Crystal Structure of Isoaspartyl Aminopeptidase in Complex with L-Aspartate

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Karolina Michalska, Krzysztof Brzezinski, and Mariusz Jaskolski

From the Department of Crystallography, Faculty of Chemistry, A. Mickiewicz University, Poznan 60-780, Poland and the Center for Biocrystallographic Research, Institute of Bioorganic Chemistry, Polish Academy of Sciences, Poznan 61-704, Poland

The crystal structure of Escherichia coli isoaspartyl aminopeptidase/asparaginase (EcAIII), an enzyme belonging to the N-terminal nucleophile (Ntn)-hydrolases family, has been determined at 1.9-Å resolution for a complex obtained by cocrystallization with L-aspartate, which is a product of both enzymatic reactions catalyzed by EcAIII. The enzyme is a dimer of heterodimers, (αβ)2. The (αβ) heterodimer, which arises by autoproteolytic cleavage of the immature protein, exhibits an αββα-sandwich fold, typical for Ntn-hydrolases. The asymmetric unit contains one copy of the EcAIII:Asp complex, with clearly visible L-aspartate ligands, one bound in each of the two active sites of the enzyme. The L-aspartate ligand is located near Thr179, the N-terminal residue of subunit β liberated in the autoproteolytic event. Structural comparisons with the free form of EcAIII reveal that there are no major rearrangements of the active site upon aspartate binding. Although the ligand binding mode is similar to that observed in an L-aspartate complex of the related enzyme human aspartylglucosaminidase, the architecture of the EcAIII active site sheds light on the question of substrate specificity and explains why EcAIII is not able to hydrolyze glycosylated asparagine substrates.

Proteins undergo several age-dependent spontaneous modifications that can limit their useful lifetime. In particular, deamidated, racemized, or isomerized derivatives can be formed in an intramolecular succinimide-mediated rearrangement involving L-asparagine or, in a 13-36-fold slower reaction (1), L-aspartyl residues (2). The major product is an L-isoaspartyl (iAsp)1-containing protein, in which the peptide backbone has been transferred to the side chain forming a β-peptide. Such a serious structural rearrangement usually leads to protein dysfunction. Two mechanisms have been proposed by which organisms may handle the useless proteins and prevent accumulation of the harmful iAsp (3-5).

One well defined mechanism involves the repair of some products of spontaneous damage to intracellular proteins. It is based on the enzyme L-isoaspartyl(D-aspartyl)-O-methyltransferase (O-MT), which is found in organisms ranging from bacteria to mammals and plants and initiates the repair pathway by methylation of L-isoaspartyl residues (and L-aspartyl residues in racemized derivatives) (6, 7). Despite the quite wide range of recognized substrates, the repair activity of O-MT is limited (8, 9). The damaged proteins that are not identified and repaired by the enzyme are degraded by cellular proteases to free amino acids and to the relatively stable iAsp-containing di- and tripeptides. To prevent accumulation of those harmful isoaspartyl peptides, specialized enzymes with peptide activity are required (10). Enzymes with isoaspartyl peptide activity (EC 3.4.19.5) were initially isolated from mammals (10) and bacteria (11). Further studies have revealed the existence of two classes of isoaspartyl peptidases. The first class includes metallopeptidases represented by the Escherichia coli (12) and Salmonella enterica (5) proteins, both encoded by the iadA gene. Sequence analysis of the E. coli IadA protein suggested its similarity to bacterial dihydroorotases and imidases, which are involved in the metabolism of pyrimidines (12). The crystal structure of E. coli IadA confirmed its structural homology to these enzymes, as well as the presence of zinc cations (13, 14).

The second class of isoaspartyl peptidases is represented by the E. coli iaaA gene product and its S. enterica homolog (5). These enzymes belong to the family of Ntn (N-terminal nucleophile) hydrolases, first classified by Brannigan et al. (15), which, according to a recent study by Elkins et al. (16), may actually form a subclass of Ntn-enzymes with more general (not necessarily hydrolytic) activities. Ntn-hydrolases are expressed as enzymatically inactive precursors that become activated upon autoproteolytic cleavage, which generates two subunits, α (N-terminal) and β (C-terminal). The N-terminal residue of subunit β, a threonine, serine, or cysteine, acts as the active nucleophile in the catalytic mechanism. The crystal structures reported for several members of this family, including ornithine acetyltransferase (16), human (17), and Flavobacterium meningosepticum aspartylglucosaminidas (18, 19), the proteasome β-subunit (20), glutamine 5-phosphoribosyl-1-pyrophosphate amidotransferase (21), acylases of penicillin G (22) and penicillin V (23), as well as cephalosporin acylases (24, 25), have revealed that, although the amino acid sequences of these proteins vary considerably, they all have the same fold. The polypeptide chains of these proteins are organized into a sandwich of two extended parallel β-sheets, flanked on both sides by α-helices. This topology is usually described as the αββα-fold (26), a term that illustrates the spatial organization of the secondary structure elements. Each of the two active
sites (at the N-terminal nucleolipides of subunits \( \beta \)) of an Ntn heterotrimer is located between the \( \beta \)-sheets.

The \( iaaA \) gene product from \( E. \) coli was originally classified as a new, plant-type asparaginase, and named EcAIII (27–29), because of high sequence homology to enzymes believed to function as plant \( \lambda \)-asparaginases. It also shows sequence similarity to aspartylglucosaminidases (AGAs) (30), which are involved in the breakdown of glycosylated proteins. However, the recently reported kinetic data indicate that the dominant activity of both EcAIII and genuine plant asparaginases is connected with the hydrolysis of isoaspartyl dipeptides, whereas their affinity for \( \lambda \)-asparagine is relatively weak, particularly when compared with the affinity of classic bacterial asparaginases (30). There is also no detectable activity toward \( N \)-acetylglucosaminyl-\( \lambda \)-asparagine, a typical substrate of aspartylglucosaminidases (5, 30). In addition, it has been shown that the homologous enzyme from \( S. \) enterica is not a rigorous dipeptidase, because it is also capable of hydrolyzing tripeptides with an N-terminal isoaspartyl residue (5). Among dipeptides, iAsp-Leu is recognized with the highest affinity.

The structure of free EcAIII has been determined and deposited in the Protein Data Bank (PDB) independently by two groups. Borek et al. (31) determined the structure of EcAIII at low and high calcium concentration (PDB accession codes 1K2X and 1JN9, respectively), and later Prahler et al. (32) reported a structure that is essentially identical to the former (1T3M). Here, we describe the crystal structure of EcAIII in complex with \( L \)-aspartate (PDB accession code 1SEO), which is a product of the two reactions catalyzed by the enzyme (Fig. 1), as well as a mimic of the substrate of the \( \lambda \)-asparaginase reaction. We report the ligand binding mode and investigate the conformational changes in the active site and in the overall architecture of the protein that occur upon ligand binding. Finally, the structure of the EcAIII-Asp complex provides insights into the catalytic mechanism and substrate specificity of the enzyme.

MATERIALS AND METHODS

Crystallization—EcAIII was produced and purified as previously described (30). Single crystals of a complex between EcAIII and \( L \)-aspartate were obtained using a modification of the protocol developed for the free enzyme (33). The crystals were grown by the hanging drop, vapor diffusion method at room temperature. The precipitant solution contained 17% polyethylene glycol 4000, 13% polyethylene glycol 400, 80 mM CaCl\(_2\), 100 mM Tris/HisCl, pH 8.5, and 100 mM sodium \( L \)-aspartate. The molar excess of \( L \)-aspartate was about 200-fold. The crystals reached their maximum size of \( 0.2 \times 0.2 \times 0.2 \) mm in 12 days. They belong to the orthorhombic space group \( P2_12_12_1 \).

Data Collection and Processing—X-ray diffraction data extending to 1.9-Å resolution were collected for a single crystal flash-frozen at 100 K in a gas nitrogen stream using the mother liquor as cryoprotectant. Synchrotron radiation generated at the MAX-lab (Lund, Sweden) beamline 1711 was used. The data were processed and scaled with DENZO and SCALEPACK from the HKL suite (34). Table I lists the data collection and processing statistics.

Structure Determination and Refinement—The structure of the EcAIII-Asp complex was solved by molecular replacement using the program MolRep (35). The search model, consisting of the complete (\( \omega \beta \)), heterotrimer, was generated from the coordinates of the free enzyme deposited in the PDB under the accession code 1K2X (31). The molecular replacement approach gave a clear solution characterized by an \( R \) factor of 36% and a correlation coefficient of 67%. The presence of the ligand molecules in the two active sites was clearly visible in the preliminary \( F_o - F_c \) electron density maps calculated with phases calculated from the protein model only. Residues 1 and 162–178 from chain A (subunit \( \alpha \)) and 158–178 from chain C (subunit \( \alpha \)), and 314–321 from chains B and D (subunits \( \beta \)) were not visible in the electron density maps even at the conclusion of the refinement. Cysteine 63 in both subunits \( \alpha \) (chains A and C) carries a clear covalent modification at the \( \gamma \) atom, which according to the appearance of the electron density maps has been modeled as a \( \beta \)-mercaptoethanol residue. As in the free enzyme, the \( \alpha \) subunits coordinate a structurally important sodium cation. At the initial stage of the refinement, the CNS program (36) was used. The final refinement was carried out in Refmacs (37) using the maximum-likelihood target. TLS parameters (38) defined for each of the polypeptide chains were also optimized in the refinement. 270 water molecules were included in the final model. The final \( R \) and \( R_{free} \) factors converged at, respectively, 16.4% and 18.9%, and the model is characterized by very good geometry (r.m.s.d. from ideal bond lengths 0.014 Å, from ideal bond angles 1.38°). Manual modeling was done using the Xfit program from the XtalView package (39). The refinement statistics are given in Table II.

Protein Data Bank Accession Code—The atomic coordinates and structure factors have been deposited in the RCSB Protein Data Bank with the accession code 1SEO.

RESULTS AND DISCUSSION

Structure Solution and Crystal Packing—EcAIII in complex with \( L \)-aspartate crystallizes in the orthorhombic space group \( P2_12_12_1 \), with unit cell dimensions \( a = 49.9, b = 77.3, c = 147.5 \) Å. The asymmetric unit contains the \( \omega \beta \) assembly, i.e. a dimer of heterodimers (Fig. 2a), corresponding to a Matthews volume of 2.3 Å\(^3\)/Da and solvent content of 46.4% (41). Because one of the polymorphs of the free enzyme (1K2X) crystallizes in the same space group with nearly identical cell parameters \( (a = 50.3, b = 77.6, c = 148.2 \) Å) and the same cell contents (31), it seemed natural that an electron density map calculated with the “complex” amplitudes and “free” phases should provide the structure solution. However, such a map was not interpretable. For that reason the structure was solved by the molecular replacement method with the free enzyme as the probe. The solution revealed that, despite apparent crystallographic similarities, these two crystals have quite different molecular packing.

Description of the Structure—The molecular structure is created from four polypeptide chains, which have arisen from the
TABLE II

Refinement statistics

| Parameter                      | Value     |
|--------------------------------|-----------|
| Resolution (Å)                 | 20.0–1.9  |
| No. of reflections in working set | 42,200    |
| No. of reflections in test set  | 1372      |
| R(Fo−Fc) (%)                   | 16.4/18.9 |
| No. of atoms (protein/ligand/solvent/Na+ / Ca2+) | 4267/18/270/6/1/1 |

* R = Σ||Fo|| − |Fc||/Σ|Fo||, for all reflections, where |Fo|| and |Fc|| are observed and calculated structure factors, respectively. Rfree is calculated analogously for the test reflections, randomly selected, and excluded from the refinement.

The biological assembly is, therefore, a dimer of heterodimers, the Sα-helices forming a broad parallel β-sheet structure (Fig. 2).

In the final model built into electron density maps, chain A corresponds to the full mature subunit α, residues 2–161, whereas four residues are missing from the C terminus of chain C (2–157), presumably as a result of disorder. Both β subunits, chains B and D, are composed of residues 179–313, i.e. they have two residues missing at their C termini. In both α subunits, the Sα atom of Cys83 is chemically modified by a covalent S–S attachment of β-mercaptoethanol (used in the protein preparation protocol), resulting in a modified residue annotated as Cme63. The modification does not seem to have any structural or functional consequences.

The topology of the EcAIII (αβ)2 heterodimer is similar to that of other Ntn-hydrolases, with a typical αβ/α-layer structure (26). The protein core is composed of two open β-sheets, positioned to face each other, with their strands following the same general direction. The smaller one consists of four antiparallel β-strands belonging to subunit β. The larger β-sheet is composed of eight β-strands contributed by both subunits and an additional (ninth) strand (Sβ3) contributed by the other heterodimer. The overall antiparallel character of this β-sheet is violated by strand S4α, which is parallel to S3α. The layer of helices that pack on the outside of the nine-stranded β-sheet consists of five α-helices and of two 3α helices. The four-stranded β-sheet is flanked by four α-helices forming a broad crossover loop between the antiparallel strands S5β and S6β.

Although EcAIII has a general fold similar to aspartylglucomaminidases, structural alignments reveal several important differences visible especially in the organization of the secondary structure elements of subunit α (Fig. 2c). In particular, the N-terminal elements S1α and H2α of EcAIII are much longer, placing the loop connecting them ~17 Å away from the corresponding AGA loop.

Metal Coordination—The loop between H3α and S2α comprises 19 residues and has a complicated conformation supported by a metal cation with a coordination geometry that is close to octahedral. The metal is chelated exclusively by mainchain C=O groups provided by residues Leu66, Gln61, Cme63, Phe66, Ala68, and Ile670 in this loop (Fig. 3a). By analogy to the structure of the free enzyme (31), the metal cation has been identified as Na+. This interpretation has been confirmed by (i) unacceptable B factors refined for species with significantly different numbers of electrons and by (ii) the coordination geometry, in particular when interpreted by the bond valence method (43). The coordination spheres of both Na+ centers are very similar, with the Na+…O distances ranging from 2.2 to 2.7 Å, and the O…Na+…O angles distributed within the intervals 81–107° and 159–173°.

Quaternary Structure of EcAIII—In analogy to aspartylglucomaminidases, EcAIII forms a dimer of heterodimers (αβ)2. The interface between the two (αβ) heterodimers involves a number of hydrogen bonds and hydrophobic contacts. The main interactions are found between helices Hβ2 and between strand S4α from one (αβ) unit and strand S3β from the other. This antiparallel interaction (which occurs twice in the heterotetramer) extends the eight-stranded β-sheet formed within one (αβ) heterodimer into a nine-stranded structure, within which three out of the four polypeptide chains of the (αβ)2 heterotetramer meet. Additional hydrogen bonds responsible for oligomerization are formed between helix H1β and two loops from the complementary (αβ) unit. The first loop connects helix H4α with strand S4α, whereas the second loop is a hairpin connecting strands S2α and S3α. Similar regions determine the quaternary structure of AGAs, but the fragment classified here as S3β (44) was interpreted as a loop in the aspartylglucomaminidase fold (17, 18). The Sβ3 element belongs to the most conserved region of the amino acid sequences of the compared enzymes (Gly206–Pro212 in EcAIII numbering). The main difference between the interfaces found in the present isoaspartyl peptidase and in aspartylglucomaminidases is the presence in EcAIII of a pair of interacting Tyr251 residues at the C termini of the H2β helices, which bring these helices closer together.

The tetrameric structure of EcAIII is found both in crystal and in solution (30). This is also true for human AGA (17), whereas for the bacterial enzyme no (αβ)2 tetramers have been observed by size exclusion chromatography (19). The lower stability of F. meningosepticum AGA tetramers cannot be attributed to the absence of a random coil domain at the C terminus of subunit α (19), because no such domain is present in EcAIII.

The non-crystallographic symmetric of the molecule corresponds to a nearly ideal 2-fold rotation (179.7°), as in the free enzyme, but the r.m.s.d. between the superposed Co atoms is relatively high (0.45 Å). The source of this discrepancy lies in some small but significant deviations between the α-subunits (0.50 Å), whereas the β-subunits are practically identical (0.28 Å). The differences between the α-subunits seem to be due to packing interactions and are restricted to peripheral secondary structure elements. Structural alignments of the EcAIII-Asp complex with the three free EcAIII structures deposited in the PDB (1K2X, 1JN9, and 1T3M) reveal that the arrangement of the individual subunits is well preserved and that there is no reorientation upon ligand binding. The overall r.m.s.d. for a Ca superposition with 1K2X (0.55 Å) is comparable with the values calculated in self superpositions, and the α-subunits also show higher variability. When the present EcAIII-Asp complex is compared with the t-aspartic complex of human aspartylglu-
cosaminidase (1APZ), the r.m.s.d. value is quite high (1.5 Å), indicating that, despite similar folding pattern, the two proteins show significant structural differences.

Interactions of L-Aspartate in the Active Site—
The electron density corresponding to the ligands in both active sites (Fig. 3c) provides a definition of the active site pocket of EcAIII. It is located between the two /H9251–H9252/sheets of the /H9251/H9252/heterodimer and has a funnel shape, sculpted mainly by several loops, belonging to both subunits (/H9251 and /H9252), that connect the helical layers and the /H9252/sheets (Fig. 2b). The L-aspartate ligand is located near Thr179, the N-terminal residue of subunit /H9252 created upon autoproteolysis and believed to act as the nucleophile during the catalysis (15, 17, 18, 46, 47). All residues that participate in aspartate binding (Table III), including Arg207, Asp210, Thr230, Gly231, and Gly233, as well as the catalytic Thr179 itself, are contributed by the /H9252/subunit and are absolutely conserved among Ntn-asparaginases. The other part of the active site, responsible for binding the other fragment of an iAsp-peptide substrate (corresponding to the sugar moiety in aspartylglucosaminidases) has additional contribution from the /H9251/subunit. As shown in Fig. 3c, Arg207 forms a strong salt bridge with the /H9251-carboxylate group of the aspartate (which, obviously, must be deprotonated), thus anchoring the ligand in the active site. Additionally, Gly233 forms an N–H…O hydrogen bond with one of the oxygen atoms of the /H9251-COO group. Residues Asp210 and Gly231 are responsible for anchoring the /H9251-amino group of the aspartate through O…H–N hydrogen bonds accepted at the side chain and main chain, respectively. The protonation state of the /H9251-amino group cannot be decided from the pattern of its hydrogen bonding as one of the partners is a water molecule. However, at physiological pH, or even at the pH of the crystallization conditions (8.5), the amino group of a free /H9251-amino acid
would be expected to be in the ammonium -NH$_3^+$ form. Hydrogen bonds formed by Thr$^{230}$ (side chain) and Gly$^{231}$ (N–H) fix one of the oxygen atoms of the aspartate β-carboxylate group. These two residues appear to form the oxyanion hole that stabilizes the negative charge that develops on the substrate carbonyl O atom during the formation of the tetrahedral transition state of the catalytic reaction. The segment Thr$^{230}$–Gly$^{233}$ of subunit β is thus very important for providing a number of points of attachment for the bound product, and presumably also the corresponding substrate molecule, in the active site. The conserved residues of this segment provide the appropriate side chain functionality (Thr$^{230}$) or adequate flex-

![Fig. 3.](image-url)

a, stereoview of a superposition of the active site and the sodium-coordinating loop of EcAIII-Asp complex (this work, heterodimer AB, blue) and from the free enzyme (PDB code 1K2X, heterodimer AB, orange). The sodium cation is shown as a blue sphere; the l-aspartate ligand is marked as “D”. b, stereoview of a superposition of the active sites of the EcAIII-Asp complex (heterodimer AB, gray), human AGA-Asp complex (1APZ, heterodimer AB, pink), and F. meningosepticum AGA (2GAW, heterodimer AB, yellow), with labeling of the EcAIII residues. c, stereoview of the EcAIII-Asp catalytic site (within the AB heterodimer) with a definition of the l-aspartate binding pocket. The ligand is shown in a 2F$_o$ – F$_c$ map contoured at the 1σ level. The broken lines represent hydrogen bonds.
The two L-aspartate ligands bound in the two active sites of the human AGA (1APZ) complexes

table IV

Conformation of the l-aspartate ligands in the EcAIII (1SEO) and human AGA (1APZ) complexes

For each torsion angle, two values are given, corresponding to the two ligand molecules bound in each enzyme.

| Torsion angle | 1SEO | 1APZ |
|--------------|------|------|
| O-Cα-Cβ-Cγ   | 81/88| 57/53|
| Cα-Cβ-Cγ-O62  | -178°-177° | -167/180 |
| Cα-Cβ-Cγ-O62  | 9/7 | -10/38 |

Implications for the Catalytic Mechanism——The current view of the mechanism of Ntn-hydrolases assumes that the reactive nucleophile attacking the scissile amide bond in the first step of the reaction (during which an acyl-enzyme covalent intermediate is formed) is provided by the N-terminal residue of the enzyme. The L-aspartate ligand is proposed to act as a nucleophilic activator, as in the classical serine-protease S-H-D triad model. The controversy is in fact mainly concerned with the role of the N-terminal amino group (Thr, Ser, or Cys) which is uncharged and because in its structural vicinity there is no other candidate to act as a nucleophilic activator, as in the classic serine-protease S-H-D triad model. The controversy is in fact mainly concerned with the role of the N-terminal amino group (Thr, Ser, or Cys) which is uncharged and because in its structural vicinity there is no other candidate to act as a nucleophilic activator, as in the classic serine-protease S-H-D triad model.
been proposed that the unfavorable geometry of the intramolecular hydrogen bond is relieved through the recruitment of a water molecule (22, 24), but no such mediation is observed in EcAIII. The questions concerning the nucleophilic character of Thr179 go even further as it is evidently the acceptor in the Oγ-H…Oγ hydrogen bond from Thr197 (Fig. 3c and Table III). The latter residue is an acceptor of two N-H…O hydrogen bonds from the following glycine tandem (Gly198-Gly199) so that in the third H-bond contact (with Thr179) it must act as a donor. It is puzzling why a nucleophile, which is expected to be highly polarized and engaged in interactions abstracting its own hydrogen atom, should accept an additional hydrogen bond. It could be argued that the situation in the present product complex does not reflect the situation when a substrate is bound, but it is rather difficult to envision how the above hydrogen bond network could be altered without a radical rearrangement of the geometry of the active site. Such a rearrangement is not supported by experiment, because in the unliganded enzyme the same Thr179 Oγ-H…Oγ Thr179 hydrogen bond exists (Table III). This means that a substrate approaching the active site of EcAIII encounters a catalytic residue whose nucleophilic character is rather compromised. It is of note that the same puzzling H-bonding scheme exists in the L-aspartate complex (Table III) and in the free form of human AGA (17). Interestingly, in the structures of inactive precursors of bacterial aspartylglucosaminidases this hydrogen bond is not observed (18, 51). The results of mutational studies indicate that the presence of both hydroxyl residues in the EcAIII positions 179 and 197 is essential for efficient activation and maintenance of catalytic activity in aspartylglucosaminidases (52). It appears that the proper conformation of Thr197, allowing the Oγ…Oγ interaction in the mature protein, is secured by its additional hydrogen bond with the main-chain amides of Gly198 and the conserved Gly199. The backbone torsion angles of Gly199 are abnormal not only in EcAIII (ψ = 96°, ω = −145° in both chains) but also in human and bacterial aspartylglucosaminidases. When the Gly199 equivalent of human AGA is mutated to Asp, the substitution limits the maturation of the protein, which remains inactive but is correctly folded and transported to the lysosomes (47). A substitution by Ala, on the other hand, leads to a molecule with aberrant fold, which accumulates at the endoplasmic reticulum (53).

The puzzle concerning Thr179 does not end here, because the Oγ atom is in a very close contact (2.5 and 2.7 Å in the two active sites) with one of the oxygen atoms (O62) of the β-carboxylate of the bound product, which at the pH of the crystallization conditions (8.5) obviously must be deprotonated. In the complex of human aspartylglucosaminidase, these contacts are somewhat longer but still very close (Table III). To explain such short contacts, one is forced to accept that they are hydrogen bonds. In this scheme, however, the proton of the hydroxyl group of Thr179 is not pulled away from the scissile-bond electrophile but directed toward it. Such an interpretation would eliminate the possibility of the intramolecular Oγ-H…NH2 hydrogen bond of the Thr179 residue. This would be still compatible with the general scheme of hydrogen bonds at the Thr179 α-amino group, because one of the ligands is a water molecule (Fig. 3c), but could not be reconciled with the accepted mechanism of Ntn-hydrolases. A way out of this dilemma would be to assume that the product-enzyme interactions are different from the situation with a substrate bound in the active site. However, the (Thr179) Oγ…O62 (L-aspartate) contact is even more intriguing, because its detailed geometry is not consistent with a classic hydrogen bond. The reason is that the Thr179 Oγ atom is not approaching the O62 “acceptor” along one of the preferred directions defined by the lone-pair sp2 orbitals (i.e. in the COO plane, 120° relative to the Cγ-O62 bond), but it is posed above the β-carboxylate plane along an Oγ…O62 line that is nearly normal (within 18/14°) to this plane. With a distance to the Cγ atom of only 2.8/2.9 Å, this geometry would actually mimic in a way the situation during the nucleophilic attack on a substrate bound in the active site (54). If, however, the hydroxyl H atom of Thr179 were not directed to the free amino group but to the β-carboxylate of the product molecule, one would have to accept that this interaction represents a non-classic O-H…π hydrogen bond.

This stereochemically puzzling observation has a precedent in the structure of an L-aspartate complex of EcAII, where one of the threonine residues in the active site (Thr89) makes an almost identical “perpendicular” Oγ…O62 contact with the β-carboxylate group of the ligand (48). The recurrence of this short “perpendicular” contact between a component of the enzymatic apparatus and the β-carboxylate of the L-aspartate product in different asparaginases indicates its importance for the catalytic mechanism. The similarity between EcAIII and EcAII is also interesting from the point of view of the mechanism of action of the latter enzyme, where two active-site threonine residues (Thr89/Thr12), with a spatial disposition relative to the ligand resembling the present Thr179/Thr2390 pair, have been considered in the role of the nucleophile. If the structural analogy with EcAIII could be given a mechanistic interpretation, this argument would favor Thr89 of EcAII as the catalytic nucleophile.

If, as in the case of classic bacterial L-asparaginases, the hydrolysis reaction is reversible, the present structure would illustrate the reverse reaction. However, it should be stressed that the electron density maps do not suggest any covalent link between the Thr179 Oγ and the ligand (Fig. 3c). Obviously, more studies are needed, especially at the atomic level, to resolve the puzzles about the mechanism of this and of similar enzymes.

Finally, the interactions of the catalytic Thr179 residue include a direct hydrogen bond of the free α-amino group to the main-chain carboxyl of Asn67. The role of this contact may be to facilitate proton abstraction from Thr179 or to provide proper orientation of the catalytic residue, i.e. a perpendicular line of attack on the substrate amide plane. A similar interaction of the free α-amino group of subunit β with an adjacent carboxyl group is also present in human and bacterial aspartylglucosaminidases, although the residue functioning as the acceptor is not conserved. Human and bacterial AGAs have a serine in this position, while in some other species, like mouse, rat, Spodoptera frugiperda, Caenorhabditis elegans, and Drosophila melanogaster, a threonine replacement is present (47). In EcAIII, Asn67 is one of the residues forming the loop between H3α and S2α, whose conformation is stabilized by the coordinated sodium cation (Figs. 2b and 3a). It appears that this characteristic conformation is necessary for enabling the interaction of Asn67 with the α-amino group of Thr179. In human and bacterial aspartylglucosaminidases, the serine residue involved in this hydrogen bond also belongs to the H3α/S2α loop. However, in those enzymes an additional, shorter hydrogen bond with the free α-amino group is formed by the side chain of this residue (Table III). In human AGA, the H3α/S2α loop conformation, with partial DSSP classification (44) as a 3α helix, is stabilized by a disulphide bond. Substitution of either one of the bridging cysteines resulted in defects in catalytic activity (55). It is not clear what factors are responsible for the conformation of this loop in bacterial AGA, because neither a metal cation nor a disulphide bridge was found there. Experiments with mutants of human AGA revealed that substitution of the serine by alanine partially reduced the enzyme activity, whereas autopropeolysis and $K_m$ remained unaffected. How-
ever, an aspartylglucosaminuria-associated, naturally occurring Ser → Pro mutant of human AGA is not activated intracellularly (45). Apparently, the loop structure is unable to accommodate the rigid proline residue without disturbing the local conformation, although the overall fold seems to be unaffected as the protein is phosphorylated and targeted to the lysosomes (47).

**CONCLUSIONS**

We have described the crystal structure of *E. coli* isoaspartyl aminopeptidase in complex with d-aspartate, which is the product common to both reactions catalyzed by this enzyme, i.e. hydrolyses of isoaspartyl peptides and of asparagine. The protein is folded as a αβα-sandwich, characteristic for Ntn-hydrolases, with the catalytic Thr197 located at the N terminus of subunit β, generated during an autoproteolytic event of the maturation process. The product molecule is bound in the two active sites of this (αβ)2 dimeric enzyme, in a manner previously observed in human aspartylglucosaminidase, with which the isoaspartyl aminopeptidase shares 42% sequence similarity. The binding mode, in particular a strong salt bridge between the α-carboxylate and Arg327, explains the observed specificity of the enzyme, which is limited to substrates with a free α-carboxyl group (30). Additionally, this interaction limits the space that can be used by potential substrates and prevents unsuitable substrates from presenting the scissile bond to the catalytic nucleophile by sliding along the catalytic cavity. For instance, because of the anchoring of the α-carboxyl end of the substrate, longer molecules, such as β-glutamine, cannot be hydrolyzed (30). Moreover, the system of hydrogen bonds formed at the free α-amino group of the ligand shows why the enzyme is an aminopeptidase. Significant differences in the overall shape of the active site cavity between EcAIII and other aspartylglucosaminidases explain why isoaspartyl aminopeptidase has an overall shape of the active site cavity between EcAIII and enzyme is an aminopeptidase. 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Crystal Structure of Isoaspartyl Aminopeptidase in Complex with L-Aspartate
Karolina Michalska, Krzysztof Brzezinski and Mariusz Jaskolski

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