Site-directed Mutagenesis of Essential Residues Involved in the Mechanism of Bacterial Glycosylasparaginase*

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Flavobacterium glycosylasparaginase was cloned in an Escherichia coli expression system. Site-directed mutagenesis was performed at residues suggested to be important in the catalytic mechanism based on the crystal structure of the human enzyme and other biochemical studies. In vitro autoproteolysis allowed the mutant enzymes to be activated, including those that were slow to self-cleave. Based on the activity of the mutant enzymes, six catalytically essential amino acids were identified: Trp-11, Asp-66, Thr-152, Thr-170, Arg-180, and Asp-183. Kinetic analysis of each mutant further defined the function of these residues in substrate specificity and reaction rate. Mutagenesis of the N-terminal nucleophile residue Thr-152 confirmed the key function of its side-chain hydroxyl group. Partial activities of mutants T152S/C were in agreement with the general mechanism of N-terminal nucleophile (Ntn)-amidohydrolases. The side-chain hydroxyl of Thr-170 contributes to the reaction rate based on studies of mutants T170S/C/A. Residues Asp-183 and Arg-180 were found to H-bond, respectively, with the charged o-amino and o-carboxyl group of the substrate (Asn-GlcNAc). Mutants R180Q/L and D183E/N had greatly decreased substrate affinity and reduced reaction rates. Kinetic studies also showed that Trp-11 is involved in regulation of the enzyme reaction rate, contradictory to a previous suggestion that this residue is involved in substrate binding. Asp-66 is a new residue found to be important in enzyme activity. The overall active site structure involving these catalytic residues resembles the glutaminase domain of glucosamine 6-phosphate synthase, another member of the Ntn-amidohydrolase family of enzymes.

Glycosylasparaginase (glycoasparaginase, N\(^1\)-(β-N-acetyl-β-glucosaminyl)-l-asparagine amidohydrolase) is a widely distributed amidohydrolase involved in the ordered degradation of N-linked glycoproteins. It cleaves the Asn-GlcNAc linkage that joins the oligosaccharide to the protein (1, 2), and genetic deficiency of this enzyme causes the human lysosomal storage disease aspartylglucosaminuria (2, 3). Reaction of glycosylasparaginase with the linkage unit Asn-GlcNAc produces asparagine and 1-amino-GlcNAc, which is further hydrolyzed non-enzymatically to ammonia and GlcNAc (4). Previous studies have shown the enzyme has a strict substrate specificity that requires both a free o-amino and o-carboxyl group on the asparagine residue. In contrast, relatively flexible substrate requirements exist at the sugar portion of the substrate. Substrates having mono- or di-, or even longer saccharides are effectively hydrolyzed although the presence of α-L-fucose on the 6-hydroxyl of the N-acetylgalactosamine prevents the enzyme reaction (5–7). A recent report suggests that the o-amino- and o-carboxyl group of the asparagine component of the substrate may not be strictly required for enzyme activity (8).

Glycosylasparaginases have been purified or cloned from seven biological sources, and all of them are encoded by a single gene (9–11). After translation, the nascent single polypeptide is cleaved into two nonidentical α and β subunits by an autoproteolytic procedure (12). This self-cleavage produces the catalytically active glycosylasparaginase heterodimer (13, 14). Glycosylasparaginases from all the different species show a high sequence homology and conservation of biochemical properties (9, 15), and they are expected to share a similar three-dimensional structure. Human glycosylasparaginase is folded into four layers of secondary structure, α-helices/β-sheet/β-sheet/α-helices (16), and shares unusual structural features of a recently recognized enzyme superfAMILY of amidases, the N-terminal nucleophile (Ntn)\(^1\) amidohydrolases (17, 18). The open cleft between the two central layers of antiparallel β-sheets is the position that incorporates the active site of these enzymes (18). This protein fold is present in penicillin acylase (PA) (19), the proteasome (PRO) (20), the glutaminase domain of glutamine phosphoribosylpyrophosphate amidotransferase (GAT) (21), and glucosamine 6-phosphate synthase (GLMS) (22), and it likely occurs in other predicted family members. These enzymes are expected to manifest a common catalytic mechanism. First, a unique property of Ntn hydrolases is that the N-terminal amino acid acts as the reaction nucleophile: O\(_{\text{y}}\) of SerB1 in PA; S\(_{\text{y}}\) of Cys-1 of GAT and GLMS (17, 18). Second, the free o-amino group of these same N-terminal residues serves as a base to enhance the nucleophilicity of their side chain hydroxyl or thiol group. The x-ray structure of human glycosylasparaginase revealed not only its active-site N-terminal Thr nucleophile/base but also other amino acids likely involved in catalysis because the reaction product aspartic acid remained bound within the crystallized protein (16).

In regards to the proposed catalytic groups of human glycosylasparaginase, some mutagenesis and expression experiments have already been done (24); however, limited kinetic data were obtained from these studies. A major obstacle was the inability to obtain pure autoproteolyzed mutant human enzyme in sufficient quantity to distinguish between the reaction mechanism for substrate hydrolysis and the autoproteolytic

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sis required to activate the single-chain precursor. Thus, it has been noted that many prospective active site residues are also likely to be involved in enzyme autoproteolysis, since precursor and mature enzyme may share a similar structure and the enzymatic mechanisms used in these two processes may resemble one another (17, 18). In this paper, we have applied a recombinant bacterial glycosylasparaginase previously used by Guan (12) to mutate the predicted active site amino acids as well as other residues structurally close to the catalytic core. In vitro autoproteolysis and biochemical characterization of these mutants make it possible to investigate in detail the residues that are essential for precursor polypeptide folding, autoproteolytic activation, structural stability, and enzyme catalysis. Three different substrates were used to kinetically analyze the activity of these mutant forms of the enzyme, and the effects of these particular amino acid residues on either substrate specificity or glycosylasparaginase reaction rate have been measured.

MATERIALS AND METHODS

Site-directed Mutagenesis of Recombinant Flavobacterium Glycosylasparaginase—Site-directed mutagenesis (Kunkel method) was carried out by Guan (12). The Flavobacterium glycosylasparaginase coding sequence (11) was cloned into pMAL-p2 (New England Biolabs) as a maltose-binding protein (MBP) chimera. Single-stranded phagemid that harbored wild-type sequence was rescued by adding M13K07 helper phage (New England Biolabs) to liquid-cultured transformed TB1 cells and was then isolated by 20% polyethylene glycol, 2.5 M NaCl precipitation followed by phenol/chloroform extraction, twice each. This single-stranded phagemid was used as the template to construct a library of the desired position. Vectors bearing the designed substitution were selected based on positional DNA sequencing of the mutation. Subsequent sequencing of the entire protein coding region revealed no other mutations. Three different substrates were used to test the activity of these mutants. Each reaction was in a volume of 200 ml with a 1-liter culture.

p-Nitroaniline Release—Each reaction was performed in 20 ml Tris buffer, pH 7.5, containing 100 mM NaCl, and lysed by sonication. Enzymes were affinity purified over an amylose column according to the protocol of the manufacturer (New England Biolabs). After purification, the chimeric glycosylasparaginases were cleaved with Factor Xa at 10°C for 2–3 h. Cleaved MBP was removed by running the protease reaction through a high-Q-Sepharose column (Amersham Pharmacia Biotech). All purification procedures were performed at 4°C. Protein expression efficiency using this system was about 40 mg glycosylasparaginase in a 1-liter culture.

In Vitro Autoproteolysis and Quantitation of Mutant Glycosylasparaginase—The protocol used to perform in vitro autoproteolysis was done according to Guan (12). All steps were done in succession immediately after affinity purification and removal of MBP. Purified mutant glycosylasparaginases were concentrated by Centricon 10 ultrafiltration (Amicon Corp.) and were immediately analyzed on SDS-PAGE. Wild-type enzyme appeared as its separated p and b subunits having molecular weights of approximately 15 and 18 kDa. Slow cleavage precursors required incubation at 37°C for 4–6 h. Autohydrolysis of these precursors was monitored by tracking the progress at different time intervals and analyzing them on SDS-PAGE. For the slowest cleavage mutants, an intermediate level of formed glycosylasparaginase subunits was used to measure the amount of active enzyme. The cleaved enzymes were quantitated after SDS-PAGE and Coomassie Blue staining by determining their image density using the computer program NIH Image 1.62.

Enzyme Assay and Determination of Kinetic Parameters K_m and k_cat—Three different substrates were used to assay the activity of wild-type and mutant glycosylasparaginases, and the kinetic parameters K_m and k_cat were determined for each reaction. Due to very low activities of some enzymes and solubility limiting conditions for some substrates, not all mutant enzymes were tested with all three substrates.

Asn-GlcNAc As Substrate Measured by Release of GlcNAc—The standard reaction was 2.5 mM substrate in 20 ml of 20 mM sodium phosphate buffer, pH 7.5, incubated with enzyme for an appropriate time at 37°C (25). The reaction was stopped by adding 50 ml of 250 mM sodium borate buffer, pH 8.8, followed by boiling for 3 min. Released N-acetylglucosamine was assayed by the Morgan-Elson reaction (26). The K_m and k_cat values of mutants R180K/Q/L and D183E/N were measured using this method, but the results were variable due to substrate heterogeneity. Therefore, 12–15 different concentrations of Asn-GlcNAc from 1 to 20 mM with R180K, 1 to 50 mM with D183E, and 10 to 100 mM with all other mutants. Substrate concentrations higher than 100 mM were also tried; however, less accurate results were obtained due to the level of free GlcNAc in this amount of substrate. Initial rates of GlcNAc release were measured when less than 5% of total substrate was hydrolyzed, and these values were used to determine K_m and k_cat.

Asn-GlcNAc and Asparagine As Substrates Measured by the Release of Aspartate—The above traditional colorimetric method measuring GlcNAc has an assay limit at about 0.1 mM which was not sensitive enough to do a precise kinetic study of many of the mutant enzyme forms. Therefore, enzyme activity was also assayed by a coupled enzymatic procedure originally described by Tarentino and Maley (27) and based on: 1) the hydrolysis of the two substrates by glycosylasparaginase with the release of aspartate; 2) the subsequent transamination of aspartate to oxaloacetate by glutamate-oxaloacetate transaminase (GOT) in the presence of α-ketoglutarate; and 3) formation of NAD⁺ after malate dehydrogenase (MD) reduction of oxaloacetate to malate with NADH. Formation of NAD⁺ was then measured fluorometrically using a Perkin-Elmer LS50B Luminescence Spectrometer with the excitation wavelength at 360 nm and emission at 460 nm. Each reaction was performed in 150 ml of 20 mM sodium phosphate, pH 7.5, 0.1 mM α-ketoglutarate, 0.2 mg of NADH, 7.5 μl of each of GOT and MD with a glycosylasparaginase substrate and an appropriate amount of wild-type or mutant enzyme. All reagents and enzymes for the GOT and MD steps were obtained from Sigma. The assay mixture was incubated at 37°C and the reaction was terminated by vigorously mixing with 20 ml of 6 N HCl to destroy excess NADH followed by adding 100 μl of 10 mM NaOH (28). Samples of 200 ml from each reaction were placed in a 96-well plate to measure the fluorescence of the NAD⁺ final product. Sensitivity of this assay allows measurement of 0.002 nmol NAD⁺, which enabled accurate determination of enzyme hydrolysis over a wide range. To determine the K_m and k_cat of enzyme hydrolysis, 10–15 concentrations of substrate were used, generally ranging from 0.2 to 5 times K_m. Initial rates were calculated by terminating reactions at three to five different time points. The highest substrate concentration was used 30 mM for Asn-GlcNAc and 8 mM for asparagine. Results were not accurate at higher substrate concentrations by this method because of a high background from contaminating free asparagine.

Aspartic Acid β-(p-nitroanilide) (Asp(pNA)-OH) As Substrate Measured by p-Nitroaniline Release—Each reaction was in a volume of 200 ml of 50 mM Tris buffer, pH 7.5, containing appropriate concentrations of aspartic acid β-(p-nitroanilide) (Bachem) substrate and wild-type or mutant enzyme. Release of p-nitroaniline was monitored at 400 nm with a 37°C temperature-controlled Beckman DU640 spectrophotometer (29). The K_m and k_cat were determined by measuring the initial hydrolysis rates at 12–15 concentrations of substrate ranging from 0.2 to 5 times K_m. Because of the limiting solubility of Asp(pNA)-OH in water, the highest concentration of substrate used in this assay was 8 mM. The maximum time required for release of p-NH₂ from p-nitroanilide did not occur during this period of incubation at 37°C. Asn-GlCNac was assayed for 3 days with the T152C and T170A mutant enzymes and was stable for itself for this long incubation.

RESULTS

Generation of Active-site Mutants, in Vitro Autoproteolysis, and Activity Analysis—Residues of Flavobacterium glycosylasparaginase selected for mutagenesis (Table I) were mostly the counterparts of essential human glycosylasparaginase residues (16, 30). The expression and purification of wild-type enzyme are shown in Fig. 1a (lanes 1–5). The same protocol was used to express and purify mutant enzymes. No obvious different behavior was observed for these mutants during the procedures, and all recombinants were expressed as stable proteins. The wild-type banding pattern was observed for mutants S50A, D58N, T64A, D107N, T169A, K176A, and T203S, indicating these mutations did not interfere with polypeptide folding or their autoproteolytic activation. All other mutagenesis of

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2 Available at http://rsb.info.nih.gov/nih-image/default.html.
the targeted amino acids resulted in incomplete or poor initial autoproteolysis with an uncleaved precursor band (αβ) present at about 32 kDa. Incubation of these mutants at 37 °C allowed most of them to be cleaved into their subunits (Fig. 1b; Table I).

In the case of the R180K mutant, the majority of its precursor precipitated during the 37 °C incubation (Fig. 1c). The same phenomenon occurred with all other R180 mutants (data not shown). Mutants W11F and T152A did not undergo autoproteolysis (Fig. 1b). Activity of wild-type enzyme and each mutant was assayed colorimetrically using 2.5 mM Asn-GlcNAc as substrate after the mutant precursors had completed their autoproteolysis (Table I). Almost all mutant enzymes that were “slow” to form subunits (T1/2 > 60 min) were poorly active once autoproteolysis took place. The T203A glycosylasparaginase (T1/2 = 3.5 h) was an exception and retained substantial catalytic capacity (31% of wild-type enzyme).

**Temperature Stability of Wild-type Glycosylasparaginase and Mutants—**Glycosylasparaginases from most organisms are known to have high thermostability (15), but the *Flavobacterium* enzyme is noticeably less heat stable than the human enzyme (32). Therefore, the stability of the mutant recombinant enzymes was examined at different temperatures (Fig. 2). Wild-type enzyme began to inactivate at 55 °C and was completely inactivated at 60 °C. Mutants D107N and T169A began to inactivate about 5–10 degrees earlier than wild-type. Three other mutants, S50A, D58N, and T203A, were completely inactivated at 55 °C. D183N had an unstable structure and precipitated at 50 °C. D66N had the least stable structure and was barely active even at 40 °C.

**Kinetic Characterization of Wild-type and Mutant Enzymes—**Glycosylasparaginase kinetic parameters *Km* and *kcat* were determined using the natural substrate Asn-GlcNAc or the alternate compounds, asparagine or Asp(pNA)-OH (see “Materials and Methods”). Enzyme residues that might interact with the oligosaccharide could be studied by comparing hydrolysis of asparagine and Asp(pNA)-OH to that of Asn-GlcNAc (Table II). A typical kinetic analysis of the initial rates as a function of substrate concentration is shown in Fig. 3. Wild-type enzyme had a *Km* for the natural substrate Asn-GlcNAc of 0.085 mM, which is similar to human glycosylasparaginases and *Flavobacterium* glycosylasparaginases in *vitro* autoproteolysis of slow-cleavage mutants. The indicated recombinant forms of *Flavobacterium* glycosylasparaginase were expressed and purified as described under “Materials and Methods.” a, SDS-PAGE of concentrated wild-type and mutant enzyme supernatants immediately after protein purification (Fig. 1a). Enzyme activity using Asn-GlcNAc as substrate was compared after >90% autoproteolysis to wild-type glycosylasparaginase. WT, wild type.

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**TABLE I**

| Mutation (human counterpart) | Autoproteolysis | Autoproteolysis | Enzyme activity |
|-----------------------------|----------------|----------------|-----------------|
|  % T1/2 |  |  |  |
| Wild type | 100 | WT | 100 |
| W11F | 65 | 30 min | 1 |
| W11F | 0 | Not detectable | Not detectable |
| E48A (D47) | 80 | 20 min | 120 |
| S50A (S49) | 95 | 5 min | 18 |
| D58N (D57) | 100 | WT | 93 |
| T64A (T63) | 97 | WT | 43 |
| D66N (D65) | 70 | 20 min | 1 |
| D107N (E107) | 97 | WT | 78 |
| T152S (T183) | 60 | 25 min | 8 |
| T152A | 0 | >36 h | <0.1 |
| T152A | 0 | Not detectable | Not detectable |
| T169A (T200) | 97 | WT | 58 |
| T170S (T201) | 65 | 20 min | 85 |
| T170C | 5 | 12 h | <1 |
| T170A | 3 | 18 h | <0.1 |
| K176A (K207) | 97 | WT | 80 |
| R180K (R211) | 10 | 3.5 h | 6 |
| R180Q | 8 | 20 h | <1 |
| R180L | 3 | >30 h | <0.1 |
| D183N | 80 | 30 min | 8 |
| D183E (D214) | 80 | 30 min | <1 |
| T170C | 97 | WT | 80 |
| D66N | 80 | 30 min | <1 |
| D183A | 50 | 60 min | <0.1 |
| T203S (T234) | 100 | WT | 80 |
| T203A | 4 | 3.5 h | 31 |

Autoproteolysis was determined by SDS-PAGE of wild-type and mutant enzyme samples immediately after protein purification (Fig. 1a). Enzyme activity using Asn-GlcNAc as substrate was compared after >90% autoproteolysis to wild-type glycosylasparaginase. WT, wild type.

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![Fig. 1](http://www.jbc.org/)

**a**. Expression and purification of wild-type and mutant *Flavobacterium* glycosylasparaginases and *in vitro* autoproteolysis of slow-cleavage mutants. The indicated recombinant forms of *Flavobacterium* glycosylasparaginase were expressed and purified as described under “Materials and Methods.” a, SDS-PAGE of concentrated wild-type and mutant enzyme supernatants immediately after protein purification (Fig. 1a). Enzyme activity using Asn-GlcNAc as substrate was compared after >90% autoproteolysis to wild-type glycosylasparaginase. WT, wild type.

**b**. In vitro autoproteolysis of slow-cleavage mutants into α and β subunits. T152S, T170S/C/A, W11S, D183E/NA, E48A, D66N, and T203A; 37 °C, overnight; T152C: 37 °C, 2 days; T152A and W11F: 37 °C, five days; R180K/Q/L: room temperature, 3 days. c, stability of the single-chain αβ precursor of R180K at 37 °C. Lane 1, immediately after purification; lanes 2–5, after 2, 4, 6, and 17 h of incubation, respectively.
for the three tested substrates, especially for asparaginase or Asp(pNA)-OH, was much less affected than \( k_{\text{cat}} \). Substitution of the nucleophile hydroxyl by a thiol group (T152C) yielded an enzyme that was barely active, and the only kinetic parameter measured was the \( k_{\text{cat}} (1.5 \times 10^{-4} \text{ s}^{-1}) \) after overnight assay with saturating Asn-GlcNAc substrate.

The \( K_m \) values of T170S and T170C mutants for Asn-GlcNAc and Asp(pNA)-OH were the same as those of the wild-type glycosylasparaginase. The \( k_{\text{cat}} \) of the Ser-170 mutant was 50% that of the wild-type enzyme with Asn-GlcNAc as a substrate. Although the \( k_{\text{cat}} \) values for the two sugar-free analogues of the linkage structure were more adversely affected, significant reaction rates remained. The \( K_m \) of T170A for the natural substrate was greatly decreased (<0.01 mM) and could not be measured accurately, but the \( k_{\text{cat}} \) of T170A with Asn-GlcNAc was measured to be 5.1 \( \times 10^{-3} \text{ s}^{-1} \) (data not shown). The T170C and T170A mutants had greater than 700 and 3000-fold decreases in the \( k_{\text{cat}} \).

Mutants R180K/Q/L and D183E/N/A—Mutation of Arg-180 or Asp-183 reduced the catalytic reaction rate. These amino acids correspond to residues in the human enzyme that H-bond respectively to the \( \alpha \)-carboxyl and \( \alpha \)-amino group of the substrate. Both \( K_m \) and \( k_{\text{cat}} \) were adversely affected and the specificity constant \( (k_{\text{cat}}/K_m) \) was decreased from 200 to \( 10^6 \)-fold compared with wild-type enzyme. Mutant R180K had an 8-fold increased \( K_m \) for Asn-GlcNAc as a substrate. Mutation to a Gln (R180Q) with a polar side-chain amide caused a much greater increase in \( K_m \) (200-fold). Replacement with a nonpolar side-chain residue (R180L) further increased the enzyme \( K_m \) about 400-fold compared with that of wild-type enzyme. Mutation of Arg-180 also reduced the enzyme catalytic rate. The \( k_{\text{cat}} \) values of R180K/Q/L were at least 10-fold lower than that of wild-type glycosylasparaginase.

Similar results were seen with Arg-180 mutants occurred upon substituting amino acids for Asp-183. D183EN mutants showed an increase in \( K_m \) more than 400-fold compared with that of wild-type glycosylasparaginase. Asp-183 is also important in maintaining the enzyme reaction rate, indicated by the large reduction of \( k_{\text{cat}} \) by a factor of about 1800 in the D183N mutant. The D183E mutant that retains the negative charge at this position maintains the rate constant much better, but still at one order of magnitude below that of wild-type enzyme.

![Fig. 2. Temperature stability of wild-type and mutant glycosylasparaginases.](http://www.jbc.org/)

### Table II

Catalytic parameters of glycosylasparaginase mutants using different substrates

|        | \( K_m \) (mM) | \( k_{\text{cat}} \) (s\(^{-1}\)) | \( k_{\text{cat}}/K_m \) (s\(^{-1}\)mM\(^{-1}\)) |
|--------|----------------|----------------|----------------------------------|
| Asn-GlcNAc \(^a\) |               |                 |                                 |
| WT     | 0.085 ± 0.004 | 16.0 ± 2.0      | 190                              |
| T152S  | 0.14 ± 0.02   | (8.5 ± 0.6)\(\times 10^{-1}\) | 6.1                              |
| T170S  | 0.078 ± 0.002 | 8.0 ± 1.0       | 100                              |
| T170C  | 0.07 ± 0.01   | (2.2 ± 0.9)\(\times 10^{-2}\) | 3.1 ± 10\(^{-1}\)              |
| R180K  | 0.7 ± 0.1     | (7.2 ± 0.4)\(\times 10^{-1}\) | 1.0                              |
| R180Q  | 17 ± 2        | 1.3 ± 0.3       | 7.6 ± 10\(^{-2}\)              |
| R180L  | 34 ± 4        | 1.1 ± 0.1       | 3.2 ± 10\(^{-2}\)              |
| D183N  | 40 ± 8        | (9.5 ± 0.9)\(\times 10^{-3}\) | 2.4 ± 10\(^{-4}\)             |
| D183E  | 65 ± 5        | 1.0 ± 0.2       | 1.6 ± 10\(^{-3}\)              |
| T203S  | 0.076 ± 0.003 | 3.5 ± 0.2       | 46                               |
| T203A  | 0.04 ± 0.01   | 1.5 ± 0.3       | 39                               |
| D66N   | 2.1 ± 0.3     | (2.7 ± 0.1)\(\times 10^{-1}\) | 1.3 ± 10\(^{-1}\)              |
| W11S   | 0.010 ± 0.002 | (3.4 ± 0.5)\(\times 10^{-2}\) | 3.4                              |

|        | \( K_m \) (mM) | \( k_{\text{cat}} \) (s\(^{-1}\)) | \( k_{\text{cat}}/K_m \) (s\(^{-1}\)mM\(^{-1}\)) |
|--------|----------------|----------------|----------------------------------|
| Asp(pNA)-OH \(^b\) |               |                 |                                 |
| WT     | 0.085 ± 0.004 | 16.0 ± 2.0      | 190                              |
| T152S  | 0.14 ± 0.02   | (8.5 ± 0.6)\(\times 10^{-1}\) | 6.1                              |
| T170S  | 0.078 ± 0.002 | 8.0 ± 1.0       | 100                              |
| T170C  | 0.07 ± 0.01   | (2.2 ± 0.9)\(\times 10^{-2}\) | 3.1 ± 10\(^{-1}\)              |
| R180K  | 0.7 ± 0.1     | (7.2 ± 0.4)\(\times 10^{-1}\) | 1.0                              |
| R180Q  | 17 ± 2        | 1.3 ± 0.3       | 7.6 ± 10\(^{-2}\)              |
| R180L  | 34 ± 4        | 1.1 ± 0.1       | 3.2 ± 10\(^{-2}\)              |
| D183N  | 40 ± 8        | (9.5 ± 0.9)\(\times 10^{-3}\) | 2.4 ± 10\(^{-4}\)             |
| D183E  | 65 ± 5        | 1.0 ± 0.2       | 1.6 ± 10\(^{-3}\)              |
| T203S  | 0.076 ± 0.003 | 3.5 ± 0.2       | 46                               |
| T203A  | 0.04 ± 0.01   | 1.5 ± 0.3       | 39                               |
| D66N   | 2.1 ± 0.3     | (2.7 ± 0.1)\(\times 10^{-1}\) | 1.3 ± 10\(^{-1}\)              |
| W11S   | 0.010 ± 0.002 | (3.4 ± 0.5)\(\times 10^{-2}\) | 3.4                              |

\(^a\) Kinetic parameters for the substrate Asn-GlcNAc of wild-type (WT), T152S, T170S/C, T203S/A, D66N, and W11S enzymes were determined by measuring NADH conversion to NAD\(^+\) in a three-step coupled assay (see “Materials and Methods”). Parameters of the mutants R180Q/L and D183EN were determined by the Morgan-Elsdon color reaction. Parameters of R180K were determined by both methods of assaying the formation of NAD\(^+\) and GlcNAc. Data are the average of two to three experiments.

\(^b\) Kinetic parameters for the substrate asparaginase were determined by monitoring NADH conversion to NAD\(^+\). Parameters for mutants T152C, T170A, and R180K/Q/L and D183N/E were not able to be determined by this assay system.

\(^c\) Kinetic parameters for Asp(pNA)-OH were determined by measuring the release of pNA at 400 nm (see “Materials and Methods”). Parameters of T152C and T170A mutants were not determined because of the long time necessary for the assay. Parameters of all Arg-180 and Asp-183 mutants were not able to be determined by this system due to the limitation of the substrate solubility.
Mutagenesis of Glycosylasparaginase

**FIG. 2. Kinetic analysis of wild-type and mutant (W11S) enzyme activity.** Wild-type purified glycosylasparaginase (0.08 µg) and mutant W11S (0.5 µg) were used to determine the kinetic parameters $K_m$ and $k_{cat}$ for hydrolysis of Asn-GlcNAc. Enzymes were assayed fluorometrically (see "Materials and Methods"). Initial rates ($V$) were calculated based on reactions at five different time points: wild-type enzyme (■); mutant W11S (●, ●).

Mutants T203S/A—From the crystal structure of human glycosylasparaginase, it was predicted that the negative β-carboxyl oxygen of the Asn component of the substrate in its tetrahedral transition state is stabilized by an oxyanion hole (16). The $\gamma$-Y of conserved Thr-203 and the main-chain nitrogen of Gly-204 were suggested to play major roles in this structure by hydrogen bonding to the carboxyl oxygen (16). However, mutagenesis of bacterial Thr-203 to Ser or Ala did not substantially influence $K_m$ for any of the three substrates (Table II). With Asn-GlcNac as substrate, the mutant T203A surprisingly showed a 50% decrease in $K_m$ with only about 10-fold reduction of $k_{cat}$. The decrease in hydrolysis rate by this mutant possibly results from slowing down the deacylation step ($k_d$) of the enzyme reaction (see "Discussion"). The difference between reaction rates in having the side-chain hydroxyl of residue 203 on Ser or Thr was small in the case of Asn as substrate.

Mutants W11S and D66N—Kinetic analysis of the bacterial mutant W11S showed reductions of enzyme specificity for substrates both with carbohydrate (Asn-GlcNac) or without (asparagine or Asp(pNA)-OH). In addition, more significant changes occurred in $k_{cat}$, which was reduced more than 400-fold for Asn-GlcNac and relatively less for the other two substrates (20- and 65-fold, respectively).

Asp-66 was found to greatly affect enzyme activity. Kinetic characterization of a D66N mutant using the substrate Asn-GlcNac revealed a 1000-fold reduction in the specificity constant $k_{cat}/K_m$. This change reflects both an increase in the $K_m$ and a decrease in $k_{cat}$. However, for this same mutant, $k_{cat}/K_m$ was reduced only 12-fold when asparagine was used as substrate, which was due only to a reduction in $k_{cat}$. In contrast, the $K_m$ of D66N for Asp(pNA)-OH increased by more than 20-fold compared with that of wild-type enzyme.

**DISCUSSION**

In this study, we have used site-directed mutagenesis of a bacterial form of glycosylasparaginase to separately examine the autoproteolytic activation and reaction mechanism of an important human lysosomal enzyme that is also a member of the Ntn-amidohydrolase family of proteins (17). Flavobacterium glycosylasparaginase was originally isolated and cloned by the laboratory of Tarentino (11, 29), and it has the simplest amino acid sequence among forms of the enzyme from six different species, as it lacks about 30 amino acids at the C terminus of its α-subunit (9, 11). The same C-terminal region of the human α-subunit is cleaved in lysosomes without any known effect on the mature enzyme. The overall sequences of all known glycosylasparaginases are highly conserved, especially the amino acids predicted to be functionally essential based on the human enzyme crystal structure and other studies (9, 13, 14, 16, 23, 30). Therefore, the catalytic mechanism of the Flavobacterium enzyme should generalize to other glycosylasparaginase members.

Maturation of glycosylasparaginase is an unusual process shared by many members of the Ntn-amidohydrolase family. The nascent translated precursor folds to a conformation that allows autoproteolysis of a zymogen into α and β subunits (12, 31). This step exposes the N-terminal Thr of the β subunit to create the active site nucleophile (T152 in this study) and possibly shift other important residues into the final reaction center. Thr-152 is also the key catalytic amino acid in autoproteolysis because it nucleophilically attacks the adjacent Asp-151, allowing the peptide bond between them to be broken and an active enzyme to be formed with a free α-amino group at the N terminus of the β subunit (12, 17). Previous mutagenesis studies on the human enzyme indicated that reductions of glycosylasparaginase activity in many cases are due to a slowing down of precursor autoproteolysis such that the enzyme is incompletely activated (13, 14, 24). This complexity in glycosylasparaginase processing creates experimental difficulties when investigating the function of specific amino acids in the mechanism of enzyme catalysis.

Flavobacterium glycosylasparaginase mutants W11S/F, E48A, D66N, T152S/C, T170S/C/A, R180K/Q/L/A, D183E/N/A, and T203A slowed precursor autoproteolysis to different levels (Fig. 1). Prolonged incubation at 37 °C in vitro allowed most of them to be cleaved and activated enabling us to analyze amino acids essential to the mechanism of this enzyme. Only W11F and T152A were not cleavable in this system (Fig. 1b). In contrast, human W11F was reported to be reported when transiently expressed in COS cells (23). Mutant T152C was also very slow to undergo autoproteolysis at 37 °C, and further increase in temperature did not accelerate cleavage but instead caused precursor precipitation (data not shown). Cleavage of T152C precursor can be accelerated by hydroxylamine (12). Residue Arg-180 appears to be important in precursor peptide folding since its mutation yielded unstable precursors (Fig. 1c). In contrast, human W11F was reported to be cleaved when transiently expressed in COS cells (23). Mutant T152C was also very slow to undergo autoproteolysis at 37 °C, and further increase in temperature did not accelerate cleavage but instead caused precursor precipitation (data not shown). Cleavage of T152C precursor can be accelerated by hydroxylamine (12). Residue Arg-180 appears to be important in precursor peptide folding since its mutation yielded unstable precursors (Fig. 1c). In contrast, human W11F was reported to be cleaved when transiently expressed in COS cells (23). Mutant T152C was also very slow to undergo autoproteolysis at 37 °C, and further increase in temperature did not accelerate cleavage but instead caused precursor precipitation (data not shown). Cleavage of T152C precursor can be accelerated by hydroxylamine (12). Residue Arg-180 appears to be important in precursor peptide folding since its mutation yielded unstable precursors (Fig. 1c). In contrast, human W11F was reported to be cleaved when transiently expressed in COS cells (23). Mutant T152C was also very slow to undergo autoproteolysis at 37 °C, and further increase in temperature did not accelerate cleavage but instead caused precursor precipitation (data not shown). Cleavage of T152C precursor can be accelerated by hydroxylamine (12). Residue Arg-180 appears to be important in precursor peptide folding since its mutation yielded unstable precursors (Fig. 1c). In contrast, human W11F was reported to be cleaved when transiently expressed in COS cells (23). Mutant T152C was also very slow to undergo autoproteolysis at 37 °C, and further increase in temperature did not accelerate cleavage but instead caused precursor precipitation (data not shown). Cleavage of T152C precursor can be accelerated by hydroxylamine (12). Residue Arg-180 appears to be important in precursor peptide folding since its mutation yielded unstable precursors (Fig. 1c). In contrast, human W11F was reported to be cleaved when transiently expressed in COS cells (23). Mutant T152C was also very slow to undergo autoproteolysis at 37 °C, and further increase in temperature did not accelerate cleavage but instead caused precursor precipitation (data not shown). Cleavage of T152C precursor can be accelerated by hydroxylamine (12).
Ntn-amidohydrolases. However, depending on the individual Ntn-amidohydrolase, either Thr, Ser, or Cys functions as the nucleophile. The large activity reduction for glycosylasparaginase mutant T152S/C indicates that a precise chemistry of the nucleophile must optimize each individual active site structure in this enzyme superfamily. Residue Thr-170 has its side-chain hydroxyl closest to the nucleophile Oy of Thr-152. Previous studies suggested this hydroxyl group forms a hydrogen bond that stabilizes the activated Oy of Thr-152 (16). Mutagenesis of bacterial Thr-170 to Cys and Ala greatly affected the enzyme hydrolysis rate (Table II). Mutant T170C had a reduction of the $k_{\text{cat}}$ with little effect on $K_m$ compared with wild-type enzyme, which suggests this rate reduction is likely in the acylation step ($k_2$) (34, 35). Mutant T170A had a further reduced value of $k_{\text{cat}}$ but was accompanied by a decrease in $K_m$, which normally occurs when the deacylation rate $k_3$ is reduced ($K_m=K_a(k_{3a}/(k_2+k_{3a})))$ (34, 35). Our study confirms that the side-chain hydroxyl of Thr-170 is essential in maintaining the enzyme reaction rate, probably influencing both acylation and deacylation steps.

Hydrolysis initiates when the substrate is bound into the active site and binding is suggested to be achieved principally by side-chains of Arg-180 and Asp-183 and the main-chain carbonyl oxygen of Gly-204 which together form hydrogen bonds to the charged groups of the substrate (16). Mutagenesis of Arg-180 and Asp-183 greatly reduced enzyme specificity, which confirmed that interactions from these charged groups are crucial for substrate binding. However, these mutations also greatly affected the enzyme reaction rate, especially in the case of Asp-183. Mutant D183N reduced the $k_{\text{cat}}$ more than 1500-fold (Table II). Asp-183 has its negative side-chain close to Gly-204, and it also may be involved in forming the transition state oxyanion hole.

The figure is produced by the program Rasmol using crystal structure coordinates of human glycosylasparaginase (16). Residues mutated in this study along with the reaction product aspartate (boxed) are indicated. Nitrogens are blue; oxygens are red. Asterisks indicate the base and nucleophile of the Ntn Thr-152.

FIG. 5. Active site structure region of glycosylasparaginase. The figure is produced by the program Rasmol using crystal structure coordinates of human glycosylasparaginase (16). Residues mutated in this study along with the reaction product aspartate (boxed) are indicated. Nitrogens are blue; oxygens are red. Asterisks indicate the base and nucleophile of the Ntn Thr-152.
binding due to its interaction with carboxydrate (16, 23). Thus, our kinetic analysis revealed that the aromatic side-chain of Trp-11 instead greatly contributes to the enzyme reaction rate. Viewing the enzyme active-site structure indicates this Trp locates outside of Thr-152 (Fig. 5), and it could function similarly to Thr-74 in glutaminase. Mutant W11S reduced both $k_a$ and $k_{cat}$, suggesting this Trp is probably involved in regulating the enzyme deacylation rate $k_a$ (Table II) (34, 35). Residue Asp-66 is geometrically close to the side-chain of Arg-180, (Fig. 5) and it may stabilize hydrogen bonding between the positively charged side-chain of Arg-180 and the $\alpha$-carboxyl group of the substrate (Table II). How this residue affects the reaction rate constant $k_{cat}$, how it affects the binding of the nitroaniline substrate analogue, and whether glycosylasparaginase has a similar gate conformation mechanism remains unclear. However, it was noted that substitutions at both Trp-11 and Asp-66 affected enzyme activity less when asparagus was used as substrate rather than Asn-GlcNAc (Table II). Residue Asp-66 was also shown to be very important in maintaining intact tertiary structure of the enzyme. Changing Asp-66 to its amide was also shown to contribute to enzyme thermostability.

Residue Glu-38 is the counterpart of human glycosylasparaginase Asp-47 and is not conserved in the rat enzyme which has an Ala at this position (9). Glu-48 together with two charged residues, Lys-176 (human Lys-207) and Arg-180 (human Arg-211), were suggested by ab initio quantum mechanics and molecular dynamics calculations to be the most important amino acids for stabilizing the transition state (30). However, the Flavobacterium mutant E48A showed an increased enzyme activity of about 120% (Table I). Our study also indicated that Lys-176 is not essential because mutant K176A retained almost full activity (Table I). Ser-50 is the shortest distance from Oy of nucleophile Thr-152 (30), and enzyme activity of the mutant S50A was reduced to about 20% (Table I). This residue probably stabilizes the nucleophile Thr-152 by hydrogen bonding with the positive $\alpha$-amino group (24), and yet based on the relatively small change in enzyme activity, this role is not critical for catalysis. Mutagenesis of two other Asp (Asp-58 and -107) and Thr (Thr-169, Asp-183, and Thr-203) were also shown to contribute to enzyme thermostability.

Our results from site-directed mutagenesis of Flavobacterium glycosylasparaginase combined with the previous crystal structure (16) and biochemical (13, 14, 24) and theoretical studies (30) of the human enzyme identify the main catalytic residues at the active site of this Ntn-amidohydrolase to be Trp-11, Asp-66, Thr-152, Thr-170, Arg-180, and Asp-183 (Fig. 5). Still lacking is detailed information about enzyme specificity and mechanism, including residues involved in both acylation and deacylation steps and the subtle structural changes that occur during these parts of the reaction. Further three-dimensional structural information about wild-type and mutant enzymes is required to define the precise mechanism of this enzyme. As a model member of the very interesting Ntn-amidohydrolase superfamily, all biochemical aspects of glycosylasparaginases, from autoproteolytic activation to catalysis, are important to characterize and understand.

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