Characterization and Functional Analysis of the Cis-autoproteolysis Active Center of Glycosylasparaginase*

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Glycosylasparaginase is an N-terminal nucleophile hydrolase and is activated by intramolecular autoproteolytic processing. This cis-autoproteolysis possesses unique kinetics characterized by a reversible N-O acyl rearrangement step in the processing. Arg-180 and Asp-183, involved in binding of the substrate in the mature enzyme, are also involved in binding of free amino acids in the partially formed substrate pocket on certain mutant precursors. This binding site is sequestered in the wild-type precursor. Binding of free amino acids on mutant precursors can either inhibit or accelerate their processing, depending on the individual mutants and amino acids. The polypeptide sequence at the processing site, which is highly conserved, adopts a special conformation. Asp-151 is essential for maintaining this conformation, possibly by anchoring its side chain into the partially formed substrate pocket through interaction with Arg-180. The reactive nucleophile Thr-152 is activated not only by deprotonation by His-150 but also by interaction with Thr-170, suggesting a His-Thr-Thr active triad for the autoproteolysis.

Glycosylasparaginase hydrolyzes the β-N-glycosidic bond between asparagine and N-acetylglucosamine of asparagine-linked glycans (1). Glycosylasparaginases from different sources consist of two non-identical α- and β-subunits held together by strong non-covalent forces (2, 3). Glycosylasparaginase is encoded by a single gene and is initially synthesized as a single polypeptide that is post-translationally processed (2). This processing is an obligatory step in the production of active enzyme, and cleavage of a single peptide bond is responsible for this activation (3, 4). The newly formed N-terminal threonine residue of the β-subunit functions as both the active base and nucleophile for the enzyme activity, which places glycosylasparaginase in the N-terminal nucleophile-hydrolase family (Ntn-enzyme)3 (5–7).

The three-dimensional structure of human glycosylasparaginase has been published (8). The enzyme active center has been characterized and is illustrated in Fig. 1A. The proposed reaction mechanism is as follows. The hydroxyl of the N-terminal nucleophile Thr-183 is probably activated by its own α-amino group and attacks the β-carbonyl of the asparagine part of the substrate to form the tetrahedral transitional intermediate. The negatively charged carbonyl oxygen of the transitional state is stabilized by hydrogen bonds from the hydroxyl of Thr-234 and the main chain nitrogen of Gly-235. The transitional intermediate collapses into the acyl-enzyme intermediate and releases the sugar part of the substrate. The acyl-enzyme intermediate is hydrolyzed by water. Besides the interactions mentioned above, the substrate binding is achieved by hydrogen bonding of the α-carboxylate and the α-amino group of the substrate to Arg-211 and Asp-214, respectively. The hydrogen interaction between Thr-183 and Thr-201 is also important for the enzymatic activity.

In a previous study (9), using a cloned glycosylasparaginase from Flavobacterium meningosepticum (10, 11), we have demonstrated that the activation is an intramolecular autoproteolytic processing event. Based on the experimental data, we proposed a cis-autoproteolysis model for glycosylasparaginase activation as illustrated in Fig. 1B. Since Ntn-enzymes have in common an unusual fold and the active N-terminal nucleophile is most often generated by autoproteolytic processing (5), we suggested that the cis-autoproteolysis model may also, with some variations, be held for processing of other Ntn-enzymes. Cis-autoproteolytic processing is a newly found post-translational modification that is involved in many important cellular processes, such as enzyme activation, proteasome biogenesis (12), hedgehog protein maturation (13), and protein splicing (14). A general catalytic mechanism of cis-autoproteolysis may be shared by many polypeptide main-chain modification pathways in living cells. Characterization and functional analysis of the autoprocessing active center in glycosylasparaginase should reveal the molecular mechanism of cis-autoproteolysis. This will provide valuable information for understanding these related cellular processing functions.

At present, the three-dimensional structure of glycosylasparaginase precursor is not available. Identification and functional analysis of the active residues for activation based on a three-dimensional structure of the precursor is not feasible. However, both the enzyme reaction and the autoproteolysis resemble the reaction by serine/cysteine proteases, and a single hydroxyl residue, Thr-152 in the bacterial or Thr-183 in the human protein, is used as the reactive nucleophile for both the reactions. Therefore, some other residues involved in the enzymatic reaction may also be involved in the autoproteolysis. On the other hand, the two reactions are chemically different. The two active centers should also be different, both structurally and functionally, although the two active centers may share some part of their structures. For instance, the C-terminal residues of the α-subunit are essential for activation but play

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1 The abbreviations used are: Ntn-enzyme, N-terminal nucleophile-hydrolase; AspNHeClNac, N-carboxy-N′(β-N-acetylgulosaminyl)-t-aspartic acid; MBP, maltose-binding protein; PAGE, polyacrylamide gel electrophoresis.
FIG. 1. A, the structure of the active center of human glycosylasparaginase. The figure was generated by computer using the coordinates of code 1APZ in the Protein Data Bank, Brookhaven National Laboratory, deposited by C. Oinonen et al. (8). There is an aspartic acid as the enzyme reaction product bound in the active center. B, the cis-autoproteolysis model for glycosylasparaginase activation. When the precursor is properly folded, the active hydroxyl of Thr-152 is deprotonated by the proton acceptor/donor His-150 and launches a nucleophilic attack on the \((\pm)\)-a-carbonyl carbon \((\pm)\)-carbonyl of Asp-151) to form a transitional tetrahedral intermediate associated with a 5-member heterocycle. The tetrahedral intermediate collapses by protonation of the amino group of Thr-152 to yield an ester intermediate involving the \(\alpha\)-carbonyl of Asp-151 and the hydroxyl of Thr-152 via an N-O acyl rearrangement (N-O shift). The final step is hydrolysis of the ester by water and the dissociation of the carboxyl portion of the \(\alpha\)-subunit from the active center.
no roles in enzyme reaction. Therefore, in this study, not only were the residues involved in the enzyme active center analyzed, but other conserved residues that may be involved in the autoproteolysis were also under investigation.

By studying in vitro autoprocessing of mutant precursors, we demonstrate that the activation of glycosylasparaginase follows the kinetics of cis-autoproteolysis. Site-directed mutagenesis and in vitro activation of purified precursors show that two residues, Arg-180 and Asp-183, which are involved in binding of substrate in the mature enzyme, are also involved in binding of free amino acids on mutant precursors. Binding of amino acid ligands on these precursors can either inhibit or stimulate their processing, depending on the individual mutants and amino acids. The results from this study suggest that Asp-151 plays an important role in activation similar to the P1 residue of substrates in protease reactions and that the partially formed substrate binding pocket on the precursor functions similarly to the S1 site of proteases. A charge interaction between Asp-151 and Arg-180 in the precursor is implicated. The data also suggest a possible His-Thr-Thr active triad in glycosylasparaginase activation.

**EXPERIMENTAL PROCEDURES**

**Materials**—The maltose-binding protein (MBP) protein fusion expression and purification system including plasmids pMAL-c2 and pMAL-p2, the *Escherichia coli* host strain TB1, amylase resin, Factor Xa protease, restriction enzymes, T4 DNA ligase, T4 DNA kinase, DNA polymerases, and synthesized oligonucleotides were obtained from New England Biolabs. The bacterial glycosylasparaginase clones used in this study were kindly provided by Dr. A. Tarentino (Wadsworth Laboratories, Albany, NY).

**Enzyme Assay**—The glycosylasparaginase assay was based on colorimetric measurement of N-acetylglucosamine released from the substrate N-α-(N-acetylglucosaminyl)-l-aspartic acid (AspNHClcNac) (Sigma) using the Reissig modification of the Morgan-Elson reaction as described previously (11).

**Recombinant DNA and Mutagenesis**—DNA manipulation and site-directed mutagenesis (Kunkel methods) were carried out as described previously (9). All site-directed mutagenesis was performed using either the pMAL system or the Litmus system (New England Biolabs Inc.). DNA sequence analysis was carried out using an ABI automated DNA sequencer.

**Gene Expression and Protein Purification**—Expression and purification of gene products using the MBP fusion and expression system (15) with modifications were described previously (9), except that for expression of the glycine inhibition-minus mutants such as the T125S/D183N double mutant, a lower induction temperature (15 °C, overnight) or shorter induction time (3 h, 30 °C) was used.

**In Vitro Autoproteolysis**—Amylase-purified fusion proteins stored at −70 °C in the reaction buffer (20 mM Tris, pH 7.4, 50 mM NaCl, 1 mM EDTA) were thawed and diluted in the same ice-cold buffer to the desired concentrations and then shifted to an appropriate temperature to start autoproteolysis. At various times, aliquots were withdrawn and subjected to SDS-PAGE analysis. For some Thr to Cys mutant proteins, 5 mM dithiothreitol was included in the reaction buffer to prevent oxidation during long incubations. Protein gels were stained with Coomassie Blue R-250, and protein bands were quantified by gel scanning with a Microtek Scanmaker III, Adobe Photoshop®, and NIH Image 1.57.

**Protein Analysis**—Protein concentration was determined by the Bio-Rad protein assay using bovine serum albumin as standards. N-terminal protein sequence analysis was performed on an Applied Biosystems 610A as described previously (9). Molecular weight determination was carried out by SDS-PAGE analysis or electrospray mass spectrometry.

**Kinetic Analysis**—The cis-autoproteolytic processing of the glycosylasparaginase proenzyme is described by the following reaction,

\[
\begin{align*}
R_1\text{-CONH}-R_2 & \xrightarrow{k_1} R_1\text{-COO}-R_2 \xrightarrow{k_2} R_1\text{-COOH} + R_2\text{-NH}_2 \\
[N] & \quad \alpha \quad \beta \\
\text{REACTION 1}
\end{align*}
\]

where \(k_1\), \(k_2\), and \(k_{-1}\) are, respectively, the N-O shift rate constant, the O-N shift rate constant, and the ester intermediate hydrolysis rate constant. \([N]\) and \([O]\) stand for the precursor and the ester intermediate concentrations, respectively.

\[
d[N]/dt = -k[N] + k_{-1}[O] \quad \text{(Eq. 1)}
\]

Since cis-autoproteolysis is not an enzymatic processing, the overall processing rate is constantly changing and a steady state of reaction cannot be reached. However, the experimental data indicate that the overall processing follows first-order kinetics and \([O]/[N] \to 0\). Therefore,

\[
d[N]/dt = -k[N] \quad \text{(Eq. 2)}
\]

where \(k\) is the overall rate constant. Combining Equations 1 and 2, we have the following.

\[
d[N]/dt = -k_{1}k_{2}/k_{-1} + k_{2})[N] \quad \text{(Eq. 3)}
\]

The overall rate constant \(k = k_{1}k_{2}/k_{-1} + k_{2}\) is determined by the N-O shift rate constant \(k_{1}\), the O-N shift rate constant \(k_{2}\), and the ester hydrolysis rate constant \(k_{-1}\). Ester hydrolysis is the rate-limiting step for the processing (In this processing, the N-O shift, O-N shift, and ester hydrolysis all are the rate determining steps. By definition, the last rate determining step, ester hydrolysis, is the rate-limiting step (16)).

When 0.25 \(m\) hydroxylamine is used, the overall rate constant is given by the following,

\[
k' = k_{1}k_{2}/(k_{-1} + k_{2}) \quad \text{(Eq. 4)}
\]

where \(k'_{2}\) is the hydroxylaminolysis constant, given \(k'_{2} \gg k_{2}\).

If both the O-N shift rate constant \(k_{1}\) and the hydroxylaminolysis (NH2OH = 0.25 \(m\)) rate constant of the ester intermediates \(k_{2}\) in the tested mutants are similar, then

\[
k' = \beta k_{1} \quad \text{(Eq. 5)}
\]

where \(\beta\) is a constant. We have used \(k'\) to estimate the relative N-O shift rates.

\[
\text{kh} = k_{1}(1/k_{-1} + 1/k_{2}) = \delta k_{1} \quad \text{(Eq. 6)}
\]

When \(k_{-1} \gg k_{2}\), i.e. for slow ester hydrolysis mutants

\[
\text{kh}' = k_{1}(1/k_{-1} + 1/k_{2})
\]

where \(\delta\) is a constant. In any case \(kh'\) increases as \(k_{2}\) increases. We have used \(kh'\) or \((\text{NH}2\text{OH})/r(\text{H}2\text{O})\) to estimate the relative ester hydrolysis rates, where \((\text{NH}2\text{OH})/r(\text{H}2\text{O})\) are the half processing times with and without 0.25 \(m\) NH2OH.

The inhibition constant \(K_{i}\) of amino acid is determined as described previously (9).

**RESULTS**

We fused the genes of the *E. coli* MBP and glycosylasparaginase, termed MG, and expressed the gene fusion in *E. coli*. The amylase-purified MBP product consists of two polypeptides. One contained a fusion between MBP and the α-subunit, termed MGoα, with a molecular mass of 60 kDa. The second was the glycosylasparaginase β-subunit with a molecular mass of 15 kDa. The two polypeptides were in tight association, termed MGαβ, and co-purified on amylase resin. Protein fusions between MBP and mutant glycosylasparaginases were termed in the single-letter format for amino acids as in the following example: MBP-glycosylasparaginase(T152A) was termed MG(T152A) where 152 indicates the residue number based on wild-type glycosylasparaginase. The precursor of MG(T152X) protein was termed pre-MG(T152X), and the activated protein was termed MG(T152X)αβ.

**The Role of the N-O Shift in the Processing Reaction**—Our previous study (9) showed that pre-MG(T152C) was processed very slowly (the half activation time \(t_{50} \approx 48 h, 3 \times 10^{-4} \) of the rate for wild-type) because a slowly hydrolyzed thioester intermediate was involved. This intermediate could be rapidly resolved by adding the strong nucleophile hydroxylamine (NH2OH), which was highly reactive with an ester or a thio-
ester. In the presence of 0.25 M NH2OH, the half activation time \( t \) of pre-MG(T152C) was only about 30 min. When purified pre-MG(T152C) was first denatured with SDS followed by incubation with NH2OH, no precursor processing was observed (see Fig. 2A, lanes 1 and 2). This indicated that no detectable theta intermediate existed in the protein sample before incubation. When pre-MG(T152C) was first incubated for 3 h at 37 °C and then SDS and NH2OH were added, followed by incubation for an additional 1 h, the processing results were the same as in samples treated identically for 3 h but without SDS and NH2OH. Only about 5% of the precursor was processed (lanes 3 and 4). When NH2OH was included in the reaction during incubation, more than 95% of the precursor was processed after 3 h at 37 °C (lane 5). These results indicate that although the theta intermediate forms rather rapidly, no accumulation of the hydrolysis-resistant intermediate takes place during the autoproteolytic processing. Thus, the N-S shift step in the autoproteolytic processing was reversible. His-150 is the proton acceptor/donor for the autoproteolysis (9), and therefore activation of pre-MG(H150S) is very slow (\( t \approx 5–7 \) days, compared with 1–2 min for pre-MG) because of the very slow N-O shift rate. However, the processing rates of pre-MG(H150S) could still be increased 3–5-fold when 0.25 M NH2OH was added (Fig. 2B). This rate increase was similar to that of the fast processing pre-MG(T152S) (\( t \approx 20 \) min) without and \( t \approx 4 \) min with NH2OH. This indicates that the hydrolysis step in activation of pre-MG(H150S) is still the rate-limiting step. It cannot be reassigned from the ester hydrolysis step to the N-O shift step simply by decreasing the N-O shift rates. This mechanistically characterizes a reversible N-O shift step being involved in the autoproteolysis (16) (also see “Experimental Procedures”).

Characterization of the Amino Acid Binding Site on Precur-

sor—The activation of pre-MG(T152S/C) is inhibited by small \( \alpha \)-amino acids such as glycine and not by glycosylasparaginase substrate (AspNHGlcNAc) or inhibitor (Aspartate) (9), indicating that the amino acid binding site on these precursors is different from the substrate binding site of the mature enzyme. The location and biological function of this amino acid binding site was unknown. To address these questions, we undertook characterization of the residues involved in binding of the amino acid ligands on the precursor. Secondary mutations were introduced into the glycine-sensitive MG(T152S) mutant. We searched for suppressor mutants in which the activation was no longer inhibited by glycine. Two residues, Arg-180 and Asp-183, were identified as the suppressor mutation sites (Fig. 3). The freshly purified MG(T152S/D183N) gene product consisted of about 20% precursor and 80% processed protein. The half processing time \( t \) of pre-MG(T152S/D183N) was about 40 min, similar to that of pre-MG(T152S) (\( t \approx 20 \) min). The purified MG(T152S/R180Q) was mainly precursor, and the half-processing time \( t \) of pre-MG(T152S/R180Q) was more than 72 h. The activation of both pre-MG(T152S/D183N) and pre-MG(T152S/ R180Q) was not inhibited by glycine, suggesting that Arg-180 and Asp-183 were involved in amino acid binding on the precursor. The activation of other double mutants such as MG(T152S/T170A), MG(T152S/T203A), MG(T152S/T64A), MG(T152S/D58N), and MG(T152S/C68S) were still inhibited by glycine. For example, the autoprocessing of pre-MG(T152S/T203A) was severely inhibited by 10 mM glycine (Fig. 3), suggesting that Thr-203 did not play significant roles in amino acid binding on this precursor.

Analysis of Aspartic Acid Mutants—In serine proteases, the active triad Ser-His-Asp forms a charge relay system to increase the nucleophilic activity of the active serine and to stabilize the transitional state of the reaction (17). To investigate whether glycosylasparaginase activation involves a similar charge relay system, we compared the sequences of six glycosylasparaginases from different sources (7) and identified seven conserved aspartate residues; Asp-33, -58, -66, -71, -151, -183 and -194. These seven aspartate residues were individually replaced with asparagine by site-directed mutagenesis. The freshly purified MG(D33N), MG(D58N), MG(D66N), MG(D71N), and MG(D194N) were present mainly as the processed \( \alpha/\beta \)-subunit form with 0–10% of the protein presented as precursors (Fig. 4A). The precursors were completely processed after incubation for 2–3 h at 37 °C (Fig. 4B). These results suggest that Asp-33, -58, -66, -71, -183, and -194 do not play significant roles in the activation. For MG(D183N), 70% of
the purified protein was processed and 30% existed as pre-MG(D183N). The processing rate of pre-MG(D183N) was about 100-fold reduced ($\tau \approx 2$ h) relative to the wild-type. Purified MG(D183E) was already processed to MG(D183E)aβ. The enzymatic activities of MG(D183N)/Eaβ were greatly reduced. Pre-MG(D151N) was not processed. Asp-151 was further replaced with different residues by site-directed mutagenesis using a synthesized DNA oligo-mixture where codon 151 was randomized. Thirteen mutations were isolated; Asp-151-Ala/Leu/Val/Met/Pro/Gly/Asn/Ser/Thr/Arg/His/Glu. All the mutant gene products were purified as precursors. Purified MG(D151E) existed mainly as pre-MG(D151E). The processing rate of pre-MG(D151E) was decreased about 1000-fold ($\tau \approx 20$ h). Pre-MG(D151G) was processed very slowly. Since MG(D151E)/Gaβ was fully active, we were able to estimate the processing rate of pre-MG(D151G) by measuring the increase in enzyme activity. The processing rate was about $3 \times 10^{-5}$ of the wild-type. All other mutants were inactive in autoprocessing. Taken together, these results indicate that Asp-151 is virtually required for activation but not for enzyme activity. Asp-183 is important for enzyme activity but not for activation.

Isolation of Mutants with Amino Acid-sensitive Activation—The activation in wild-type is not inhibited by free amino acids ($K_r$ of glycine $\gg 10$ m$m$) suggests that the amino acid binding site is covered or sequestered in the wild-type precursor. To investigate the residues that are involved in sequestering the amino acid binding site, we set out to isolate mutants whose processing rates were inhibited by exogenous amino acids. Several dozen single and double mutants at 22 residues, including 16 conserved ones (Asp-33, -58, -66, -71, -107, -151, -183, and -197; His-150; Thr-64, -152, -170, and -203; Arg-180; Trp-11; Glu-97, and -121; Cys-68, -81, -168, -232, and -270) were constructed, and the gene products were analyzed. Among all of the characterized mutants, only in those mutated at His-150 or Thr-152 was the activation of the gene products inhibited by glycine (data not shown). For example, the activation of pre-MG(T152S/C) or pre-MG(H150S/K/W) was almost completely prevented by 10 mM glycine, although some of the precursors were processed very slowly (Fig. 2B). The glycine-sensitivity of activation in MG(H150X) mutants was irrelevant to the size, charge, hydrophathy, and geometry of the replacement residues.

Stimulation of Autoproteolysis by Glycine—The low processing rate of pre-MG(D151G) increased only slightly (2-3-fold) in the presence of 0.25 M NH$_2$OH, indicating that a slow N-O shift step involved in the processing (see “Experimental Procedures”). Stereochemically, it is unlikely for Asp-151 to form a charge relay system with His-150 and Thr-152 because of the physical position of Asp-151. A possible role for Asp-151 is to anchor its side chain into a pocket through charge interaction to produce the correct conformation for the processing site polypeptide, i.e.
**Analysis of Thrreonine Mutants**—We have shown that the reactive nucleophile Thr-152 for enzyme activity is also the reactive nucleophile for autoproteolysis (9). Two other conserved threonine residues in the enzyme active center, Thr-170 and Thr-203 (see Fig. 1A), were also investigated for their possible function in the activation. The purified MG(T203S) was already processed to MG(T203S)Δβ, which retained 50% of the enzyme activity. The processing rate of pre-MG(T203A) was about 100–200-fold reduced (τ = 3 h) relative to the wild type. MG(T203A)Δβ was about 30% active. These results suggest that Thr-203 may not play essential roles either in the precursor activation or in the enzyme activity. Thr-170 was replaced with Ser, Cys, and Ala, respectively, and the activation of each mutant gene product was analyzed. The processing rate of pre-MG(T170S) was reduced 10–20-fold relative to the wild-type. The processing rates of pre-MG(T170A/C) were reduced about 1000-fold. To understand these profound effects of Thr-170 on activation, we investigated the possible interaction in the mutants that lacked hydroxyl residues at one or both of these positions. (a) Comparing the k′ values, which indicated the N-O or N-S shift rates (see “Experimental Procedures”), among the different mutants showed that the lower processing rates in T170A/C single or in T152S/T170A/C double mutants were due to lower N-O shift rates since the relative hydrolysis rates (k/k′) for these mutants were actually the same (0.2–0.3) (T170A was an exception, the k/k′ = 0.04). (b) The processing rates in T152C and T152C/T170A/C/S were slow but could be greatly accelerated by adding NH$_2$OH (k/k′ = 0.01–0.03). In contrast to N-O shifts in mutants with hydroxyl 152, the N-S shift rates in mutants with thiol 152 became independent of the presence of a hydroxyl group on residue 170. As a whole, the results showed that the presence of both hydroxyl residues 152 and 170 was required for the efficient activation and maintenance of the enzyme activity.

| Mutant       | Processing constant | Enzyme activity |
|--------------|---------------------|-----------------|
|              | k/min$^{-1}$ | k′/min$^{-1}$ | k/k′ |
| WT           | 6.9 × 10$^{-1}$ | NA             | 100  |
| T170S        | 5.0 × 10$^{-2}$  | 2.3 × 10$^{-1}$ | 0.2  |
| T170C        | 1.2 × 10$^{-3}$  | 5.8 × 10$^{-3}$ | 0.2  |
| T170A        | 2.3 × 10$^{-4}$  | 5.8 × 10$^{-3}$ | 0.04 |
| T152S        | 3.5 × 10$^{-2}$  | 1.7 × 10$^{-1}$ | 0.2  |
| T152S/T170S  | 3.5 × 10$^{-2}$  | 1.7 × 10$^{-1}$ | 0.2  |
| T152S/T170C  | 4.8 × 10$^{-4}$  | 1.4 × 10$^{-3}$ | 0.3  |
| T152S/T170A  | 2.3 × 10$^{-4}$  | 7.2 × 10$^{-4}$ | 0.3  |
| T152C        | 2.3 × 10$^{-4}$  | 2.5 × 10$^{-2}$ | 0.01 |
| T152C/T170S  | 9.5 × 10$^{-4}$  | 3.5 × 10$^{-2}$ | 0.03 |
| T152C/T170C  | 2.3 × 10$^{-4}$  | 2.5 × 10$^{-2}$ | 0.01 |
| T152C/T170A  | 2.3 × 10$^{-4}$  | 7.7 × 10$^{-3}$ | 0.03 |

**DISCUSSION**

The experimental data from in vitro activation of glycosylasparaginase indicate that activation of glycosylasparaginase is an intramolecular autoproteolytic event (9), and the experimental results from analysis of the mammalian 20 S proteasome biogenesis are consistent with a cis-autoproteolysis model (12). On the other hand, a study on autocatalytic processing of the 20 S proteasome from *Thermoplasma acidophilum* suggests that this processing is probably intermolecular (18). Therefore, more studies on the autoprocessing mechanism in different Ntn-enzymes are needed to determine if there is a common one for their activation. In this study we characterized the autoprocessing catalytic center of glycosylasparaginase. The results provided further evidence for cis-autoproteolytic processing of this Ntn-hydrolase.

Two types of intramolecular autoproteolysis have been reported. In the first type, the active hydroxyl or thiol residue attacks a distant peptide carbonyl on the same polypeptide to initiate autoproteolytic processing. Autocleavage of LexA repressor is an example of this type of reaction. This intramolecular processing has been successfully converted to an intermolecular one by genetic manipulation (19, 20). In the second type, so-called cis-autoproteolysis, the active nucleophile attacks the carbonyl at its N-terminal side (−1 carbonyl) to start the processing. Activation of glycosylasparaginase is such a cis-autoproteolytic processing. Cis-autoproteolytic processing is not enzymatic but instead is a catalyzed chemical reaction. In contrast to proteolysis by serine/cysteine proteases, in cis-autoproteolysis, formation of the ester or thioester intermediate via an N-O or N-S shift is a reversible step. The released amino group cannot diffuse from the catalytic center and therefore is ready to attack the ester carbonyl to restore the peptide linkage. The equilibrium is favorable for peptide bond formation via an O-N or S-N shift (21, 22), and therefore no significant amounts of ester or thioester intermediates are accumulated in slow ester hydrolysis mutants. The overall rate of cis-autoproteolysis is determined not only by the N-O or N-S shift rate but also by the O-N or S-N reverse shift rate and the hydrolysis rate of the ester intermediate. The hydrolysis of the ester intermediate is the rate-limiting step. The overall processing follows first-order kinetics. The transitional tetrahedral intermediate engaged by cis-autoproteolysis is shared by many post-translational modification pathways in living cells such as autocleavage (23), autoproteolysis (13), protein splicing (14), and formation of oxazole and thiazole structures (24).

In this study, two residues, Arg-180 and Asp-183, were identified as being involved in binding of amino acids such as glycine to certain mutant precursors. The enzyme $K_m$ values in R180Q and D183N mutants were greatly increased (27). The three-dimensional structure analysis showed that two corresponding residues, Arg-211 and Asp-214, in human glycosylasparaginase were involved in binding of the α-carboxyl and the α-amino group, respectively, of the aspartic acid as product co-crystallized in the enzyme active center (8) (see Fig. 1A). Thus, our original suggestion that the amino acid binding site found on the precursor is the partially formed substrate binding pocket for the enzyme has now been proven (9).

Mutations at His-150/Asp-151/Thr-152 were able to produce amino acid-sensitive mutants (Figs. 2B, 3, and 5), suggesting that this processing site tripeptide is involved in sequestering the amino acid binding site on the wild-type precursor. Mutations at His-150, disregarding the size, charge, hydrophathy, and geometry of the replacement residues (Ser, Lys, or Trp), produced the same glycine-sensitive gene products, suggesting that His-150 may specifically interact with another residue, most likely Thr-152, to prevent free amino acids from access to...
the binding site rather than directly interfering with the amino acid binding. This is also consistent with the fact that His-150/Thr-152 is the active base/nucleophile pair for autoproteolysis.

For a hydroxyl or thiol side chain to attack the adjacent –1 carbonyl carbon, certain Ramachandran angles around the cleavage site dipeptide are required. Replacement of Asp-151 with a non-acidic residue other than glycine resulted in an inactive precursor because an incorrectly folded processing site may result. Pre-MG(D151G) could slowly undergo the autoproteolysis because a side chain-free residue, Gly-151, may fit into the correct processing site conformation with less free energy costs than other non-acidic residues. This is consistent with the fact that glycine residues are frequently found at –1 position in many similar bioprocessing systems (12–14, 24).

Among the seven conserved aspartate residues of glycosylasparaginase, only mutations at Asp-151 produced profound effects on activation (Fig. 4). The experimental data suggest that Asp-151 most likely plays a role in the activation similarly to the P1 residue of substrate in the reaction by trypsin, where the interaction between the P1 residue of substrate and the S1 site of the enzyme is the primary determinant in the enzyme-substrate binding. We proposed that Asp-151 side chain was anchored into the partially formed substrate pocket (the S1 site) by interacting with Arg-180. Possible interaction between the –1 residue and the substrate pocket was also suggested in an attempt to account for proenzyme activation in Ntn-hydrolases solely based on their unusual folding (5). But the experimental evidence was lacking. On the other hand, based on our hypothesis, the molecular mechanism for inhibition of the activation by amino acids can be well explained. Binding of an amino acid would interrupt the interaction between Asp-151 and Arg-180 and displace the side chain of Asp-151 from the pocket, and this would introduce conformation change and abolish the autoproteolysis. This hypothesis is further supported by the following facts. 1) Mutations at Arg-180 caused significant reductions in the processing rates and often resulted in misfolded precursors (25, 27), while mutations at Asp-183 did not profoundly affect the activation. 2) The partially formed substrate pocket on the wild precursor was sequestered by the processing site His-150/Asp-151/Thr-152 tripeptide. 3) Asp-151 was virtually required for activation. 4) Instead of inhibition of the activation, glycine actually stimulated the activation of pre-MG(D151G) (Fig. 5), which is very similar to the observation that methyamine inhibits trypsin hydrolysis of specific substrates and stimulates the enzyme activity toward nonspecific substrates (26). This also suggested that the charge interaction between Asp-151 and Arg-180 had a role in keeping the correct conformation of the precursor. As predicted, other amino acids were still able to inhibit the activation of pre-MG(D151G) as long as they could access the binding site.

The experimental data showed that the N-S shift rates of pre-MG(T182C) and pre-MG(T152C/T170A/C) were similar (Table I), which indicated that hydroxyl 170 was unlikely to take part in stabilization of the oxazin at the transitional state of autoproteolysis. Thus, the fact that the N-O shift rates in the mutants with hydroxyl residue 170 are significantly higher than in the mutants with non-hydroxyl residue 170 may suggest that hydrogen bonding between the two hydroxyl residues 152 and 170 also exists in the precursor and plays an important role to increase the nucleophilic activity of Thr-152. The N-S shift rates in the mutants with thiol residue 152 were independent of hydroxyl 170 because thiol group hydrogen bonds poorly.

Tackling all available experimental data together, we proposed a model for the interactions of functional side chains involved in glycosylasparaginase activation as illustrated in Fig. 6. In a properly folded precursor, the polypeptide chain at the processing site adopts a specific conformation that makes possible the functional interactions between active side chains and allows the occurrence of an efficient N-O shift between Asp-151 and Thr-152. Thr-152 is activated not only by deprotonation by His-150, but may also by interaction with Thr-170. The side chain of Asp-151 is anchored into the partially formed substrate pocket, possibly through a charge interaction with Arg-180. This interaction helps to stabilize the processing site conformation. Arg-180 and Asp-183 in the partially formed substrate pocket are involved in binding of free amino acids to sensitive mutant precursors. Binding an amino acid to this site will displace the Asp-151 side chain from the pocket and thereby block autoproteolysis. In the wild-type precursor, interaction between His-150 and Thr-152 prevents free amino acids from binding to this site. Because formation of the ester intermediate via an N-O shift is a reversible reaction, with the equilibrium favoring the peptide bond formation, an efficient ester hydrolysis step is essential for this cis-autoproteolysis.

Glycosylasparaginase activation provides a unique example that one catalytic center can be converted to another with different activity in a single protein through main chain modification and conformation change. X-ray three-dimensional structure analysis on the properly folded precursor should be able to reveal the detail conformation changes in this conversion.

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