Crystal Structures of Flavobacterium Glycosylasparaginase
AN N-TERMINAL NUCLEOPHILE HYDROLASE ACTIVATED BY INTRAMOLECULAR PROTEOLYSIS*

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Glycosylasparaginase (GA) is a member of a novel family of N-terminal nucleophile hydrolases that catalytically use an N-terminal residue as both a polarizing base and a nucleophile. These enzymes are activated from a single chain precursor by intramolecular autoproteolysis to yield the N-terminal nucleophile. A deficiency of GA results in the human genetic disorder known as aspartylglycosaminuria. In this study, we report the crystal structure of recombinant GA from Flavobacterium meningosepticum. Similar to the human structure, the bacterial GA forms an αββα sandwich. However, some significant differences are observed between the Flavobacterium and human structures. The active site of Flavobacterium glycosylasparaginase is in an open conformation when compared with the human structure. We also describe the structure of a mutant wherein the N-terminal nucleophile Thr152 is substituted by a cysteine. In the bacterial GA crystals, we observe a heterotetrameric structure similar to that found in the human structure, as well as that observed in solution for eukaryotic glycosylasparaginases. The results confirm the suitability of the bacterial enzyme as a model to study the consequences of mutations in aspartylglycosaminuria patients. They also suggest that further studies are necessary to understand the detailed mechanism of this enzyme. The presence of the heterotetrameric structure in the crystals is significant because dimerization of precursors has been suggested in the human enzyme to be a prerequisite to trigger autoproteolysis.

Eukaryotic glycosylasparaginase (glycoasparaginase, N1- (βN-acetyl-b-glucosaminyl)-1-asparaginase, 1-aspartamido-β-N-acetylgalosamine amidohydrolase, aspartylglycosaminase, aspartylglucosaminidase, EC 3.5.1.26) is a well known lysosomal enzyme that cleaves the amide bond of asparaginyl-linked glycoproteins (1). It is widely distributed in vertebrate lysosomal enzyme that cleaves the amide bond of asparagine-aspartylglucosaminidase, aspartylglycosaminidase, EC 3.5.1.26) is a well known enzyme, and an inhibitor specifically reacts with the N-terminal threonine for activation. Neither the single chain precursor (6, 7) nor the isolated subunits (8) are enzymatically active by themselves.

Expression of the α- and β-subunits of GA on separate DNA constructs showed that independently folded subunits lack enzyme activity, and even when co-expressed in vitro they fail to produce an active heterodimer (9). A common feature of GA from different species is a new N-terminal threonine of the C-terminal product (the β-subunit) resulting from the autoproteolytic activation (10). A study demonstrated that an irreversible inhibitor specifically reacts with the N-terminal threonine on the β-subunit of the human leukocyte enzyme via an α-keotorone ether linkage with the hydroxyl side chain (8), indicating that this N-terminal threonine acts as a nucleophile during substrate hydrolysis.

The crystal structure of human GA shows a topology similar to other N-terminal nucleophile hydrolases (11, 12) and reveals interactions between the N-terminal threonine and aspartate, one of the reaction products (13).

GA from Flavobacterium meningosepticum is the only prokaryotic homolog characterized so far. It differs from the human counterpart in several aspects: (i) sequence alignment of these two enzymes reveals only about 30% sequence identity and shows a difference in one gap/insertion of 31 residues (3, 4); (ii) part of the 31-residue insertion in the human enzyme is removed from the new C terminus of the α-subunit in the lysosome (6); no trimming occurs in the bacterial enzyme; (iii) the human enzyme contains N-linked glycans on both the a- and b-subunits (Asn15 and Asn285) (14), whereas the bacterial enzyme is nonglycosylated (9); (iv) according to previous sequence alignments (3, 4), neither the position nor the pattern of disulfide bridges is conserved between these two enzymes. The

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The atomic coordinates and structure factors (codes 2GAW and 2GAC) have been deposited in the Protein Data Bank, Brookhaven National Laboratory, Upton, NY.

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1 The abbreviations used are: GA, glycosylasparaginase; AGU, aspartylglycosaminuria; MIR, multiple isomorphous replacement; r.m.s., root mean square.
**TABLE I**

| Resolution (Å) | Wild type | T152C | Derivatives |
|----------------|-----------|-------|-------------|
| 2.2            | 2.1       | 2.5   | HG(OAc)    |
| 42,281/23,999  | 59,059/30,530 | 50,741/18,514 | CH₃HgCl  |
| 6.9            | 8.3       | 9.5   | K₂PtCl₄    |
| 86.7           | 96.2      | 97.7  | KAuCN₂     |

Number of crystals (12–2.5 Å) (MLPHARE)

- Overall figure of merit of 0.785.

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**RESULTS**

**Description of the Structure**—The enzymes crystallized in space group P2₁, with unit cell constants a = 46.2 Å, b = 97.3 Å, c = 61.8 Å, and β = 90.3°. The initial phases were obtained by MIR method with four heavy atom derivatives (Table I). The wild type structure has been determined at 2.2 Å and refined to an R_{free} (21) of 29.70% and an R_{cryst} of 24.65% with all reflections (Table II). There are two a/b heterodimers per asymmetric unit. In the final model, each heterodimer comprises 136 residues (3–138) of the a-subunit and 139 residues (152–290) of the b-subunit. No electron density is observed for the 13 residues spanning the segment (139–151) that connects the a- and b-subunits in the precursor protein. In the crystal, this linker segment appears to face into the solvent channels. 93% of the nglycine residues fall in the most favored regions of Ramachandran plot, as defined in PROCHECK (22), and no residues are in the disallowed regions.

Overall, the topology of *Flavobacterium* GA is very similar to its human counterpart (13). Both the a- and b-subunits together form a four-layer α-β-α-β structure (Fig. 1), with two β-sheets packed against each other to form a core that is “sandwiched” by two layers of α-helices. Eight β-strands from both the a- and b-subunits form the first β-sheet, with topology aS4, aS3, aS2, bS2, bS1, aS1, bS7, and bS8. All these β-strands...
formed by five lix layer packed against the outside of the eight-strand sheet is in the asymmetric unit. The double mutant (human Arg138 to Gln and Cys140 to Ser) mutations onto the structure-based sequence alignment. Only known as AGU (1). In Fig. 2, we have mapped five known AGU maps outside of the shared secondary structural elements. This is significantly larger than the r.m.s. deviation of 0.26–0.45 Å found between the two human structures (13). Moreover, the r.m.s. deviation between our two bacterial structures is 0.22 Å (see below), similar to that observed between two heterodimers in the asymmetric unit (Table II). A number of peptide fragments within the structure deviate by more than 2 Å (Fig. 2); most of them are in loops connecting elements of secondary structure. The largest difference of 8.5 Å is near the 7-residue insertion in the bacterial structure. Deviations greater than 2 Å are also observed in the common secondary structural elements (see below). These data are consistent with the observation that molecular replacement using the human structure proved difficult with the data of Flavobacterium GA.3 The human enzyme contains four disulfide bonds (Fig. 2) that are important for protein folding, autoproteolysis, and enzyme activity (15). These four disulfide bonds are conserved among mammalian enzymes. The insect enzyme retains all but one (Cys283,Cys285) of the disulfide bonds (3). However, no conserved disulfide bond is found between the Flavobacterium and eukaryotic GA. Indeed, there are no disulfide bonds among the five cysteines in the bacterial a/b heterodimer. One cysteine pair in the bacterial structure (Cys68,Cys168) has side chains in close proximity that may potentially form a disulfide bond, but this was ruled out based on several observations: (i) Cys68 and Cys168 bind to heavy atoms Hg(OAc)2 and CH3HgCl, respectively; (ii) the initial MIR map indicates that the side chain of Cys168 point away from Cys68; (iii) the simulated annealed omit maps also show these two side chains to be in nonbridged conformations; (iv) a Cys to Ser mutation at either of these two cysteines does not significantly affect either protein stability or enzymatic activity; and (v) the a- and b-subunits can be separated on a nonreducing SDS protein gel (data not shown).

Although the overall protein folds are similar in the bacterial and human structures, the location or length of some secondary structural elements differ. For example, the bacterial enzyme has a unique 310 helix (bH4), whereas the human enzyme carries a C-terminal additional loop on its a-subunit (Fig. 1c). Furthermore, in the bacterial structure, the insertion of Gly14 extends helix aH1 at its N-terminal end by two residues. At the C-terminal end of helix aH1, Ser286 is designated as part of the helix in the human structure, but the equivalent Lys27 in the bacterial structure is not assigned as part of the helix by PROCHECK (22). This is apparently because of a significant deviation of the main chain traces between these two structures (Fig. 3). When these two structures are superimposed by their common secondary structural elements, Ca of Lys27 deviates by 3.5 Å from its equivalent atom in the human structure. The C-terminal end of aH2 helix also deviates by more than 2 Å. No crystal contact either in the human or bacterial structure can account for these deviations. In the bacterial structure, the 7-residue insertion in the b-subunit also extends the bH3 helix by 4 residues at its C-terminal end.

Active Site and Mechanism—The loops connecting different layers of a-helices and b-sheets form a deep funnel-shaped active site centered at the N-terminal Thr152 of the b-subunit (Fig. 1). The funnel in the bacterial enzyme is wider than that of the human enzyme, mainly because of deviation of the loop between helix aH2 and strand aS2 as well as lack of the
FIG. 1. The structure of glycosylasparaginase from *F. meningosepticum*. *a*, stereo ribbon representation of the *Flavobacterium* GA structure. One heterodimer is shown with α-(red) and β-subunits (green). The active site is at *top center* of the structure toward the viewer and around the N-terminal end of the β-subunit (green) (labeled Nb in *light blue*). *b*, stereo diagram of Ca traces of *Flavobacterium* GA. *c*, stereo diagram
FIG. 2. Structure-based sequence alignment between the Flavobacterium (Flavo) and human glycosylasparaginase (Human). The dashed lines represent incorporated gaps that bring the sequences into alignment. The vertical lines represent identical matches. The bold black arrow represents the autoproteolytic site. Yellow shading shows conserved residues in the active site (see Fig. 4 and text). Purple shades highlight the mutations identified in human AGU disease (1); not conserved residues in the active site (see Fig. 4 and text).

C-terminal loop in the a-subunit (Fig. 1c). Several conserved residues surround the nucleophilic center Thr172 of the bacterial active site, including Thr170, Arg180, Asp183, Thr203, and Gly204, which are highlighted in yellow in Fig. 2. These residues had been described to interact with aspartase, one of the two reaction products (13). As depicted in Fig. 4a, the human equivalent to Arg180 forms hydrogen bonds with the α-carboxyl group of aspartate. Both human equivalent residues of Asp183 and Gly204 make hydrogen bonds with the α-amino group of aspartate. Human residues equivalent to Thr172, Thr203, and Gly204 also form hydrogen bonds with the O61 of aspartate. In addition, human residue equivalent to Thr172 makes a hydrogen bond with the Oγ of Thr152.

Additional conserved residues not described previously also might participate in either ligand binding or catalysis. Residue Trp11 has a putative role in carbohydrate binding (13). It is also near the N-terminal nucleophile and may participate in catalysis, possibly through a bridging water molecule. In line with this suggestion, mutation of Trp11 to Ser (W11S) affects enzyme specificity for substrates with or without carbohydrate moiety (23). Furthermore, the kcat of the W11S mutant was reduced by more than 400-fold, suggesting an additional role of Trp11 in regulating enzyme catalysis. Contrary to a previous suggestion (13), human Phe13 might not contribute to carbohydrate binding, because sequence alignment shows that this residue is not conserved either in the bacterial (Gln254 in Fig. 2) or insect (Met272 in Ref. 3) enzymes. Nonetheless, aromatic side chains have been suggested to be involved in protein-carbohydrate interactions (24). Here we propose that two conserved aromatic residues, Phe13 and Trp11 (Fig. 4), form part of the carbohydrate binding site.

Based on structural and biochemical studies, the reaction mechanism of GA is similar to serine proteases and hence utilizes a cycle of enzymatic acylation and deacylation. However, the free α-amino group on the N-terminal threonine acts as the base, probably through a bridging water molecule, to enhance the nucleophilicity of its own side chain hydroxyl group. This intra-residue base on the threonine replaces the well characterized histidine base in the hydroxylated triad that is present in the active site of many serine proteases (25). The activated Oγ of Thr152 attacks the amide carbon of a substrate to form a tetrahedral transition state structure that is stabilized by an oxyanion hole. The structure then collapses to form a covalent enzyme-acetyl (β-aspartyl) intermediate with release of the carbohydrate product. Decay is accomplished by a nucleophilic attack by an entering water molecule on the same carbon to release aspartate, the second product.

The identity of the oxyanion hole that stabilizes the negatively charged carbonyl oxygen on the tetrahedral transition state is still unclear in the current GA structures. Oinonen et al. (13) proposed that the side chain of human residue equivalent to bacterial Thr203 and the main chain equivalent to Gly204 act as the oxyanion hole, based on the structure of human enzyme-product complex. However, when the active sites of the bacterial and human structures are superimposed (Fig. 4a), there are conformational differences with respect to the nucleophilic Oγ of the N-terminal Thr152 (human Thr163) and the proposed oxyanion hole Oγ of Thr203 (human Thr204). We suggest that the current structure of the bacterial enzyme appears to be in an open conformation, whereas the human enzyme adopts a closed conformation that grasps the reaction product, aspartate (Fig. 4a). In the bacterial structure, the Oγ of Thr203 is displaced by 1.9 Å and the Oγ of Thr152 is shifted in the opposite direction by 0.7 Å (the r.m.s. deviation of all other residues hydrogen-bonded to Asp is 0.64 Å between the bacterial and human enzymes, and 0.25 Å between bacterial wild type and the T152C mutant). As a result, the relative distance between these two atoms has changed by 2.3 Å. In the case of isocitrate dehydrogenase (26), small changes in distance (<1.55 Å) and orientation of reacting groups results in a large reduction (10−7 to 10−5) in the reaction rate. The differences in the GA case could result from the binding of ligand (aspartate) in the human complex. However, the structure of the unliganded human enzyme (13) also has a similar closed conformation. This raises the possibility that the differences observed in the position of Thr203 in the bacterial structure may represent differences in mechanism relative to the human enzyme. Mutagenesis studies also indicate that the side chain of bacterial Thr203 may not be as important in stabilizing the negative oxyanion intermediate as previously suggested for the human enzyme (13), because replacement by Ala (T203A mutant) in the bacterial enzyme decreases kcat only about 10-fold (23). Further studies are necessary to determine whether Gly204 together with a main chain component of Thr203 (or other residues) actually form the oxyanion hole.

Structure of the T152C Mutant—Thr152 plays a key role in catalysis (4, 7, 8). Substitution of the N-terminal nucleophile Thr152 by a thiol group (T152C mutant) reduces kcat by 5 orders of magnitude (23). Autoproteolysis in this mutant is also very slow but can be accelerated by hydroxylamine (10). In this study, we have also determined the three-dimensional struc-
ture of the T152C mutant in its mature form at 2.1 Å and refined to an \( R_{\text{free}} \) of 28.06% and an \( R_{\text{cryst}} \) of 23.32% with all reflections (Table II). The structure of this mutant is essentially identical to that of the wild type enzyme with an r.m.s. deviation of 0.22 Å for all the main chain atoms and 0.25 Å for the active site atoms (Fig. 4b). This indicates that the reduction of reaction rate of this mutant is because of the change of chemical groups at the side chain of residue 152.

The active site of the T152C mutant also has the open conformation as described above. Like the wild type structure, the distance between the Cβ atoms of Cys152 and Thr203 is 2.0 Å further apart than in the human structure. Furthermore, the thiol group of Cys152 points in the opposite direction and is 2.9 Å removed from the wild type nucleophile Oγ of Thr152. This appears to be because of a favorable packing of the thiol group into a small pocket formed between side chains of Cys152 and Thr203 and main chain atoms of the β-sheet b51. Such an inactive conformation has also been observed in the glutaminase domain of glucosamine 6-phosphate synthase, where Cys1 is the wild type N-terminal nucleophile (27). In the native GA enzyme, packing of the γ-methyl group of Thr152 into this pocket, as well as a hydrogen bond formation between Oγ of Thr152 and Oγ of Thr170 (Fig. 4α), positions the nucleophile in an active conformation. We propose that in the presence of substrate, the thiol group switches to the active conformation by a rotation of 120 ° around the Cα-Cβ bond and a small angular adjustment around the Cα-C bond. Further studies are needed to determine whether the Cys152 adopts our proposed active conformation in the presence of substrate.

*Quaternary Structure of GA—* Bacterial GA forms a dimer of \( \alpha/\beta \) heterodimers in the crystals (Fig. 5a). A similar quaternary structure is also observed in the crystals of human GA in different crystal packings (13). The surface interactions between pairs of heterodimers are extensive and mainly involve hydrogen bonds and hydrophobic contacts (Fig. 5, b and c). Basically, both heterodimers use the same hydrophobic surface...
The physiological importance of the glycosylasparaginase is revealed by the occurrence of a human genetic disorder, known as AGU, because of a deficiency of this lysosomal hydrolase (1). Many mutations in the GA gene that cause AGU have been reported, and more are likely to be found. However, a major obstacle to studying the consequences of these mutations is the difficulty to obtain recombinant human enzyme in sufficient quantities (28). In this study, four known AGU single mutations have been mapped onto the shared secondary structural elements between the bacterial and human enzymes (Fig. 2). A double mutant (human Arg138 to Gln and Cys140 to Ser) maps outside of the secondary structural elements and appears to result from the loss of a disulfide bond (Cys140-Cys156) that stabilizes a unique loop in the human enzyme. Thus, our work confirms the suitability of the bacterial enzyme as a model to analyze the consequences of mutations in AGU patients at the molecular level.

**DISCUSSION**

**Aspartylglucosaminuria**—The physiological importance of the glycosylasparaginase is revealed by the occurrence of a human genetic disorder, known as AGU, because of a deficiency of this lysosomal hydrolase (1). Many mutations in the GA gene that cause AGU have been reported, and more are likely to be found. However, a major obstacle to studying the consequences of these mutations is the difficulty to obtain recombinant human enzyme in sufficient quantities (28). In this study, four known AGU single mutations have been mapped onto the shared secondary structural elements between the bacterial and human enzymes (Fig. 2). A double mutant (human Arg138 to Gln and Cys140 to Ser) maps outside of the secondary structural elements and appears to result from the loss of a disulfide bond (Cys140-Cys156) that stabilizes a unique loop in the human enzyme. Thus, our work confirms the suitability of the bacterial enzyme as a model to analyze the consequences of mutations in AGU patients at the molecular level.

**Structural Comparisons**—Glycosylasparaginase belongs to a newly classified family of enzymes that have a novel N-terminal threonine, serine, or cysteine that provides the nucleophile in their reaction mechanism (11). Previously reported structures of this family include glutamine 5-phosphoribosyl-1-pyrophosphate amidotransferase from *Bacillus subtilis* (29), *Escherichia coli* penicillin amidohydrolase (30), the 20 S proteasome from the archaeabacterium *Thermoplasma acidophilum* (31) and yeast (32), human glycosylasparaginase (13), and the glutaminase domain of *E. coli* glucosamine 6-phosphate synthase (27). All of these enzymes have a similar protein fold comprised of a sandwich of antiparallel β sheets surrounded on either side by layers of α helices. Many of these enzymes are activated by cleavage of the peptide bond to free the α-amino group to form the N-terminal nucleophile (10). A different protein fold has recently been described for the autoprocessing domain of *Drosophila* Hedgehog protein (33). It is an all β structure that is distinct from the GA structure but is related to the intein domain of PI-SceI endonuclease (34, 35).

**Enzyme Mechanism**—Crystal structures described in this study, on the other hand, also raise questions about the detail mechanism of GA. Structure of the wild type GA from *F. meningosepticum* in its mature form has been determined at 2.2 Å resolution. Although the topology of the bacterial enzyme is very similar to that of the human structure, several significant differences have been observed. The active site of *Flavobacterium* GA is in an open conformation, whereas the human enzyme adopts a closed conformation that grasps the reaction product, aspartate (Fig. 4a). Moreover, the side chain of Thr203 may not be as important in stabilizing the negative oxyanion intermediate as previously suggested (13). This is consistent with a mutagenesis study in which replacement of Thr203 by Ala (T203A mutant) in the bacterial enzyme does not dramatically decrease the reaction rate (23). A three-dimensional structure of the enzyme-substrate complex is necessary to clarify the role of Thr203 side chain in the enzymatic mechanism.

In addition, we also report the structure at 2.1 Å resolution of a T152C mutant wherein the N-terminal nucleophile Thr152 of the b-subunit is replaced with Cys. The T152C mutant has a dramatically reduced rate of autoproteolysis or enzyme catalysis (23) and thus is a good candidate for future crystallographic studies of the precursor structure and enzyme-substrate complex. Similar to the glutaminase domain of glucosamine 6-phosphate synthase, Cys152 in the T152C mutant appears to be in an inactive conformation (27). We propose that binding of substrate would switch the thiol group into an active conformation.

**Autoprocessing for Enzyme Activation**—Cis-autoproteolysis involves the intramolecular catalytic cleavage of a peptide bond and is required to activate many enzymes (12). In addition to GA, these include penicillin acylase (30), proteasomes (31, 36, 37), as well as the hedgehog family of eukaryotic developmental
regulatory proteins (38). Autoproteolytic cleavage also serves as a mechanistic component for protein splicing (35). In contrast to the activation ofzymogens, such as chymotrypsinogen and trypsinogen through proteolysis by another trypsin molecule, the autoproteolysis of GA is an intramolecular reaction (10). Human GA is also believed to undergo autoproteolysis to form the active enzyme but with some differences. First, the disulfide bridges in the human enzyme are essential for early folding and for autoproteolytic processing (15). In contrast, there are no disulfide bridges in the bacterial enzyme. Furthermore, part of the 31-residue insertion in the human enzyme is removed from the C terminus of the autoproteolyzed a-subunit in the lysosome by a second cleavage (6). No such trimming occurs for the bacterial enzyme.

This work reveals an (a/b)2 quaternary structure that has been observed in solution or crystals of the eukaryotic GA. Furthermore, an amino acid substitution (equivalent to bacterial GA.5 Nonetheless, a dimer of a/b heterodimers exists in the crystals of bacterial GA (Fig. 5a). In contrast, only the a/b dimer formation of the precursor protein and also prevents proteolytic activation of the enzyme (15). Therefore, it appears that in the human enzyme dimerization of precursors is a prerequisite to trigger autoproteolysis. In contrast, only the a/b heterodimer is observed on sizing gels and columns for the bacterial GA.5 Nonetheless, a dimer of a/b heterodimers exists in the crystals of bacterial GA (Fig. 5a) that is similar to the quaternary structure observed in the crystals of human GA (13). This raises the possibility that dimerization of bacterial GA, although it has not been observed yet, might also occur in solution.

Further studies are necessary to determine whether dimerization of the single chain precursor proteins occurs and, if so, to determine the significance of this dimerization in autoproteolysis. Unless there is a large conformational change as a result of autoproteolysis, the location of the key cleaved Thr152 in the enzyme active site suggests that the autoproteolytic site is near or overlaps with the active site. In line with intramolecular autoproteolysis, the two active sites in the dimer of GA are facing apart with the autoproteolyzed N-terminal threonine 32 A away (arrows in Fig. 5a). The size and shape of the active site funnel also appear to be difficult for any proteolytic enzyme to approach Thr152 for peptide bond cleavage. However, it remains unclear how this dimerization triggers autoproteolytic activation of these enzymes. It is still possible that dimerization of precursor proteins results in a conformational change to trigger autoproteolysis. In our group, crystallographic studies on the precursor proteins are underway.

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