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Enhancement of Catalytic Activity by Gene Truncation: Activation of L-Aspartase from *Escherichia coli*

Maithri M. K. Jayasekera, A. Sami Saribaş,[1] and Ronald E. Viola[2]
Department of Chemistry, University of Akron, Akron, Ohio 44325-3601

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Aspartase from *Escherichia coli* is activated by proteolysis at the carboxy-terminal. A systematic study has been undertaken with the goal of identifying the amino acids in this region that influence the catalytic activity of aspartase. Stop codons have been introduced at various positions to prematurely truncate the *aspA* gene that encodes for aspartase by sequentially eliminating each of the polar and charged amino acids in this region. The affinity of the enzyme for its substrate aspartic acid decreases systematically as each functionally significant amino acid is eliminated. However, enhanced catalytic activity (up to 2.5 times the k_{cat} for native aspartase) is observed for those truncation mutants that end in a positively charged carboxy-terminal amino acid. The precise position of the proteolytic activation of aspartase has been defined, and this covalent activation has been shown to be independent of the allosteric activation of aspartase that is also observed.

Aspartase (L-aspartate ammonia-lyase, EC 4.3.1.1) catalyzes the reversible deamination of L-aspartic acid to yield fumarate and ammonium ion. The high-resolution structure of the enzyme from *Escherichia coli* has recently been determined, and reveals an elongated subunit consisting of three distinct domains (1). Site-directed mutagenesis studies (2) have identified an intersubunit cleft as the location of the enzyme active site, and a putative set of active site amino acid functional groups have been identified (2,3). There are, however, some important features of aspartase that have not yet been revealed by structural studies. Divalent metal ions have long been known to play a role in the catalytic activity of this enzyme (4,5). NMR relaxation studies have established the site of metal ion binding as being separate and distinct from the enzyme active site (6), and kinetic studies have shown the presence of a separate site on the enzyme that binds the substrate, aspartic acid, as an activator (7,8). In addition to this non-covalent activation, aspartase is also activated by proteolytic cleavage (9,10). Cleavage of one or more peptide bonds near the carboxy-terminal results in a several-fold activation of aspartase activity (11). This proteolytic activation has been shown to be non-specific, with the action of several different proteases leading to enhanced catalysis by aspartase (12).

This study has been undertaken to precisely define the sites of covalent activation, to determine the kinetics of the activated enzyme, and to examine any relationship between proteolytic activation and allosteric activation of aspartase. Site-directed mutagenesis has been used to truncate the *aspA* gene at several sites located near the carboxy-terminal. The resulting modified enzymes have been overexpressed, purified, and examined.

EXPERIMENTAL PROCEDURES

Materials. L-Aspartic acid, fumaric acid, and substrate analogs were obtained from Sigma-Aldrich. The enzyme aspartase was purified by the procedure of Karsten et al. (13) from an overproducing strain of *Escherichia coli* (3). The Y467stop mutant has low affinity for the red-A agarose column that was used as the final purification step. This mutant was purified by a modified procedure that incorporates a KCl gradient elution from DEAE-sepharose and a Sephacryl-300 gel filtration step (14). DNA polymerase and the PCR optimization kit were obtained from Stratagene.

Production of mutants. The preliminary mutants that defined the region of aspartase activation were produced by the method of Kunkel (15). Subsequent site-directed mutagenesis was carried out by using the recombinant circle PCR (RC-PCR) method (16,17). This method gives a high mutation efficiency and requires little DNA template. The primers are designed so that after PCR the products are double-stranded, linear DNA molecules. The linear DNA molecules are then combined, denatured and reannealed to form double-stranded DNA molecules with discrete, cohesive, single stranded

[1] Present address: Department of Biology, University of Pennsylvania, Philadelphia, PA 19104.
[2] Corresponding author: Department of Chemistry, University of Akron, 190 East Buchtel Commons, Akron, OH 44325-3601. Fax: (330) 972-370. E-mail: rviola@uakron.edu.

Abbreviations used: 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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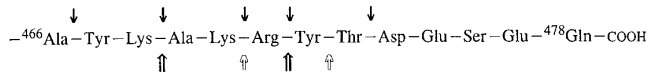

FIG. 1. Carboxy-terminal sequence of aspartase with the truncation sites (↓) and the proposed sites of proteolytic cleavage by trypsin (⇑) and by subtilisin (⇧) indicated.

ends. These DNA molecules will form recombinant circles that mimic circular DNA which were then transformed into either TG-1 or the JRG-1476 (*aspA*$^-$) cell line (18).

The four primers that are required for this method are incubated in sets of two, with two of the primers required for each mutation consisting of two to three base pair mismatches, located close to the 5′ end. These primers generate the stop codons and also, to facilitate screening, generate an alteration in a restriction enzyme site at that loci. For the mutation at position 469 these changes resulted in the destruction of a *Bsp*WI site. The mutations at 471 and 472 resulted in the creation of an *Acc*I site, however, the mutation at 472 required the creation of a silent mutation, one residue away from the mutated site. The mutations at positions 467 and 474 involved the creation of an *Afl* II site. Initial screening for the presence of mutagenic colonies was accomplished by restriction enzyme mapping, with the subsequent DNA sequencing of the mutated region.

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. For the examination of activation by substrate and metal ions the enzyme was assayed in the amination direction as previously described (8). The kinetic data were fitted by using the Enzyme Kinetics Package software (SciTech International, Chicago, IL) to obtain the kinetic parameters.

RESULTS

Aspartase has previously been shown to be activated by several different proteases acting to remove one or more small peptides from the carboxy-terminal (11,12). Several primary and secondary sites of proteolysis have been identified (Fig. 1), however it is not clear whether cleavage at a single site is responsible for the enhanced catalytic activity of aspartase. We have undertaken a systematic study of the carboxy-terminal region of aspartase with the goal of identifying the amino acids in this region that influence the activity of the enzyme.

Kinetic characterization of the truncation mutants. Stop codons have been introduced at specific positions in the carboxy-terminal region that had previously been identified, from peptide mapping studies of partially proteolyzed aspartase, as affecting enzyme activity. The position of these truncation mutations was designed to sequentially eliminate a single charged or polar amino acid when compared to an adjacent mutation site. Each of these truncation mutants was expressed, purified and characterized in order to ascertain the role of the amino acid that has been eliminated. The kinetic parameters of this family of truncation mutants are summarized in Table 1. Elimination of a 12-amino acid peptide from the carboxy-terminal (Y467stop) lead to an enzyme with significantly weaker affinity for its substrate and with decreased catalytic efficiency. Extending this truncated enzyme by two amino acids to a C-terminal lysine (A469stop) leads to recovery of the catalytic activity to native enzyme levels, but leaves an elevated Michaelis constant for aspartic acid. Addition of another dipeptide keeps the C-terminal amino acid as lysine, but results in an enzyme (R471stop) with significantly enhanced catalytic activity over that of native aspartase (Table 1). Extending the enzyme by a single arginine (Y472stop) causes a further enhancement in catalysis, and a tightening of the K_m for aspartic acid, to yield a modified aspartase with a k_{cat}/K_m that has been optimized beyond that which has been achieved by the native enzyme. Extending the C-terminal by another dipeptide leads to an enzyme form (D474stop) that terminates in a threonine and has returned to native-like kinetic parameters.

Activation by aspartic acid and divalent metal ions. At pH values above neutrality aspartase must be activated by aspartic acid and by divalent metal ions in order to catalyze the addition of ammonium ion across the double bond of fumarate (9,10). The truncation mutants were examined for their affinity for Mg^{2+} as the divalent metal ion activator. Native aspartase had previously been determined to have a K_a for Mg^{2+} of 5 μM at pH 7.0 (6). All of the truncation mutants have K_a values for Mg^{2+} in the range of 3 to 6 μM, with no obvious trend that correlates with either the identity of the C-terminal amino acid or with the kinetic parameters for the mutants. Activation by aspartic acid was examined in the concentration range from 0.1 to 5 mM and appears to be similar, for all of the truncation mutants, to the activation observed with the native enzyme.

DISCUSSION

Proteolytic activation is a common means for converting an inactive precursor to a catalytically active enzyme. While there are numerous examples of the non-covalent activation of enzymes by various effectors, enhancement of the catalytic potential of an already active enzyme by proteolytic cleavage is un-

TABLE 1

Kinetic Parameters of Truncated Aspartases

Mutant	k_{cat} (min^{-1})	Percent k_{cat}	K_m (mM)	k_{cat}/K_m
Wild-type	40.5 ± 1.6	100	1.8 ± 0.1	22.6 ± 0.9
D474stop	42.7 ± 7.6	105	2.8 ± 1.1	15.3 ± 2.9
Y472stop	97.2 ± 6.1	240	3.3 ± 0.8	29.5 ± 1.8
R471stop	70.7 ± 2.9	175	4.5 ± 0.5	15.7 ± 0.6
A469stop	39.6 ± 1.0	98	10.2 ± 0.7	3.9 ± 0.1
Y467stop	2.5 ± 0.8	6.1	31 ± 8.2	0.08 ± 0.003

usual. Phenylalanine and tyrosine hydroxylases have both been reported to be activated by partial proteolysis. Activation of phenylalanine hydroxylase by chymotrypsin involves the removal of a 15 kDa domain (19), while trypsin activation of tyrosine hydroxylase leads to significant alteration in the size of the protein (20). More recent studies have shown that deletion mutations of up to 165 amino acids from the amino-terminal results in over a 2-fold increase in the catalytic activity of tyrosine hydroxylase (21). The chymotrypsin-catalyzed activation of glutamate dehydrogenase leads to a 3-fold enhancement of V_{max} (22), while trypsin activation results in the removal of a 39 amino acid peptide from the amino-terminal and an 8-fold activation of glutamate dehydrogenase (23). In each of these cases proteolytic activation has been achieved through the removal of a significant region of the protein.

Proteolytic activation of aspartase. In most cases when proteolytic enhancements of catalysis have been observed the specificity and the site(s) of proteolysis have not been established, and the structural and mechanistic effects of these covalent changes have not been elucidated. This is also true for the proteolytic activation of aspartase, where several different peptides resulting from the partial proteolysis by trypsin and subtilisin have been identified (Fig. 1). However, errors in the earlier carboxy-terminal sequence, and amino acid compositions for the released peptides that do not match the terminal sequence, have hampered a definitive assignment of how the amino acids in this region influence the catalytic activity of aspartase.

Region of covalent activation of aspartase. The location of the region of activation at the carboxy-terminal has been defined by the boundaries of the truncation mutations at positions 467 and 474. The introduction of a stop codon at position 467 leads to an enzyme with significantly impaired catalytic function (Fig. 2). Further truncation of the enzyme is unlikely to lead to any improvement in catalysis. Truncation at position 474 has only a modest effect on the catalytic parameters of aspartase. Extending the gene from this point to the native carboxy-terminal end would not be expected to alter enzyme activity. Thus, these two positions define the boundaries of the region where altering the length of the enzyme affects catalysis. The 7 amino acids in this region consist of three positively charged amino acids, a polar amino acid, and two aromatic residues. Truncation mutations have been introduced that lead to the sequential removal of each of the polar and positively charged amino acids.

It is apparent that the presence of a positively charged amino acid at the carboxy-terminal of aspartase leads to a dramatic enhancement in catalytic activity. The addition of a dipeptide ending in lysine to the Y467stop mutant results in a 16-fold enhancement of the k_{cat} of this kinetically compromised mutant (Fig. 2). Extension of the enzyme by another dipeptide to a new C-terminal lysine causes a further 2-fold enhancement in catalysis. The inclusion of an additional arginine at the C-terminal increases k_{cat} by another 37% to an enzyme form with 2.5 times the catalytic activity of the native aspartase. In contrast to the results observed with aspartase, the sequential removal of amino acids from the carboxy-terminal of firefly luciferase leads to a stepwise loss of bioluminescent activity without regard to the nature or identity of the amino acid that is removed (24).

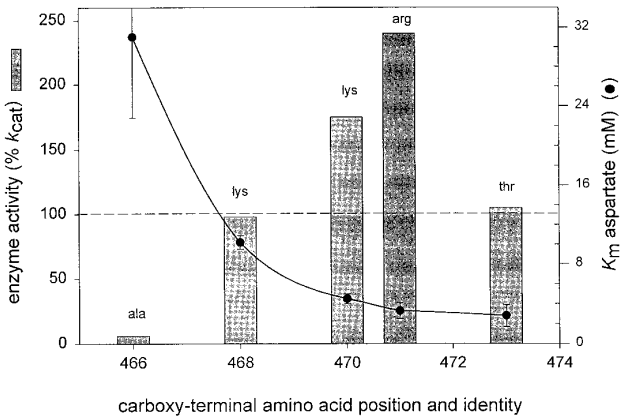

FIG. 2. Relationship of the kinetic parameters of aspartase to the identity and position of the carboxy-terminal amino acid. The K_m values of the truncation mutants (●) are shown along with the associated standard errors, and the catalytic acitivity of the mutants (%k_{cat}) are compared in a bar graph to the activity of the native enzyme.

From an examination of these truncation mutants we can conclude that the proteolytic activation that was achieved upon treatment of aspartase with trypsin is caused by the cleavage of the peptide bond between arg471 and tyr472 (Fig. 1). The resulting peptide is the primary peptide product that was isolated after trypsin-activation (11). The proteolytic activation by subtilisin results from the cleavage of the peptide bond between lys470 and arg471, and this resulting peptide is also the major peptide product that was isolated (12).

Mechanism of covalent activation. How these structural modifications in the carboxy-terminal region cause the observed alterations in the catalytic activity of aspartase is as yet undetermined. The conformation of the C-terminal region of the native enzyme is highly disordered, with no clearly identified electron density beyond amino acid 460 in the crystal structure (1). This disorder may reflect a highly flexible region of the protein that can alter its conformation in response to binding at the active site of the enzyme. One possible explanation for the alterations in catalytic activity is that the carboxy-terminal can directly participate in substrate binding or in catalysis. The affinity of the enzyme for aspartic acid (as measured by the K_m for aspartate)

decreases systematically as functionally significant amino acids are successively removed from the C-terminal. Enhanced catalytic activity is observed for those truncation mutants that end in a positively charged carboxy-terminal amino acid (lys 468, lys 470 and arg 471). These functional groups may act to optimize the orientation of the substrate aspartic acid at the enzyme active site.

Alternatively, the creation of a new negative charge (the α-carboxyl group) adjacent to a positively charged amino acid side chain will cause some reorientation of the adjacent charged and neutral amino acids to accommodate this new charge. These resulting structural changes may be sufficient to alter the conformation at the active site of aspartase, leading to improved catalysis. Crystallization trials are currently underway in order to determine a structural basis for the enhanced catalytic activity of these truncation mutants of aspartase. An understanding of the structural basis of enhanced catalysis in this highly specific enzyme should provide some general insights into the refinement and enhancement of enzyme-catalyzed reactions.

Allosteric activation of aspartase. The required activation of aspartase at alkaline pH is unaffected by the truncations at the C-terminal. The affinity of the enzyme for divalent metal ions and for aspartic acid as an activator are unchanged across the series of truncation mutants, which have k_{cat} values ranging by nearly 40-fold and k_{cat}/K_m values varying over 350-fold. Thus it appears that the proteolytic activation of aspartase and the allosteric activation of aspartase are separate and distinct events.

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