Evaluation of Functionally Important Amino Acids in L-Aspartate Ammonia-Lyase from *Escherichia coli*†

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ABSTRACT: The high-resolution structure of L-aspartate ammonia-lyase from *Escherichia coli* has recently been determined [Shi, W., Dunbar, J., Jayasekera, M. M. K., Viola, R. E., & Farber, G. K. (1997) *Biochemistry 36*, 9136−9144]. An examination of the putative active site has been carried out, with the active site located in a cleft that contains the functionally significant lysine 327. A list of potential active site residues has been generated based on their proximity to this active site lysine, sequence homology comparisons with other members of the aspartase−fumarase enzyme family, and the necessity for chemically reasonable functionalities for the proposed roles. The five most likely candidates in the putative active site cleft have been examined by site-directed mutagenesis to test their feasibility for either substrate binding or acid−base catalytic roles. Arginine and lysine residues have been identified that appear to function in the orientation and binding of aspartic acid at the enzyme active site. Some tentative assignments have also been made of the acid and base catalytic groups that are proposed to be involved in the deamination reaction.

Aspartase[1] (L-aspartate ammonia-lyase, EC 4.3.1.1) catalyzes the reversible deamination of L-aspartate to yield fumarate and ammonium ion. This enzyme is highly selective with L-aspartic acid, and the recently identified L-aspartate β-semialdehyde, as the only amino acid substrates that are recognized by aspartase (Schindler & Viola, 1994). Chemical modification and pH profile studies have previously suggested the potential importance of cysteine (Mizuta & Tokushige, 1975; Ida & Tokushige, 1985), histidine (Ida & Tokushige, 1984; Karsten & Viola, 1991), and lysine residues (Karsten & Viola, 1991) in the activity of aspartase. However, site-directed mutagenesis of a cysteine identified in the earlier studies (Cys 430) did not confirm the essentiality of this functional group (Murase et al., 1991). Mutagenesis of a highly conserved cysteine, which is present throughout the fumarase−aspartase family of enzymes (Takagi et al., 1986; Woods et al., 1986, 1988), also had no effect on the activity of aspartase (Saribas et al., 1994). Recent site-directed mutagenesis studies have identified a specific lysyl residue (Lys 327) that appears to be involved in the active site of the enzyme (Saribas et al., 1994), and peptide mapping has identified a cysteinyl and a second lysyl residue of aspartase that have been modified by a mechanism-based inactivator (Giorgianni et al., 1995, 1997). While this lysine (Lys 139) is present in *Escherichia coli* fumarase C this is not a highly conserved residue in the fumarase−aspartase family. The adjacent cysteine (Cys 140) is not present at this position in any of the homologous family members.

Our recent determination of the high-resolution structure of aspartase (Shi et al., 1997) has enabled a careful examination of the putative active site and the selection of a number of possible residues for further study. Several candidates have been identified by site-directed mutagenesis that appear to play significant roles in the activity catalyzed by aspartase, and several prominent functional amino acids in the vicinity of these active site residues have been eliminated from consideration.

EXPERIMENTAL PROCEDURES

Materials. L-Aspartic acid, fumaric acid, and substrate analogs were obtained from Sigma-Aldrich, except L-aspartic acid amide which was purchased from Novabiochem. The enzyme aspartase was purified by the procedure of Karsten et al. (1985) from an overproducing strain of *Escherichia coli* (Saribas et al., 1994). DNA polymerase and the PCR optimization kit were obtained from Stratagene.

Gene Amplification. The conditions for PCR were optimized by using gene amplification to allow proper annealing and elongation of primers using a twelve buffer optimization kit that varied the sensitive parameters including [Mg^{2+}], [K^{+}], and pH. Two primers were used for this purpose, each to anneal to either end of the aspartase gene inserted into the pTZ18R expression vector. Native *pfu* DNA polymerase was used for its proofreading ability and for producing less contamination than either recombinant *pfu* or tac polymerases. Thin-wall tubes were used for effective heat transfer with a fresh deoxynucleotide mix. Gene amplification was determined according the protocol indicated in the Opti-Prime PCR optimization kit.

Mutagenic Methods. Site-directed mutagenesis was carried out by using two different methods, two-step PCR and

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[1] Abbreviations: aspartase, L-aspartate ammonia-lyase; PCR, polymerase chain reaction; RC-PCR, recombinant circle-polymerase chain reaction; YT, yeast-tryptone growth media.

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Table 1: Construction and Screening of L-Aspartase Mutants

mutation	DNA sequence[a]
D10A	GAA GcT CTG (*Bgl* II)
D10N	GAA aaT CTG (*Bgl* II)
R15A	ACC gcG GAA *Sac* II
H26Q	CAg ACT CTG *Alw*N I
R29A	CTG gcA GCG (*Dde* I)
S143G	CAG ggC ACT *Bsp* 1286 I
S143T	CAG aCC ACT ACC GAt GCC (*Bsa*H I)

[a] The mismatched bases to create the mutagenic replacements are shown in lower case. The restriction endonuclease sites are underlined and annotated to indicate either the introduction of a new site, e.g., *Sac*II, or the removal of an existing site, e.g., (*Bgl*II).

recombinant circle PCR (RC-PCR). In the two-step PCR method (Barik, 1993) a megaprimer is first produced between one of the flanking primers and the mutagenic primer. In the second step the megaprimer is used with the remaining flanking primer to obtain the full gene. The primers consisted of one or two base pair mismatches, located close to the 5′ end, to generate the desired amino acid replacements and also to cause either the introduction of a new restriction enzyme site at that loci or the removal of an existing site. The specific changes that were introduced are listed in Table 1 for each of the mutants that have been prepared, along with an annotation of the restriction endonuclease sites that have either been created or destroyed through the use of these primers. Initial screening for the presence of mutagenic colonies was accomplished by restriction enzyme mapping, with subsequent DNA sequencing of the mutated region to confirm the amino acid replacements. For transformation, 100 μL of a stock TG-1 cell culture was used to infect a 25 mL culture of YT media which was grown at 37 °C until $A_{600} = 0.6$. The culture was transferred to prechilled centrifuge tubes, left for 10 min, and then centrifuged at 5000 rpm for 5 min. The pellet was resuspended in 10 mL of 100 mM $CaCl_2$ and placed on ice for 45 min before being centrifuged at 2500 rpm for 10 min. The pellet was resuspended in 2 mL of cold 100 mM $CaCl_2$ and used after 12 h.

Mutagenesis at the Asp 10 site posed an unusual problem. After creating the mutagenic gene during PCR the full gene is inserted back into the expression vector by cutting at unique *Kpn*I and *Hin*dIII sites. However, the *Kpn*I site, located within the aspartase gene close to the ATG start codon, cuts at a position thirteen residues after the N-terminal methionine. This location prevents any mutation created before this position during PCR from being transferred into the expression vector. This difficulty was overcome with the use of recombinant circle PCR. The RC-PCR method (Tarragona-Fiol et al., 1993) utilizes four primers that are designed to generate double-stranded, linear DNA molecules with blunt ends. Once combined, denatured, and reannealed, this linear DNA produces double-stranded DNA with discrete cohesive single-stranded ends, in addition to the previously made blunt end products. However, only the former product will anneal to form recombinant circles of DNA that can effectively mimic circular DNA that is required to be transformed into TG-1 cells. RC-PCR eliminates the insertion of the aspartase gene back into the expression vector by using *Kpn*I, thus enabling the creation of a mutation within the first thirteen residues of the aspartase gene. However, the design of primers was restricted due to limited information about the nucleotide sequence in the region immediately upstream of the ATG start codon and the apparent presence of secondary structure in this region that competes with primer binding. Thus utilization was constrained to a limited area of 27 bases, which starts from the ATG start codon to the Asp 10 codon, for annealing of one complete primer and a significant part of the second primer. Within these restrictions this method was successfully utilized for the production of the linear DNA which then allowed the creation of a mutagenic site at any location within the aspartase gene without further limitations.

Enzyme Purification. Aspartase was purified according to the previously published protocol (Karsten et al., 1985). When the red A-agarose dye ligand column was washed after loading protein, the aspartase mutants were found to bind very tightly with no leakage of the enzyme observed. Approximately 30% of the total amount of protein loaded on the column washed through, indicating that a significant fraction of the contaminating proteins in this high expression system have no affinity toward this dye. The bound proteins were eluted with a KCl gradient, the fractions were pooled based on specific activity, and the enzyme was concentrated by using 25 kDa Microcon filters. In the case of low activity mutants collection of the fractions was dependent on the protein concentration and their location in the salt gradient. Purity was examined by SDS−PAGE and, in all cases, the enzyme was found to be 90% pure or greater.

Fluorescence Spectroscopy. The fluorescence emission spectra of aspartase, and the series of mutants that were produced, were examined on an Aminco SPF-500 spectrofluorometer. The enzyme samples, in the absence and presence of aspartic acid, were excited at 260 nm and the emission spectrum of each was scanned from 285 to 390 nm.

Enzyme Assay. The activity of aspartase was determined spectrophotometrically by measuring the formation of fumarate at 240 nm ($E_{240} = 2.53$ mM^{-1} cm^{-1}). A standard assay mix contained 30 mM Hepes buffer, pH 7.0, 10 mM Mg acetate, and varying concentrations of L-aspartic acid at 30 °C. Inhibition constants were determined by varying the concentrations of aspartate and the inhibitor, and fitting the competitive inhibition pattern. A constant ionic strength buffer system (Ellis & Morrison, 1982) consisting of 50 mM Hepes and 50 mM Tris was used to cover the pH range from 6.0 to 10.5 for the pH profile studies. The kinetic data were fitted by using the Enzyme Kinetics Package software (SciTech International, Chicago, IL) to obtain the kinetic parameters and to model the pH variation of these parameters.

Table 2: Candidates for Functionally Important Amino Acids in Aspartase

amino acid	distance from Lys 327 (Å)	homology[a] (%)	putative role
His 26	14	20	general base catalyst
Asp 10	9	100	general base catalyst
Ser 143	7	100	general acid catalyst
Thr 233	12	70	general acid catalyst
Thr 104	10	100	general acid catalyst
Arg 29	8	70	substrate binding
Arg 15	15	20	substrate binding
Lys 327	−	100	substrate binding

[a] Homology is the percent identity at this position based on a sequence alignment among *E. coli* aspartase, *Pseudomonas fluorescens* aspartase, *H. influenza* aspartase, *Bacillus subtilis* fumarase, *E. coli* fumarase, yeast fumarase, human fumarase, δ-crystallin, yeast, and human argininosuccinases.

RESULTS

Selection of Mutagenic Targets. The three-dimensional structure of aspartase (Shi et al., 1997) was carefully examined with the aim of identifying a set of functional amino acids that are potentially involved in substrate binding and in catalysis. Lys 327[2] has been previously identified both by chemical modification studies (Karsten & Viola, 1991) and by site-directed mutagenesis studies (Saribas et al., 1994) as playing a significant role in the catalytic activity of aspartase. This locus was the focal point in a search for potential amino acid targets which are located within a 15 Å radius. At least 30 functional amino acids on three of the four subunits were found which are proximal to Lys 327. This list was narrowed to a preliminary set of targets based on their relative proximity, sequence homology within the fumarase−aspartase family, and possession of a reasonable structure to provide a chemical basis for the proposed role (Table 2). This list of potential targets consists of amino acids that come primarily from the subunit adjacent to that which contributes Lys 327 to this intersubunit cleft. Other prospective candidates were eliminated from this initial consideration because they did not optimally conform to the selection criteria.

Characterization of Aspartase Mutants. The fluorescence spectra of the aspartase mutants were measured to assess the structural rearrangements that may have occurred as a consequence of the amino acid replacements. In the absence of tryptophans in aspartase (Takagi et al., 1985) tyrosines are the primary amino acid fluorophore. The fluorescence spectrum of the wild-type enzyme has an emission maximum at 300 nm that is typical of tyrosines. The mutant enzymes each give a similar spectrum in this region (data not shown), with fluorescent intensities that are within 10% of that observed for the wild-type enzyme. Thus, the mutant enzymes appear to retain an intact overall structural integrity.

The mutants that were produced were purified by using the standard purification protocol (Karsten et al., 1985). The kinetic parameters were determined for each mutant and compared to those of the wild-type enzyme (Table 3). Replacement of several prominent amino acid targets, including His 26 and the arginines at positions 15 and 29, had no effect on the catalytic potential of aspartase. However, the replacement of Arg 29 with alanine does lead to a 40-fold increase in the Michaelis constant for aspartic acid. Aspartic acid analogs that were modified at either the α- or the β-carboxyl groups were examined to determine the orientation of the substrate at the active site relative to the location of Arg 29. Analogs in which the β-carboxyl group is replaced by either a phosphate or a phosphonate group are competitive inhibitors *versus* aspartate with K_i values ranging from 0.13 to 2.3 mm (Table 4). For the R29A mutant these compounds are also competitive inhibitors, but with inhibition constants that have been weaken by a factor of 2.5−10 compared to the native enzyme. Substrate analogs in which the β-carboxyl group has been more extensively modified, either by esterification or by replacement with neutral or positively charge functional groups, are not inhibitors of either the native enzyme or the R29A mutant. Modification of the α-carboxyl group to an amide leads to an analog that is only weakly inhibitory toward the native enzyme, and does not inhibit the R29A mutant.

Table 3: Kinetic Parameters of Aspartase Mutants

mutant	k_{cat} (min^{-1})[a]	percent k_{cat}	K_m (mM)	k_{cat}/K_m
wild-type	40.6	100	1.8 ± 0.1	22.6
H26Q	40.9	101	5.7 ± 1.0	7.9
R29A	55.3	136	80 ± 12	0.7
R15A	52.5	129	4.9 ± 1.4	10.7
S143G	4.4	11	7.3 ± 1.4	0.6
S143T	0.6	1.4	5.3 ± 1.4	0.1
D10N	7.5	19	6.3 ± 1.3	1.2
D10A	0.003	0.007	nd	nd

[a] Standard errors on k_{cat} are typically less than 10%, except for the D10A mutant which has a standard error of about 40%.

Replacement of Ser 143 with glycine causes a modest increase in $K_{aspartate}$ and a more substantial 10-fold decrease in k_{cat}, while replacement with threonine results in an additional 10-fold decrease in k_{cat}. A similar effect on k_{cat} is observed when Asp 10 is replaced with asparagine. However, substitution with an alanine at this aspartate position leads to a dramatic loss of catalytic activity (Table 3). The pH profile of the D10N mutant has the same overall shape as that of the wild-type enzyme (data not shown), with p*K* values at 7 on the acid side and near 10 on the base side that result in loss of catalytic activity.

DISCUSSION

Examination of Active Site Functional Groups. The deamination of aspartic acid is proposed to involve acid−base catalysis by *E. coli* aspartase, with base catalyzed removal of the *pro-R* proton on carbon 3 followed by protonation and expulsion of ammonium ion (Nuiry et al., 1984; Schindler & Viola, 1994). The location of the active site in the structure of aspartase has been determined from the position of Lys 327, an amino acid that had previously been shown to be involved in the activity of this enzyme (Saribas et al., 1994). Chemical modification and sequence homology studies have suggested the potential involvement of a number of other amino acids in the activity of this enzyme. However, in most of these cases, replacement of these amino acids by site-directed mutagenesis has either eliminated them from consideration or has relegated them to an indirect role in binding or catalysis in aspartase. The determination of the high-resolution structure of this enzyme

[2] The amino acid numbering scheme has been incremented by one from that reported in previous publications to reflect the presence of the *N*-terminal methionine in the aspartase structure.

Table 4: Binding of Aspartic Acid Analogs to Aspartase

substrate analog	structure[a] R_1	structure[a] R_2	inhibition constant, K_i (mm) native	inhibition constant, K_i (mm) R29A
O-phospho-L-serine	$^{2-}O_3P-O-$	$-COO^-$	2.3 ± 0.5	6.0 ± 1.0
O-phospho-D-serine	$^{2-}O_3P-O-$	$-COO^-$	0.13 ± 0.01	0.8 ± 0.1
2-amino-3-phosphonopropionic acid	$^{2-}O_3P-$	$-COO^-$	0.20 ± 0.04	1.8 ± 0.3
2-amino-4-phosphonobutyric acid	$^{2-}O_3P-CH_2-$	$-COO^-$	1.2 ± 0.3	12.0 ± 1.4
L-aspartate-α-amide	$^-OOC-$	$-CONH_2$	63 ± 16	no inhibition

[a] Parent structure: $R_1\text{-}CH_2\text{-}\underset{\displaystyle NH_3^+}{\underset{|}{CH}}\text{-}R_2$

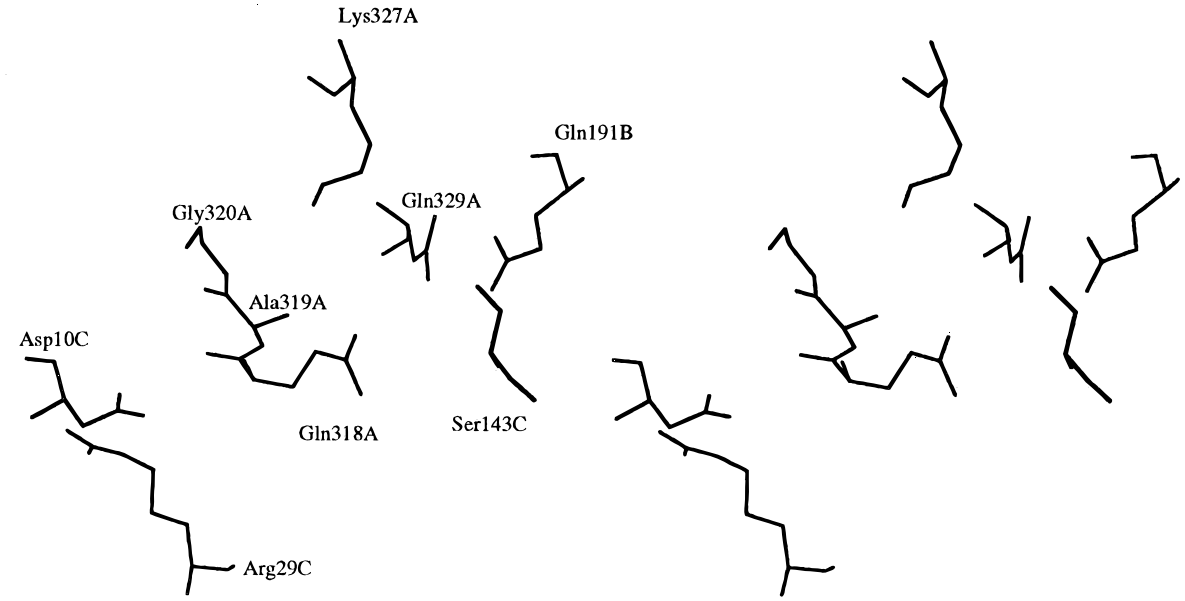

FIGURE 1: Stereoview of some amino acid residues in the active site region of aspartase.

(Shi et al., 1997) has now allowed the identification and examination of an additional set of potential candidates for these roles that had not been identified by previous studies.

A similar approach has been used, through the identification of the homologous conserved lysine, to locate the active site in the structure of the related enzyme fumarase from *E. coli* (Weaver et al., 1995). Unfortunately, comparison of the putative aspartase active site (Figure 1) to that of fumarase makes it clear that the residues that have been identified as important in fumarase are not the same ones that are involved in aspartase catalysis. The glutamate and threonine that have been assigned a hydrogen bonding role in fumarase are Gln 318 and Val 331 in the equivalent positions in aspartase. It is interesting to note that the Glu 315 mutation to glutamine that abolishes activity in fumarase is in fact the wild-type residue that is found at the corresponding position (Gln 318) in aspartase. Furthermore, the putative catalytic histidine of fumarase is Gln 191 in aspartase, and therefore could not be involved in the proposed charge relay system (Weaver et al., 1995). In fact, in the region surrounding Lys 327 in aspartase there are a number of residues that are well positioned for binding the substrate, but there are a minimal number of candidates that possess the expected chemical functionality to catalyze the deamination reaction.

Identification of Substrate Binding Groups. In the absence of guidance from an enzyme−substrate complex structure there are a number of ways in which aspartic acid can be docked at the putative active site to produce favorable binding interactions with the functional groups that are present. We expect that binding of this dicarboxylic acid substrate will involve an electrostatic attraction, with positively charged amino acids or with helix dipoles (Hol et al., 1978), in the substrate binding site. There are several potential candidates that have been examined. Previous studies have shown that alteration of the lysine at position 327 results in a 6-fold loss of binding affinity and over a 300-fold decrease in catalysis (Saribas et al., 1994). Additional substrate binding candidates that have been examined include the arginines at positions 15 and 29. Arg 15 is not conserved in the other aspartases, while Arg 29 is conserved in all of the aspartases and fumarases that have been sequenced. Removal of the positively charged functionality at either of these positions by replacement with an alanine has no effect on enzyme catalysis. In the case of the less conserved Arg 15, mutagenesis also had only a negligible effect on the K_m for aspartic acid. However, alteration of Arg 29 results in a 40-fold increase in K_m. Weaker binding by the R29A mutant is also supported by an observed increase in the K_i values for a number of competitive inhibitors of aspartase. The importance of the substrate carboxylate functional groups as binding determinants is demonstrated by the failure of substrate analogs to bind to the enzyme when the β-carboxylate group has been modified. Modification at the α-carboxyl group also results in a large decrease in binding affinity. These studies have lead to the assignment of a substrate binding role for Arg 29, and have

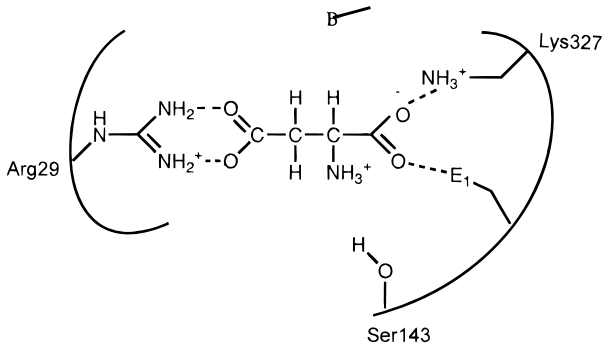

FIGURE 2: Cartoon of the active site of aspartase. Arg 29 and Lys 327 have been assigned to binding roles, with Arg interacting with the β-carboxylate of the substrate and Lys binding to an oxygen of the α-carboxylate. The high substrate specificity of aspartase suggests the presence of additional binding groups such as E_1, but no additional candidates have been definitively identified from the apostructure. The identity of the catalytic base is uncertain although two obvious candidates, Asp 10 and His 26, have been eliminated from consideration by site-directed mutagenesis studies. Ser 143 is suggested as the catalytic acid.

suggested the β-carboxyl group of the substrate as the most likely site of interaction (Figure 2).

Identification of Active Site Catalytic Groups. Among the preliminary candidates for the role of the base catalyst that is responsible for removal of the proton at carbon 3 of the substrate, His 26 with its higher p*K* value is better suited for this task than is Asp 10 (Table 2). While the latter functional group is fully conserved in the entire enzyme family, His 26 is found at this position only in aspartases from *E. coli* and *Haemophilus influenza*. Replacement of His 26 with glutamine has no effect on catalytic efficiency and only a modest effect on substrate recognition. Replacing the conserved aspartate with asparagine causes the same small increase in K_m but, in addition, results in a 5-fold decrease in k_{cat}. However, the observation of identical p*K* values for the D10N mutant and the wild-type enzyme indicate that the protonation of Asp 10 is not responsible for the observed loss of activity below pH 7. Complete removal of the functionality at this position by replacement with an alanine leads to a decrease in catalysis by over 4 orders of magnitude. This loss of activity is consistent with what would be expected upon removal of an essential catalytic residue. However, on the basis of the enhancement observed with an asparagine mutation at this position, the most likely role for Asp 10 is to participate in a hydrogen-bonding interaction either directly with the substrate or to help orient a catalytic group at the active site of aspartase.

No significant changes have been observed in the fluorescence spectra of these mutants. In contrast, the subunit dissociation and unfolding of aspartase that occurs upon addition of 4 M guanidine hydrochloride causes a 40% decrease in the fluorescence emission spectrum of the wild-type enzyme (Imaishi et al., 1989). These observations demonstrate that the loss of activity resulting from replacement of the amino acid at these positions is not a consequence of the loss of structural integrity of the enzyme.

The question of the difference in p*K* values between this putative active site base and the substrate proton that must be removed has not been resolved. Removal of a proton α to a carboxylate is a catalytic challenge that is accomplished by a wide variety of enzymes (Babbitt et al., 1996). Several suggestions have been made, including tight binding of an intermediate in the reaction and the formation of strong, low-barrier hydrogen bonds in the transition state (Cleland, 1992; Cleland & Kreevoy, 1994), to explain how a catalytic residue with a low p*K* value can remove a quite basic proton. Structural analogs such as 3-nitro-2-aminopropionate have been shown to bind very tightly to aspartase as transition state or transition intermediate analogs (Porter & Bright, 1980), however, this increased binding affinity is not sufficient by itself to account for the energy difference required for proton abstraction.

Among the candidates for the general acid catalyst that protonates the departing α-amino group are three hydroxyl-containing amino acids that are partially or fully conserved in the homologous enzyme family (Table 2). Ser 143, which is fully conserved and optimally positioned to carry out this role, was examined by site-directed mutagenesis. Removal of this functionality by replacement with threonine leads to an enzyme that has only 1% of the wild-type k_{cat}, lending support to this assignment.

It has been proposed, from an examination of the pH profile of monovalent cations interacting with aspartase from *Hafnia alvei*, that the amino group is eliminated in this enzyme as the neutral species and is then protonated to produce the final ammonium ion product (Yoon et al., 1995). The loss of Ser 143 as a general acid would eliminate this mode of catalysis in the *E. coli* enzyme, but, analogous to the proposed mechanism for the enzyme from *H. alvei*, the remaining catalytic potential of this enzyme may still leave a very efficient catalyst.

In summary, the current model of aspartase supports the previously suggested location of the active site. If the proposed mechanism of fumarase (Weaver et al., 1995) is correct, then aspartase must act by a different means since most of the equivalent residues are missing the required functionality. While not inconceivable, it does seem unlikely that two enzymes with similar overall structures, and binding the same substrate (fumarate), would use two disparate mechanisms in the same region of the protein to accomplish equivalent reactions. The active site and a constellation of functionally important amino acids have been identified in aspartase, however the exact positioning of the substrates and products at the active site and a more precise assignment of the functional roles of each amino acid must await the structural determination of the corresponding enzyme complexes. It is reasonable to expect some significant differences between the structure of the apoenzyme and the enzyme−substrate complex. Work is currently in progress on the determination of these structures.

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