Activation of Glycosylasparaginase

FORMATION OF ACTIVE N-TERMINAL THREONINE BY INTRAMOLECULAR AUTOPROTEOLYSIS*

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The activation mechanism of glycosylasparaginase of Flavobacterium meningosepticum has been analyzed by site-directed mutagenesis and activation of purified precursors in vitro. Mutation of Thr-152 to Ser or Cys leads to gene products that are not activated in vivo but are activated in vitro because processing of the mutant precursors is inhibited by certain amino acids in the cell. Kinetic studies reveal that activation is an intramolecular autoproteolytic process. The involvement of His-150 and Thr/Ser/Cys-152 in activation suggests that autoproteolysis resembles proteolysis by serine/cysteine proteases. Multiple functions of the highly conserved active threonine residue are implicated.

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 (4). This processing is an obligatory step in production of active enzyme and cleavage of a single peptide bond is responsible for this activation (5–7). The newly formed N-terminal threonine of the β-subunit plays an essential role in enzyme activity (8).

Aspartylglycosaminuria (AGU) (McKusick 20840), which is caused by a deficiency of glycosylasparaginase (9). The incidence of AGU is high in Finland; in eastern Finland the frequency of defective gene carriers was estimated to be about 1 in 40 (10). Most cases of AGU are caused by a mutation in the gene that results in a failure to activate the enzyme (7, 11). A closely related glycosylasparaginase occurs in Flavobacterium meningosepticum and its gene has been cloned into Escherichia coli. The cloned enzyme was efficiently processed and fully active (12, 13). Since the bacterial enzyme and its mammalian counterparts have the same enzymatic specificity, have similar heterodimer structures, are activated in the same way, cross-react with common antibodies, and share extensive similarities in their polypeptide sequences (12, 13), it is likely that their molecular mechanisms of activation are identical. However, little is known about the nature of the activation process. In this study we have used the cloned bacterial enzyme to investigate the mechanism of glycosylasparaginase activation.

EXPERIMENTAL PROCEDURES

Materials—Plasmids pMAL-c2 and pMAL-p2, E. coli host strain TB1, Factor Xa protease, amyllose resin, restriction enzymes, T4 DNA ligase, T4 DNA kinase, DNA polymerases, CDE 20 mutagenesis kit, and synthesized oligonucleotides, ATC directed mutagenesis (Kunkel method) were carried out as described by Sambrook et al. (14). The DNA encoding the mature glycosylasparaginase was cloned into the Xmn I and Hind III site of pMAL-p2 plasmid as described previously (15). The unique EcoRV site in the lacI gene on the pMAL-plasmids was removed by site-directed mutagenesis without changing the polypeptide sequence of LacI protein. The codon ACT for the threonine residue at position 152 of glycosylasparaginase was removed and a EcoRV site was generated by site-directed mutagenesis using the synthesized oligonucleotide GTA AAT ATC GAA AAC CAT GAT GAT GTG ATA GCC TTA GAT. The generated EcoRV site was used for inserting the codons for each of the other 19 amino acids using the CODE 20 kit as described by the manufacturer (New England Biolabs). The H150S mutation in the glycosylasparaginase gene was introduced by site-directed mutagenesis with the oligonucleotide GTA AAT ATC GAA AAC CAT GAT GAT GTG ATA GCC TTA GAT. All of the introduced mutations were confirmed by DNA sequence analysis.

Gene Expression and Protein Purification—Expression and purification of gene products using the maltose-binding protein (MBP) fusion and expression system were described by Riggs (15), with modifications. Briefly, E. coli strain TB1 harboring a pMAL plasmid carrying the MBP-glycosylasparaginase fusion was grown in LB medium to OD600 = 0.5 and induced with 0.5 or 1 mM isopropyl-1-thio-β-D-galactopyranoside for 4–6 h at either 25 °C or 30 °C. For expression of the MBP wild type protein fusion, buffered glycine was added to 50 mM at the same time as the isopropyl-1-thio-β-D-galactopyranoside. The cells were harvested by low speed centrifugation (3,000 x g, 10 min). The cell pellets were resuspended in 20 mM Tris, pH 7.6, 50 mM NaCl, 1 mM EDTA, 10 mM glycerine and lysed by sonication. The cell debris was removed by high speed centrifugation (20,000 x g, 20 min) and the clarified crude extracts were applied to an amylose column. The column was then washed, first with the sonication buffer, then with the same buffer without glycerine. The fusion protein was eluted with buffer containing 10 mM maltose. The purified protein was stored at ~70 °C until use. The whole purification process was carried out at 0–4 °C as quickly as possible.

For preparation of the nonfusion active mutant precursors of glycosylasparaginase, 10 mM glycerine was included in all of the extraction and purification buffers to inhibit autoproteolysis. The amylose-purified fusion protein was digested with 2% Factor Xa in the presence of 10 mM

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1 The abbreviations used are: AGU, aspartylglycosaminuria; MBP, maltose-binding protein; PAGE, polyacrylamide gel electrophoresis; DGNV, 5-diazo-4-oxo-L-norvaline; HA, hydroxylamine; IAA, iodoacetamide.
glycine for 1 h at 37°C. The nonfusion precursor was then separated from MBP and Factor Xa by FPLC with a Mono Q column. The glycine in the purified precursors was removed by dialysis against the reaction buffer (20 mM Tris, pH 7.6, 50 mM NaCl and 1 mM EDTA) with a Pierce Slide-A-Lyzer dialysis cassette, 4 changes in 60 min at 0°C.

In Vitro Autoproteolysis—Both amylase-purified fusion proteins and nonfusion precursors stored at −70°C were thawed and diluted in the ice cold reaction buffer at different concentrations, then shifted to an appropriate temperature to start autoproteolysis. At various times, an aliquot was withdrawn and subjected to SDS-PAGE. The protein gel was stained with Coomassie Blue R-250, and the protein bands were quantified by gel scanning using a Microtek Scanner III, Adobe Photoshop, and NIH Image 1.57.

Protein Sequencing—Amylose-purified fusion proteins were first incubated at 37°C to allow autoproteolysis. The treated samples were subjected to electrophoresis on precast 12% SDS-PAGE gels and stained with Coomassie Blue R-250. The gels were subjected to fluorography and analyzed for determination of the molecular weight of properly folded nonfusion precursors. Protein samples (0.2 ml, 0.3−1.0 mg) were applied to a Superose 12 column (1 × 30 cm, Pharmacia Biotech Inc.) equilibrated with 20 mM Tris, pH 7.6, 100 mM NaCl, 1 mM EDTA, with or without 10 mM glycine at 4°C. A calibration curve was prepared by measuring the elution volumes of standard proteins (Pharmacia low molecular weight Superose 12 column (1 cm × 30 cm) equilibrated with 20 mM Tris, pH 7.6, 50 mM NaCl, 1 mM EDTA, 4 changes in 60 min at 0°C).

For determination of the molecular weight of properly folded nonfusion precursors, Protein Sequencing—Amylose-purified MBP-mutant glycosylasparaginase (T152S) fusion protein, called MG(T152S), was diluted into the buffers with different pH values. For pH 4.0−7.5, 50 mM citric acid-Na2HPO4 buffers were used. For pH 6.0−10.0, 50 mM bis-Tris-propane buffers were used. The autoproteolysis reaction rates (k) at different pH values were compared by measuring the half-life (t1/2) of the fusion precursor pre-MG(T152S) at 37°C in the tested buffer. Half-lives were determined by gel scanning of SDS-PAGE gels as described above. For a first-order reaction, k = (ln 2)/t1/2.

To test inhibitors of autoproteolysis, amylose-purified MG(T152S) protein was diluted to a concentration of 5 μM in a reaction buffer containing an inhibitor (amino acids) at different concentrations. The inhibition constant K was determined by the equation:

\[ K = [I] / [E] = [E]^* \] (Eq. 1)

where [I] = the inhibitor concentration and [E] = the enzyme concentration. Let [E]^* and [E]^* equal the initial inhibitor and enzyme concentration, respectively. For a first-order reaction, the concentration of the inhibitor that doubles the half-life of the pre-MG(T152S) equals the inhibition constant K. Thus

\[ [E] = [E]^* = [E]^*/2 \] (Eq. 2)

\[ K = [I] = [I]^* − [E]^* \] (Eq. 3)

If [I]^* >> [E]^*, then K ≈ [I]^*.

Kinetic Analysis—The first-order reaction constant k was measured by plotting In(C0/Ct) versus time, where C0 and Ct are the quantities of proteins in the precursor bands at 0 time and at time t (s) as estimated by SDS-PAGE and gel scanning, the slope of the line is k. The relationship between k and the activation energy is given by:

\[ \ln k = - \Delta H^\ddagger/RT + \Delta S^\ddagger/RT + \ln A \] (Eq. 4)

where \( \Delta H^\ddagger \) is the activation enthalpy and \( \Delta S^\ddagger \) is the activation entropy of autoproteolysis. R is the gas constant, T is the absolute temperature, and ln A is the frequency factor and can be taken as a constant under the experimental conditions. Plotting k against 1/T (Arrhenius plots), the slopes of the linear plots provide an estimate of the activation enthalpies (−ΔH^\ddagger/RT).

\[ \ln k_1 - \ln k_2 = - (\Delta H^\ddagger_1 - \Delta H^\ddagger_2)/RT + (\Delta S^\ddagger_1 - \Delta S^\ddagger_2)/RT \] (Eq. 5)

\[ \Delta S^\ddagger = (ln k_1 - ln k_2)/R + \Delta H^\ddagger/RT \] (Eq. 6)

where k1 and k2 are the rate constants of the wild type and the mutant fusion precursors. \( \Delta S^\ddagger \) is the difference of activation entropies of autoproteolysis between the wild type and the mutant proteins.

Figure 1 shows the pattern of autoproteolysis of amylase-purified MG(T152X), 1, freshly purified MG(T152S); 2, MG(T152S) after incubation for 1 h at 37°C; 3, freshly purified MG(T152C); 4, MG(T152C) after incubation for 36 h at 37°C; 5, freshly purified MG(T152A); 6, MG(T152A) after incubation for 36 h at 37°C. All reactions were in buffer containing 20 mM Tris, pH 7.6, 50 mM NaCl, 1 mM EDTA. SDS-PAGE was performed in a 4–20% precast gradient gel. The free MBP is the result of proteolysis in vivo and copurifies with the MBP-glycosylasparaginase fusion protein.

Autoproteolysis Is an Intramolecular Event—To determine whether the activation of glycosylasparaginase was an autoproteolytic process, we further examined the kinetics in detail. The half-life of pre-MG(T152S) was about 18 min, irrespective of the starting concentration of pre-MG(T152S) in the range from 0.1 to 10 μg/ml, characteristic of a first-order process (Fig. 2). The half-life of the nonfusion precursor glycosylasparaginase T152S mutant, termed pre-G(T152S) (see below) was the same.
as that of the fusion pre-MG(T152S) under the same conditions, showing that the presence of MBP did not affect autoproteolysis (Fig. 3). First-order reaction kinetics show that the processing does not require a separate protease, but do not necessarily prove that the process is an intramolecular event unless it is known that the precursor exists as a monomer. Therefore, we measured the molecular masses of both nonfusion precursors pre-G(T152S) and pre-G(T152C) by gel filtration. The molecular masses of both pre-G(T152S) and pre-G(T152C) were estimated as 34 kDa, indicating that both precursors existed as monomers under the experimental conditions (data not shown). Combining all of the above experimental data, we conclude that the autoproteolysis of glycosylasparaginase is an intramolecular reaction.

Inhibition of Autoproteolysis by Amino Acids—The half-life of pre-MG(T152S) was about 40 min at 30°C in vitro. In contrast to this relatively rapid in vitro processing, when MG(T152S) was prepared from cells after induction for 4–6 h at 30°C the resulting protein consisted of more than 90% of unprocessed precursor, pre-MG(T152S). A possible explanation of this apparent discrepancy between the rates in vivo and in vitro is that the cells contained an inhibitor(s) of pre-MG(T152S). Indeed, we found that several amino acids inhibited the autoproteolysis of pre-MG(T152S). Glycine, threonine, serine, cysteine, and alanine at 1 mM all inhibited the autoproteolysis of pre-MG(T152S) by more than 50%. For glycine or threonine the inhibition constants ($K_i$) were estimated to be about 30–50 $\mu$M (Fig. 4). Autoproteolysis was not affected by 1 mM of D-amino acids such as D-threonine or D-alanine, or by 10 mM of acetic acid, D- or L-lactic acid, urea, and ammonium chloride. The serine protease inhibitor phenylmethylsulfonyl fluoride (1 mM), the cysteine protease inhibitor E-64 (10 mM), the metalloprotease inhibitor EDTA (10 mM), and the aspartic protease inhibitor pepstatin (10 $\mu$M) had no effect on autoproteolysis. Glycine or threonine also inhibited the autoproteolysis of pre-MG(T152C). By adding 10 mM glycine to the growth medium and during purification, we were able to isolate the nonfusion precursors, pre-G(T152S) and pre-G(T152C). Addition of glycine to the culture medium also slowed the maturation of wild-type fusion MG in the cell. Whereas the freshly purified MG from cells cultured in LB medium contained only MG, as judged by SDS-PAGE analysis, the freshly purified protein from cells cultured in LB plus 50 mM glycine contained about 20% of pre-MG (data not shown). After eliminating the inhibitor, this pre-MG-containing MG protein could be used for kinetic analysis as described below. The results described above indicate that this inhibition of processing is specific, reversible, and requires both free $\alpha$-amino and $\alpha$-carboxyl groups on the inhibitor molecule.

Nucleophile and Proton Acceptor-Donor—Insight into the mechanism by which the hydroxyl or thiol side chain of residue 152 participates in autoproteolysis came from an experiment that took advantage of the high reactivity of thioesters with hydroxylamine. When 250 mM hydroxylamine was included in the reaction buffer, the half-life of pre-G(T152C) at 37°C was dramatically shortened from about 48 h to about 20 min. This suggested that a stable thioester intermediate was generated during autoproteolysis. When pre-G(T152C) was first incubated with 10 mM iodoacetamide, a thiol-alkylation reagent, the processing of pre-MG(T152C) was greatly inhibited even in the presence of hydroxylamine. In a control experiment, the autoproteolysis of pre-G(T152S) was not affected by 10 mM.
iodoacetamide under the same conditions (Fig. 5). These results indicate that an initial step in autoproteolysis is an N-O or N-S acyl shift at residue 152 to yield an ester intermediate. The rate of autoproteolysis of pre-G(T152S) is not profoundly affected by including 250 mM hydroxylamine in reaction buffer. This indicates that either the N-O shift at residue 152 is the rate-limiting step of autoproteolysis or the hydrolysis rate and the aminolysis rate of the ester intermediate under the experiment conditions are about the same.

The amino acid sequence HDTIG surrounding the processing site of glycosylasparaginase is conserved from humans to bacteria (13). It is known that in the human enzyme only His-204 in the above sequence is involved in activation (6). We replaced the corresponding histidine residue (His-150) in the MBP-bacterial enzyme fusion with serine by site-directed mutagenesis and examined its expression in E. coli. The purified MBP-mutant fusion protein was a single polypeptide with a mass of 75 kDa and did not undergo autoproteolysis, even in the presence of 250 mM hydroxylamine (data not shown). This suggests that His-150 of the bacterial glycosylasparaginase, like His-204 of the human enzyme, is involved in autoproteolysis, most likely in the formation of the ester intermediate. Further evidence for the involvement of histidine in autoproteolysis came from examining the effect of pH on the autoproteolysis of pre-MG(T152S). Autoproteolysis was maximal between pH 6.0 and 7.5, while below pH 4.0 or above pH 9.0 it was not detectable. This pH dependence for the involvement of histidine in autoproteolysis came barely be detected. These results suggest that His-150 does not play the same role in enzymatic activity as in autoproteolysis.

Activation Energy for Autoproteolysis—To understand the reason for the differences in the rates of autoproteolysis between the wild type and the mutant proteins, we determined the activation energies for autoproteolysis of both pre-MG(T152S) and pre-MG, respectively. The reaction rates of autoproteolysis of pre-MG(T152S) were measured at temperatures ranging from 56 °C to 10 °C. The half-lives of pre-MG(T152S) varied from 2 min at 56 °C to 16–18 h at 10 °C. Higher temperatures, e.g., 65 °C, caused denaturation of pre-MG(T152S) and aborted autoproteolysis. The activation enthalpy calculated for autoproteolysis of pre-MG(T152S) is about 22 kcal/mol. We also measured the activation enthalpy for pre-MG using the pre-MG-containing MG protein described above. The half-lives for pre-MG varied from 3 min at 30 °C to 165 min at 0 °C. At 37 °C or higher the reaction for pre-MG took place so rapidly that reliable data could not be collected. The activation enthalpy for the autoproteolysis of pre-MG is calculated as 21 kcal/mol, very similar to the value obtained for pre-MG(T152S) under identical experimental conditions (Fig. 6). The 13-fold difference in the rates of autoproteolysis between pre-MG and pre-MG(T152S) at physiological temperatures must therefore reflect the difference in activation entropies between pre-MG and pre-MG(T152S). The difference in activation entropies between pre-MG and pre-MG(T152S) is about 5 cal/degree/mol at 25 °C, which is in good agreement with internal rotation entropies of groups such as -CH2- or -COOH, 3–5 cal/degree/mol (19). These data suggest that the chemical nature of autoproteolysis in pre-MG and pre-MG(T152S) is the same, but the side chains of reactive residues in pre-MG are mechanistically better positioned for the reaction than in pre-MG(T152S).

**DISCUSSION**

Non-protease zymogens are generally considered to be processed by proteases. But in this study we have demonstrated that the precursor of glycosylasparaginase is processed by a unique intramolecular autoproteolysis.

This study has identified His-150 and the hydroxyl or thiol group of residue 152 as being involved in autoproteolysis. The optimum pH range for autoproteolysis suggests that His-150 is the proton acceptor-donor base in the reaction. A thioester intermediate is likely formed by Cys-152 of pre-G(T152C) during autoproteolysis. This would indicate that the mechanism of autoproteolysis resembles proteolysis by serine or cysteine proteases. Since glycosylasparaginase does not possess protease activity per se, the activation cannot be an autocatalytic process. It is a spontaneous intramolecular reaction with the key

![Fig. 5. Hydroxylamine (HA) effect on autoproteolysis of pre-G(T152C).](Image)

![Fig. 6. Measurement of activation energy for autoproteolysis by Arrhenius plots.](Image)
mechanistic characteristics of serine/cysteine proteases.

Based on the data presented here and in other published studies (5, 6), we propose the following model of autoproteolysis of glycosylasparaginase, illustrated in Fig. 7. When the newly synthesized polypeptide is secreted into the periplasm, the signal peptide is removed and the precursor is properly folded. The reactive hydroxyl of Thr-152 is deprotonated by His-150 (possibly mediated by a water molecule), and then launches a nucleophilic attack in cis on the α-carbonyl carbon of Asp-151 to form a transitional tetrahedral intermediate associated with a five-member ring structure. After a proton is transferred to the leaving amino group of Thr-152, the α-carbonyl of Asp-151 is shifted to the hydroxyl of Thr-152 leading to the ester intermediate via an N-O shift (20). The final step is hydrolysis of the ester by water. During this maturation process, we assume that residues close to the cleavage site or other places are reoriented to give the final structure of the active site and the correct conformation of the mature enzyme.

Even though the above model is consistent with the experimental results so far obtained, many questions remain to be answered. What is the biological significance of the apparent amino acid binding site of the precursor associated with inhibition of autoproteolysis? Residue 152 at the processing site is the active residue for both autoproteolysis and enzyme activity. Changing Thr-152 to Ser dramatically increases the inhibition of autoproteolysis by certain amino acids such as glycine. The mature enzyme is also inhibited by certain amino acids such as aspartic acid and the asparagine analog 5-diazo-4-oxo-L-norvaline (DONV). The K, of glycine for autoproteolysis of pre-MG(Thr152S), the K, of the natural substrate Asn-GlcNAc, and the K, of DONV for glycosylasparaginase activity are similar (30–100 μM) (8). Thus, the simplest explanation is that the amino acid binding site of the precursor is the partly formed substrate binding site in the mature enzyme, which implies that the processing site and the enzyme active center are located in the same area of the protein.

Why is the active threonine residue conserved from humans to bacteria? Activation of the active mutants, but not the wild type enzyme, is inhibited in vivo. One explanation is that the methyl group of Thr-152 interacts with another part of the protein and prevents inhibitors such as amino acids from accessing and binding to the partly formed substrate site and thus interfering with autoproteolysis. By the same token we can also interpret the data from the activation energies of autoproteolysis of the wild type Thr-152 and the T152S mutant. The methyl group of Thr-152, through an as-yet- unidentified interaction, could prevent free rotation about the Ca-Cβ bond of Thr-152 and place the reactive hydroxyl in a proper orientation for deprotonation and subsequent nucleophilic attack, thus reducing the activation entropy. The activation energy for shifting the α-carbonyl of Asp-151 to the primary hydroxyl of Ser-152 or to the secondary hydroxyl group of Thr-152 should be similar. These potential multiple functions of the methyl group of Thr-152 might explain why this active threonine is evolutionarily conserved among different glycoasparaginases, and why the native enzyme is more active than the serine mutant.

In human glycosylasparaginase, no histidine residues are involved in enzyme activity (6). The pH dependence of glycosylasparaginase activity is not consistent with histidine as a proton acceptor-donor. Therefore, it is very likely that glycosylasparaginase, like the β-subunits of proteasomes or the E. coli penicillin acylase, is also a single-amino acid catalytic center enzyme and the newly generated α-amino group of the N-terminal Thr of β-subunit becomes the proton acceptor-donor for enzyme activity (21, 22). Another intriguing question is what are the actual conformational changes in the protein resulting from activation that convert the the protein to glycosylasparaginase. X-ray structural analysis on both the properly folded precursor and the mature enzyme will be essential to answer these questions.

The mutations of human AGUs so far found which result in a deficiency of activation are not located at the processing site. This suggests that proper folding and the correct conformation of the precursor play an indispensable role in activation.

Autoproteolytic processes play important roles in post-translational processing of gene products. For example, the Hedgehog proteins undergo a specific autoproteolysis to realize their biological function (23, 24). The precursors of the catalytic β-subunits of proteasomes may be activated by autoproteolysis after being assembled with the α-subunits into the proteasomes (25). Autoproteolytic cleavage also serves as a mechanistic component for protein splicing (20). Understanding the mechanism of this unique post-translational process in glycosylasparaginase may not only provide valuable information for studies related to AGU, it may also shed light on autoproteolytic activations of different gene products in different biological systems.

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Fig. 7. Model for autoproteolysis of glycosylasparaginase (see text).
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