA, L-aspartate β-semialdehyde; DACM, *N*-[7-(dimethylamino)-4-methyl-5-coumarinyl]maleimide; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); FAA, fumaric acid semialdehyde; FAB, fast atom bombardment; HFIP, hexafluoro-2-propanol; PIC, phenyl isocyanate; PITC, phenyl isothiocyanate; TE, Tris−EDTA buffer; TFA, trifluoroacetic acid.

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al., 1982)] were from Sigma, and *Staphylococcus aureus* V8 protease was from Boehringer. The unpurified oligonucleotide primers for mutagenesis were ordered from Operon Technology, and were dissolved in sterile TE (Tris−EDTA) buffer, pH 7.3. All other reagents are available from commercial sources.

Enzyme Source. L-Aspartase is the gene product of the *E. coli aspA* gene which has been previously isolated and cloned into pBR322. The 1.6 kb structural gene was isolated and subcloned into the ampicillin-resistant plasmid pTZ18R, downstream from the T7 RNA polymerase promoter region, to produce the recombinant expression vector pTZ18R-ASP (Saribas & Viola, 1993). This plasmid was expressed in the L-aspartase-deficient *E. coli* strain JRG 1476 (*aspA*$^-$), a generous gift of Dr. John Guest, and the enzyme was purified according to Karsten et al. (1985). Enzyme purity was estimated to be greater than 95% by SDS gel electrophoresis.

Enzyme Assays. Enzyme activity was monitored spectrophotometrically by following the change in absorbance at 240 nm due to the formation of the double bond in the product fumarate. The typical assay buffer contained 30 mM L-aspartic acid, 10 mM $MgCl_2$ in 0.1 M HEPES−Tris buffer at pH 7.0.

L-ASA Enzyme Inactivation. L-Aspartase, at a concentration of 1.3 mg/mL in 0.4 M TAPS buffer, pH 8.5, was inactivated by the addition of L-ASA at concentrations up to 5 mM, or FAA at concentrations up to 0.5 mM, as previously described (Schindler & Viola, 1994). Inactivation was carried out at 25 °C and terminated, at various times, by rapid elution through a small G-25 gel filtration column. Protection studies against L-ASA inactivation were carried out in the presence of the product fumarate (30 mM), magnesium ions (5 mM) and α-methylaspartate (25 mM) or by the addition of glutathione (1−2 mM) to selectively react with the FAA that is produced. The sulfhydryl content was determined by using DTNB under both nondenaturing and denaturing conditions. Reactions were performed at room temperature and pH 8.5 for 10 min, using 50−100 μg of protein per 1 mL reaction volume. The reaction mixture contained 100 mM potassium borate and 0.2 mM EDTA. For the reactions conducted under denaturing conditions, 6 M guanidine hydrochloride was added to the mixture. The number of moles of reactive sulfhydryl was calculated from the increase in absorbance at 412 nm, corrected by a solvent blank, using a molar extinction coefficient of 1.36×10^4 M^{-1} cm^{-1}.

Proteolytic Digestion and HPLC Mapping. L-ASA-treated enzyme and controls were lyophilized, and then dissolved in 8 M urea containing 10 mM Tris buffer at pH 8.0. Disulfide reduction and S-carboxymethylation of the free sulfhydryl groups were carried out as previously described (Dong, 1990). Samples were then dialyzed against 50 mM ammonium bicarbonate, pH 7.6, and lyophilized. HPLC-purified trypsin was added in 10 mM Tris, pH 8.3, containing 100 mM sodium acetate and 0.1 mM calcium chloride, at a substrate to protease ratio of 100:1. Digestion was carried out at 37 °C for various times up to 24 h (with 4 h as a typical incubation time) and terminated by the addition of 10% phosphoric acid to lower the pH to 3. When necessary, some samples were briefly stored at 4 °C until the analysis could be conducted. When further proteolysis was required, the purified and dried tryptic peptides were dissolved in 100 μL of ammonium bicarbonate buffer (50 mM, pH 7.8), and from 1 to 6 μg of protease (*S. aureus* V8 protease or HPLC-purified α-chymotrypsin) was added to each sample. Digestion was carried out overnight at 36 °C. Samples were then dried and stored at −20 °C prior to analysis.

All digests were analyzed by reverse-phase HPLC on a Vydac C_{18} column (250 × 4.6 id, 5 μm particle size, 300 Å pore size). The chromatograms were developed at room temperature, at a flow rate of 0.8 mL/min, with a linear gradient from 2% to 100% solvent B (0.09% TFA, 20% water, and 80% acetonitrile) mixed with solvent A (0.1% TFA in water) over a period of 210 min. The column was then returned to 2% solvent B and held at these conditions for 15 min to allow reequilibration before the next injection. The HPLC equipment consisted of a Waters Baseline 810 Chromatography Workstation, with two pumps, a Valco electrically actuated injection valve, and an ISCO UV−visible variable detector adjusted to 216 nm for peptide detection. Eluted peptides were manually collected and concentrated by drying in a vacuum concentrator (Labconco).

Mass Spectrometry. Conventional FAB mass spectra were acquired on a VG AutoSpec tandem mass spectrometer. Vacuum-dried peptides were dissolved in water, and a 1.5 μL aliquot of the solution was loaded on a stainless-steel FAB probe tip coated with a liquid matrix (5:1 mixture of dithiothreitol and dithioerythritol). HCl (0.5 μL of a 1.0 N solution) was then added to the mixture in order to enhance protonation. Ionization of the sample was achieved using a Cs^+ ion gun operated at 20 kV, and the resulting secondary ion beam was accelerated to 8 keV. All data were collected and processed by employing the VG Opus data system. The spectra shown are averages of 3−5 scans. The tandem mass spectra were recorded with a JEOL HX110/HX110 four-sector instrument.

Peptide Sequencing. Peptide identities were confirmed by partial sequencing using the "ladder" sequencing protocol (Chait et al., 1993). Samples were dissolved in 20 μL of pyridine/water solution (1:1 v/v), and 20 μL of coupling reagent mixture, containing phenyl isothiocyanate (PITC)/phenyl isocyanate (PIC)/pyridine/hexafluoro-2-propanol (HFIP) (20:1:76:4 v/v), was added to the solution. Coupling was performed at 50 °C for 3 min; the reagents and nonpeptide products were extracted by adding 300 μL of heptane/ethyl acetate (10:1 v/v). After the two phases were separated by centrifugation, the top phase was discarded. This step was repeated once followed by washing twice with heptane/ethyl acetate (2:1 v/v). The remaining solution was dried on a vacuum concentrator. Cleavage was carried out by adding 20 μL of anhydrous TFA to the dry residue and incubation at 50 °C for 2 min. The coupling, wash, and cleavage steps were repeated for a total of 5 cycles. An additional treatment with PIC was used to convert any remaining unblocked peptides to their phenylcarbamyl derivatives. This peptide mixture was then analyzed by FAB mass spectrometry as described above.

Site-Directed Mutagenesis. Mutagenesis experiments were performed according to the method of Kunkel (1987). The template containing the (−) strand of the *aspA* gene was obtained after transfecting *E. coli* RZ 1032 cells (*ung*$^-$, *dut*$^-$) with the M13-ASP(−) vector (Saribas & Viola, 1993). Site-directed mutagenesis was carried out by designing a 24-mer oligonucleotide with a two-base mismatch. These changes led to the alteration of Cys-273, by changing the **TGC** codon to **TCG** (serine) or **GCC** (alanine), and the creation of either

an *Ava*I or a *Bsa*HI restriction endonuclease site in the *aspA* gene. Restriction enzyme analysis was used to screen for mutations. The mutated *aspA* genes were excised from the M13 vector by using a *Hind*III and *Kpn*I double restriction enzyme digestion and ligated into pTZ18R, the phagemid used for the expression of L-aspartase (Saribas et al., 1994). The mutant vectors pTZ18-S273 and pTZ18-A273 were then used to transform the JRG 1476 strain of *E. coli* (*asp A*$^-$), and the overexpressed enzymes were purified as previously reported (Karsten et al., 1985).

RESULTS

Inactivation of L-Aspartase. Incubation of L-aspartase from *E. coli* in the presence of L-ASA leads to the rapid and irreversible inactivation of the enzyme (Yumoto et al., 1982; Schindler & Viola, 1994). L-Aspartase was treated with L-ASA for a sufficient time to cause a decrease in the catalytic activity to less than 10% that of the untreated enzyme. Complete protection against L-ASA inactivation is observed when the incubation is carried out in the presence of fumarate, an activator, and magnesium ions. These conditions also protect the enzyme from inactivation when FAA is directly incubated with the enzyme. Protection against L-ASA (or FAA) inactivation of L-aspartase is also achieved when glutathione is added to the incubation mixture. Glutathione was found to specifically react with free FAA even in the presence of a 20-fold excess of L-ASA. Control studies have shown that glutathione itself has no effect on the activity of L-aspartase.

Titration of the native enzyme with DTNB determined the presence of 1.9 reactive cysteines per enzyme monomer. Sulfhydryl group titration yielded 2.1 cysteines in the L-ASA-treated, but otherwise undenatured, enzyme that are reactive toward the titrating reagent. Previous work has reported that the total number of cysteines titrated by DTNB, under denaturing conditions (6 M urea), decreased from 9 to 8 after inactivation with L-ASA (Yumoto et al., 1982). We have observed that mild denaturing conditions (0.8 M guanidine hydrochloride) allow the titration of all 11 cysteines in the wild-type enzyme. After treatment with L-ASA, the total number of cysteines titrated under these conditions decreased to 10.

Peptide Mapping. In order to obtain a complete tryptic map of native and derivatized L-aspartase, several conditions of digestion and separation were tested. It was found that the quality of the chromatograms critically depended upon the purity of the protease and the conditions of the enzyme substrate. When HPLC-purified trypsin was used on L-aspartase, which had been denatured and carboxymethylated at cysteines residues, about 40 well-resolved peaks were obtained. The total theoretical number of peptides generated from complete digestion of L-aspartase by trypsin, which could be retained on a reverse-phase column (i.e., containing more than 2 amino acid residues), is predicted to be 40.

Comparative HPLC analysis of the tryptic maps obtained from the native and L-ASA-treated enzymes (Figure 1) showed that the two chromatographic patterns are identical, with the exception of a single peak. The L-ASA-treated sample (Figure 1B) contains an additional fragment, with a retention time of 117 min, and shows the partial disappearance of another peak with a retention time of 126 min. Repetitive analysis of the tryptic digests verified the consistency of these results.

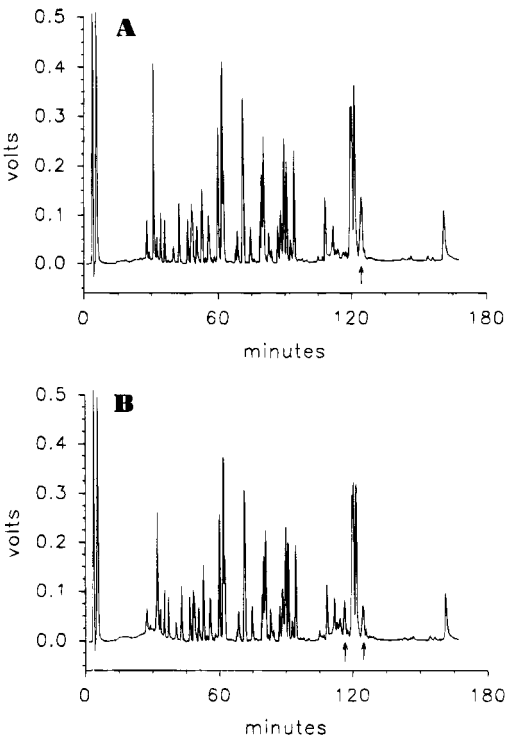

FIGURE 1: HPLC chromatogram of tryptic digests of L-aspartase. Each enzyme sample was denatured and carboxymethylated prior to treatment with trypsin. The resulting peptides were separated on a reverse-phase C_{18} column with a linear acetonitrile gradient as described under Materials and Methods. Curve A, untreated L-aspartase; curve B, FAA-modified aspartase. The altered peaks at 126 min and 117 min are indicated with arrows.

HPLC mapping of the enzyme sample treated with L-ASA in the presence of protective compounds, i.e., fumarate, an activator, and magnesium ions, shows no difference from the chromatogram of the native (unmodified) protein. This demonstrates that the shifted peptide contains a functional group which has been modified by a specific reaction with FAA, and that this modification can be prevented if the free enzyme is converted to an enzyme−product complex.

The synthesis of L-ASA leads to the production of formaldehyde as a side product (Black & Wright, 1955). Purification of L-ASA by ion-exchange chromatography removes all but trace levels of this contaminant. We have previously shown that formaldehyde does inactivate L-aspartase by reaction with specific lysyl residues on the enzyme (Karsten & Viola, 1991). Therefore, the modification that is observed by peptide mapping could be a consequence of the reaction with formaldehyde. However, tryptic mapping of formaldehyde-treated L-aspartase produced a chromatogram with an identical pattern to that of the control (native enzyme).

Hydrophobicity calculations, based upon the method of Teller (Sasagawa et al., 1982), were carried out to obtain some indications about the most likely candidates for the modified tryptic peptide. The results of these calculations indicated four possible fragments from the trypsin-generated map (Table 1), with molecular masses ranging from 3500 to 5000 daltons, which are predicted to have HPLC retention times near the region of the modified peptide. Three of them contain at least one cysteinyl residue.

Analysis of HPLC-Isolated Peptides by Mass Spectrometry. To facilitate the subsequent tandem mass spectrometry analysis, it is necessary to have peptides with a maximum

Table 1: Possible Candidates for the FAA-Modified Peptide

No.	tryptic peptide sequences	peptide mol.wt.[a]	expected fragment size[a] V8 Protease(▼)	Chymotrypsin(▲)
1	^{88}CMDQF▲PVDVY▲QGGAGTSVNMN TNE▼VLANIGLE▼LMGHQK	4138	2635, 828, 713	701, 592, 2883
2	LAE▼VTGF▲P^{260}CVPAE▼DLIE▼ATSD^{273}C GAY▲VMVHGALK	3522	332, 1077, 489, 1681	736, 1970, 854
3	E▼V^{400}CE▼GY▲VY▲NSIGIVTY▲LNPF▲ IGHHNGDIVGK	3465	148, 408, 2948	757, 281, 866, 490, 1147
4	GLLTE▼AE▼LDDIF▲SVQNLMHPA Y▲K	2603	532, 219, 1891	1336, 1160, 147

[a]After carboxymethylation of sulfhydryl groups.

molecular weight below 3000. Further proteolytic digestion of the tryptic fragments will generate species of a diagnostic size that will aid in their identification. The peaks eluted at 117 and 126 min were collected and redigested with either *S. aureus* V8 protease or chymotrypsin. The resulting peptide mixtures were then analyzed by FAB mass spectrometry.

The sample collected at 126 min yielded, after treatment with V8 protease, abundant signals at *m/z* 1548 and 1681 (Figure 2A). The peak at 1681 was identified as resulting from cleavage at Glu-268 (peptide 2 in Table 1). The signal at *m/z* 1548 arises from the failure to cleave the susceptible peptide bond between Glu-264 and Asp-265 that would have yielded fragments at *m/z* 1077 and 489. The same sample, digested with chymotrypsin, produced signals at *m/z* 854 and 2687, with the larger fragment derived from incomplete digestion between Phe-258 and Pro-259 that would have yielded fragments at *m/z* 736 and 1970. From these data, the peptide at 126 min was determined to correspond to fragment 252−284 of L-aspartase. None of the other peptide candidates that were identified by hydrophobicity calculations would produce a comparable proteolytic pattern, or have the correct overall molecular weight (Table 1). A computer survey of the entire enzyme sequence identified seven fragments that have a molecular mass within ±1 amu of 1681. However, only the peptide from positions 269 to 284 has the appropriate terminal amino acids that correspond to the specificity of both V8 protease and trypsin (Figure 3).

The mass spectra of the peptide eluted at 117 min display two major signals: at *m/z* 1548 and 1655 (for the V8-digested peptide, Figure 2A) or at *m/z* 854 and 2661 (after digestion with chymotrypsin). The peaks at 1548 and 854 are identical to those obtained for the 126 min sample. The other two fragments show a shift of 26 amu, i.e., from 1681 to 1655 and from 2687 to 2661, for the V8 protease and chymotrypsin-digested peptide, respectively. Obviously, the peptide at 117 min corresponds to a derivatized form of the peptide at 126 min. However, the mass of the modified peptide has ***decreased*** relative to the unmodified, instead of the expected increase of 100 amu upon FAA modification.

Methylation of the carboxylate groups in the V8 protease-digested fragments was carried out to determine the number of carboxylates (Falick & Maltby, 1989). Treatment of the fragment derived from the 126 min peak results in a mass increase of 42 (from 1681 to 1723) as observed by FAB mass spectrometry (Figure 2B). Analogous treatment of the V8 protease fragment from the 117 min peak results in a mass increase of only 28. These results indicate the presence of three carboxylate groups in the fragment derived from the 126 min tryptic peptide (Figure 3), and only two in the 117 min fragment. The other fragment, from Val-255 to Glu-268, shows the mass increase from 1548 to 1618 that is expected for the addition of five methyl groups (Figure 3).

The identity of the V8 protease-digested fragments was confirmed by partial sequencing using a ladder sequencing technique (Chait et al., 1993). This method generates a family of sequence-defining peptide fragments, each differing from the previous one by a single amino acid. After five cycles of derivatization and cleavage, the complete mixture was analyzed by mass spectrometry. A peptide "ladder" was obtained in the FAB spectrum, with peaks observed for four amino acids in the sequence. Each amino acid was identified from the mass difference between successive peaks, with the position in the mass spectrum data set defining the sequence of the peptide. The ladder is consistent with the sequence **TGFP**, which corresponds to residues 256−259 of L-aspartase. These results are consistent with our previous data that had indicated peptide 252−284 of L-aspartase as the site of FAA modification.

Tandem mass spectrometry (MS/MS) was also used to verify the amino acid sequence of the V8-generated peptide (MH^+ = 1655), and to identify the residue where derivatization has taken place (Biemann, 1990). The fragmentation pattern obtained from peptide 1655 confirmed its identity as a modified form of the control peptide (peptide 2 in Table 1, MH^+ = 1681). Both samples displayed an identical set of C-terminal fragments arising from dissociations of the sequence: ^{274}GAYVMVHGALK^{284}. In addition, an ion corresponding to the first two N-terminal amino acids of this peptide (Ala-Thr) is also present in both spectra. This finding leaves residues ^{271}Ser-Asp-Cys^{273} as the only possible sites of chemical modification. Indeed, an ion of the N-terminal series (fragment a_{14}), which includes these three amino acids, shows a mass difference between the control and the modified samples of 26 units, the same as the overall difference observed for the parent peptides.

Site-Directed Mutagenesis. To verify the position of chemical modification in L-aspartase, and to examine the role of this residue, site-directed mutagenesis was carried out to change cysteine-273 to a serine. The resulting enzyme retains full catalytic activity, with the kinetic parameters (k_{cat} = 210 s^{-1} and $K_{aspartate}$ = 1.3 mM) unaltered from those of the wild-type enzyme. The pH profile of the C273S enzyme is also unchanged from that observed in the wild-type enzyme (Karsten & Viola, 1991). However, the sensitivity of the C273S enzyme to inactivation in the presence of L-ASA decreased by a factor of 5, from a k_{obs} of 0.134 min^{-1} for the wild-type enzyme to 0.025 min^{-1} (Figure 4). The HPLC peak that was observed at 126 min in the chromatogram of the trypsinized wild-type enzyme is shifted to 122 min in the mutant enzyme. HPLC mapping of the FAA-treated mutant enzyme does not show the presence of the modified peptide that had previously been detected in the wild-type enzyme. To determine if the serine that was introduced at position 273 was now being modified by FAA at a reduced rate, this residue was replaced by an alanine. This C273A mutant also retained full catalytic activity, and has the identical diminished sensitivity to inactivation when incubated with L-ASA as the C273S enzyme (Figure 4).

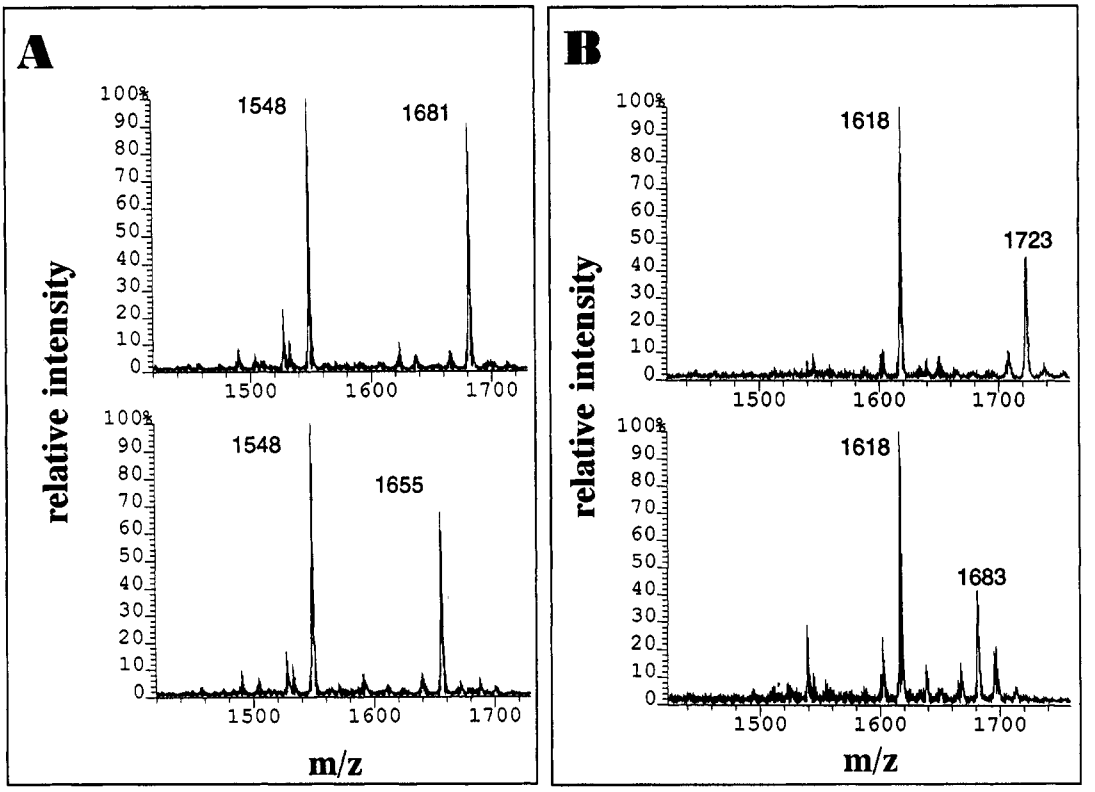

FIGURE 2: FAB mass spectra of an isolated tryptic peptide from L-aspartase that has been digested with *S. aureus* V8 protease. Panel A: Digested tryptic peptides eluted at (*upper trace*) 126 min and (*lower trace*) 117 min. Panel B: Digested tryptic peptides in panel A which have been methylated after V8 protease digestion. Peaks were eluted at (*upper trace*) 126 min and (*lower trace*) 117 min.

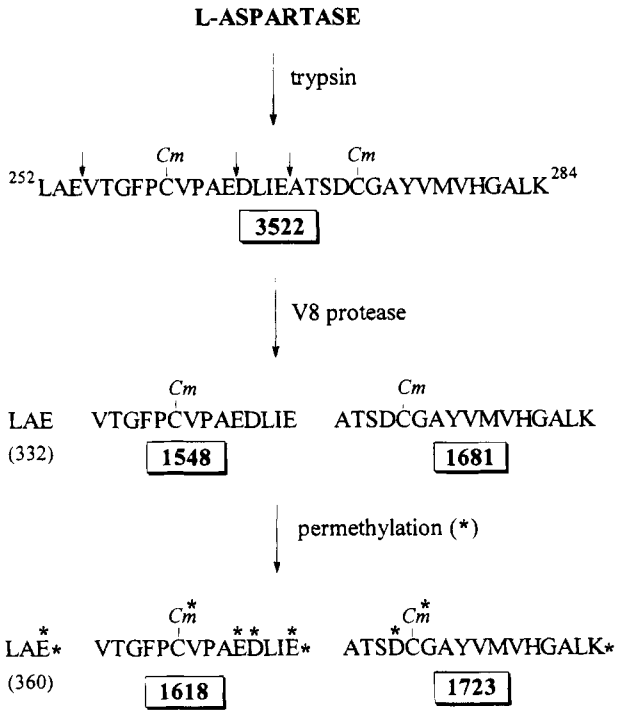

FIGURE 3: Proteolytic digestion of L-aspartase by trypsin and *S. aureus* V8 protease. The arrows (↓) indicate the expected cleavage sites for V8 protease. The calculated mass of each fragment (MH^+) is shown, with the mass in boxes indicating those fragments which have been observed by mass spectrometry, and the mass of the small, unobserved fragments given in parentheses. The sites of carboxymethylation (*Cm*) and methylation (*) are indicated.

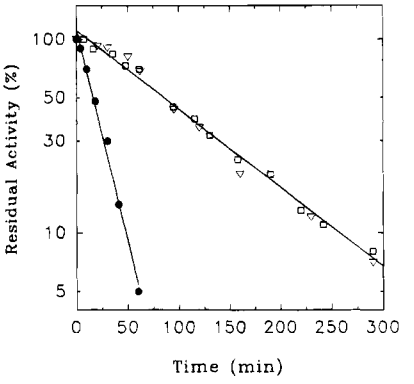

FIGURE 4: Inactivation of L-aspartase in the presence of the substrate analogue L-ASA. L-Aspartase (0.5 μM) was incubated in 0.4 M TAPS buffer, pH 8.5, at 25 °C with 5 mM L-ASA. Aliquots were removed at the indicated times and assayed for activity as described under Materials and Methods. Wild-type L-aspartase (●); C273S mutant (□); C273A mutant (▽).

DISCUSSION

Modification of L-Aspartase. Inactivation of L-aspartase from *E. coli* by the product analogue FAA is proposed to involve attack at the double bond by an enzyme nucleophile (Schindler & Viola, 1994). A decrease by one in the number of accessible sulfhydryl groups that are titrated by DTNB in the inactivated and denatured enzyme has previously suggested a cysteine as the candidate for this nucleophile (Yumoto et al., 1982). However, several cysteines in L-aspartase have already been examined by site-directed mutagenesis studies (Murase et al., 1991; Saribas et al., 1994), and have been eliminated as potential active site residues. The insensitivity of the highly homologous L-aspartase from *P. fluorescens* to inactivation when incubated with L-ASA suggests that this putative cysteine nucleophile may be absent, or have altered accessibility, in the enzyme from this species. Alignment of the amino acid sequences of these 2 L-aspartases shows that of the 11 cysteines that

are present in the *E. coli* enzyme 7 are not present in the corresponding positions in the *P. fluorescens* enzyme. Two of these nonconserved residues (cysteine-140 and -430) have already been examined (Ida & Tokushige, 1985; Murase et al., 1991), leaving five other cysteine candidates to be considered.

Protection against FAA inactivation by the addition of fumarate, an activator, and magnesium ions supports the hypothesis that modification occurs at or near the enzyme active site. Previous work has shown that fumarate binds only at the active site, and that at higher pH the binding of fumarate requires the presence of an activator and divalent metal ions (Falzone et al., 1988). The complete protection against inactivation upon incubation with L-ASA that is provided by glutathione indicates that the product FAA is released and must accumulate to some extent before binding to inactivate the enzyme. Glutathione has been shown to react stoichiometrically with any free FAA, even in the presence of excess L-ASA. This result is in contrast with our previous observation that the presence of high levels of fumarase, an enzymic-trapping system for FAA, did not affect the rate of inactivation of L-aspartase in the presence of L-ASA (Schindler & Viola, 1994).

Characterization of the Derivatized Peptide and Identification of the Modified Residue. Treatment of L-aspartase with L-ASA leads to an alteration in the retention time of a single peptide from the HPLC tryptic map (Figure 1). Hydrophobicity calculations have suggested four possible peptides with predicted retention times in this region (Table 1), and the first two of these peptides contain three of the five nonconserved cysteine candidates. Further proteolytic digestion has lead to the identification of this tryptic peptide as encompassing amino acids 252−284 of the L-aspartase sequence (peptide 2 in Table 1). A V8 protease fragment of this tryptic peptide, containing amino acids Ala-269 to Lys-284 (*m/z* 1681), has been observed to undergo a mass shift upon FAA derivatization. A chymotrypsin fragment of the same peptide, containing amino acids 252−276, has also undergone a similar mass shift. These results localize the modification of L-aspartase by FAA within the amino acid sequence between residues 269 and 276, the region of overlap between the mass-shifted V8 protease and chymotrypsin fragments:

^{269}Ala-Thr-Ser-Asp-Cys-Gly-Ala-Tyr^{276}

This fragment contains cysteine-273, one of the nonconserved candidates for this active-site nucleophile, which has been replaced by a methionine in the *Pseudomonas* enzyme. The only explanation that is consistent with the observed decrease in molecular weight of the modified peptide obtained at 117 min is that catalytic turnover of L-ASA leads to a modification on the cysteine residue at position 273. Modification at this position would prevent the subsequent carboxymethylation of cysteine by iodoacetic acid during sample preparation for trypsin digestion, resulting in a decrease of 59 amu when compared to the fully carboxymethylated control peptide. The molecular mass of the group that is present on the cysteine from reaction with FAA is calculated to be 33 amu, 59 amu for a carboxymethyl group minus 26 amu for the observed mass decrease. To verify the position of modification, both peptides 117 and 126 were methylated at carboxylate residues after digestion with V8 protease (Figure 3). FAB mass spectral analysis of these samples (Figure 2B) shows a decrease of one carboxylate group in the modified V8 protease fragment from Ala-269 to Lys-284, consistent with the inability to carboxymethylate the cysteine-273 that has been modified in this peptide fragment. The other V8 protease fragment, from Val-255 to Glu-268, shows no change in the number of carboxylate groups in the peptide derived from the L-ASA-treated enzyme. These results show that the other cysteine in peptide 2 (Cys-260) is not modified during the catalytic turnover of L-ASA, nor during the subsequent proteolyses and separations.

Peptide ladder sequencing and tandem mass spectrometry were used to confirm the sequence of the peptides. The MS/MS study also indicated that FAA modification can only occur between Ser-271 and Cys-273.

Taken together, these results prove that a single residue, cysteine-273, has been modified by L-ASA treatment of L-aspartase. The molecular mass of the modifying group, 33 amu, corresponds to the introduction of an $-O_2H$ group. This implies subsequent oxidation, following derivatization of the sulfhydryl group by FAA, leading to the production of cysteine-sulfinic acid. Alteration of the initially modified cysteine during proteolysis and purification is not unexpected (Uchida & Stadtman, 1992).

Role of the Cysteine Nucleophile. Replacement of cysteine-273 with either serine or alanine has no observable effect on the catalytic activity of L-aspartase. This establishes that this residue plays no direct role in the mechanism of the deamination of aspartic acid. However, substitution of the cysteine side chain either with a serine hydroxymethyl group or with a methyl group of alanine substantially decreases the sensitivity of this enzyme to inactivation by the alternative product FAA. These observations verify cysteine-273 as the primary site of modification. Protonation of this cysteine would also decrease the nucleophilicity of this residue, and would be expected to decrease the sensitivity of L-aspartase to modification by FAA. The enzyme has been shown to catalyze the deamination of L-ASA at pH 6 without observable inactivation by the FAA product (Schindler and Viola, unpublished results). Thus, cysteine-273 has been identified as an opportunistic active-site nucleophile that is capable of attacking an activated double bond in enzyme-bound product analogues.

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