

# A Dual Role for an Aspartic Acid in Glycosylasparaginase Autoproteolysis

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## Summary

Glycosylasparaginase uses an autoproteolytic processing mechanism, through an N-O acyl shift, to generate a mature/active enzyme from a single-chain precursor. Structures of glycosylasparaginase precursors in complex with a glycine inhibitor have revealed the backbone in the immediate vicinity of the scissile peptide bond to be in a distorted *trans* conformation, which is believed to be the driving force for the N-O acyl shift to break the peptide bond. Here we report the effects of point mutation D151N. In addition to the loss of the base essential in autoproteolysis, this mutation also eradicates the backbone distortion near the scissile peptide bond. Binding of the glycine inhibitor to the autoproteolytic site of the D151N mutant does not restore the backbone distortion. Therefore, Asp151 plays a dual role, acting as the general base to activate the nucleophile and holding the distorted *trans* conformation that is critical for initiating an N-O acyl shift.

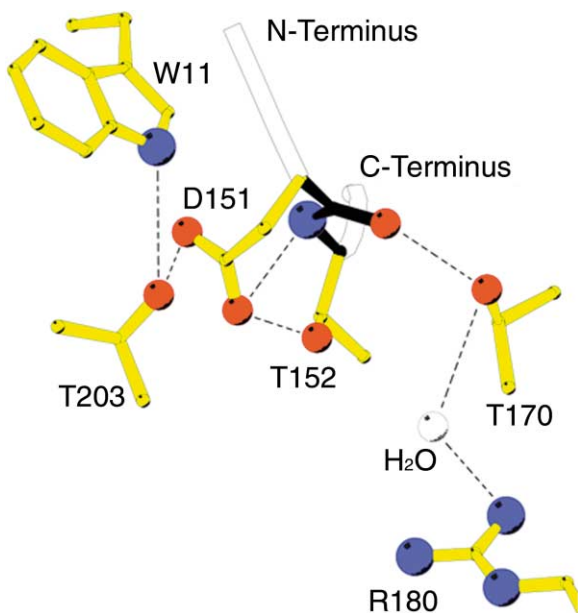
## Introduction

Autoproteolysis is one type of the protein-autoprocessing events that have emerged as novel mechanisms of posttranslational modifications to activate their biological activities. In most cases, it requires neither cofactors nor auxiliary enzymes. The reaction is initiated by a nucleophilic attack of a threonine, serine, or cysteine residue at the scissile peptide bond, leading to an N-O or N-S acyl rearrangement of the peptide bond (acyl shift). A diverse group of proteins also utilizes such an N-O or N-S acyl shift to undergo various types of self-catalyzed rearrangements, ranging from protein splicing to autocleavage of a peptide bond (Perler, 1998; Paulus, 2000). Intramolecular proteolysis, one such autoprocessing event, is utilized to cleave precursors and activate biological functions of various proteins, including an enzyme family called the N-terminal nucleophile (Ntn) hydrolases (Brannigan et al., 1995; Schmidtke et al., 1996; Guan et al., 1996). Glycosylasparaginase (GA) is a member of the Ntn family that catalytically uses an autoprocessed N-terminal threonine, serine, or cysteine as both a polarizing base and a nucleophile (Aronson,

1996). Without autoproteolysis, the GA precursor is inactive in cleaving the  $\beta$ -N-aspartylglucosylamine bond of asparagine-linked glycoproteins, resulting in a human genetic disorder known as aspartylglucosaminuria (AGU) (Mononen et al., 1993; Aronson, 1999). Autoproteolysis of GA occurs between evolutionarily conserved Asp151 and Thr152 to generate a newly exposed N-terminal threonine, which plays central roles in both enzyme activity (Karttinen et al., 1991; Tarentino et al., 1995; Liu et al., 1998) and autoproteolysis (Guan et al., 1996, 1998).

N-O and N-S acyl shifts occur adjacent to threonine, serine, or cysteine residues in polypeptides through a nucleophilic attack by the amino acid side chain at the carbonyl carbon of the scissile peptide bond, resulting in a (thio)ester bond (Albert et al., 1998; Ekstrom et al., 2001). From the (thio)ester intermediate, a diverse group of proteins undergoes various types of self-catalyzed rearrangements, by attacking the intermediate with a second nucleophile, resulting in cleavage of the linkage between the threonine, serine, or cysteine and the preceding amino acid. Usually the equilibrium of an N-O or N-S acyl shift favors the amide bond, resulting in a peptide bond that does not break often at threonine, serine, or cysteine. The fact that some proteins can self-process at these scissile peptide bonds suggests that these are not typical peptide bonds. This notion has been supported by our crystallographic analyses of autoproteolytically active GA precursors in complex with a reversible glycine inhibitor, which showed that the backbone in the immediate vicinity of the scissile peptide bond is in distorted *trans* conformation (Xu et al., 1999). Thus, the distorted conformations with higher energy than a regular peptide bond could drive the equilibrium toward the formation of an ester bond. Recently, both scissile peptide bonds involved in protein splicing of PI-Scel have also been found to be in distorted *trans* conformations (Poland et al., 2000). Some other conformational constraints have also been reported in precursors of other autoprocessing proteins (Ditzel et al., 1998; Klambunde et al., 1998). Similar to GA autoproteolysis, relieving the distorted/strained main chain backbone is believed to help in driving an N-O or N-S acyl shift in these precursors. With the help of such an unusual tight turn in the GA precursor, one carboxylate oxygen of Asp151 loops back via a six-member ring to form a hydrogen bond with the immediate downstream backbone amide nitrogen and forms a second hydrogen bond with the hydroxyl oxygen of Thr152 (Figure 1). On the basis of distances and orientations, the carboxylate group of Asp151 has been proposed to act as the general base and deprotonate the hydroxyl oxygen of Thr152 to enhance its nucleophilicity for initiating the N-O acyl shift (Xu et al., 1999). To investigate the roles of Asp151 in GA autoproteolysis and to determine whether the glycine inhibitor is capable of generating the polypeptide chain distortion, we carried out biochemical and crystallographic studies of a D151N mutation, which is essentially a substitution of a hydroxyl group with an amine group. In addition to the loss of the base essential in autoproteo-

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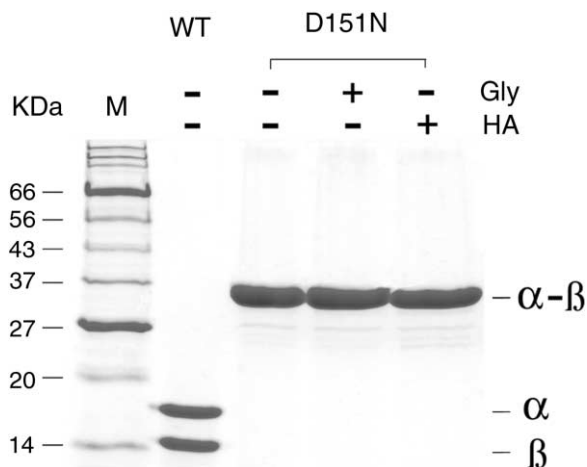
**Figure 1. Autoproteolytic Site Model of GA Wild-Type Precursor**  
Only side chain nonhydrogen atoms of the autoproteolytic site are shown, with the exception of main chain atoms near the scissile peptide bond. Bonds for side chains and main chains are colored yellow and black, respectively. Atoms are colored by type: yellow, carbons; blue, nitrogens; red, oxygens; open circle, putative water molecule. Main chain traces juxtaposing the scissile peptide bond are shown by transparent tubes, with N and C termini labeled. Hydrogen bond interactions are indicated by dotted lines (see text). This figure was made with the program MOLSCRIPT (Kraulis, 1991) and is based on information in Xu et al. (1999).

teolysis, this mutation also eradicates the backbone distortion near the scissile peptide bond. Binding of a glycine inhibitor to the autoproteolytic site of the D151N mutant does not restore the backbone distortion. Therefore, Asp151 plays a dual role, acting as the general base to activate the nucleophile and holding the distorted *trans* conformation that is critical for initiating an N-O acyl shift.

## Results and Discussion

### Loss of Autoproteolytic Activity in the D151N Precursor

The D151N mutation completely abolishes autoproteolysis. As shown in Figure 2, the wild-type GA autoproteolyzes spontaneously into two peptide fragments. In contrast, no autoproteolytic activity was detected for the D151N mutant, confirming a critical role of the Asp151 in GA autoproteolysis. This is likely due to a loss of the critical base required to activate the nucleophile Thr152. Interestingly, certain small free amino acids, such as glycine, can act either as a reversible inhibitor of autoproteolysis for some GA mutants or as an autoproteolysis accelerator for other GA mutants (Guan et al., 1996, 1998). We have previously proposed that glycine inhibits autoproteolysis by blocking a charge relay system that is critical to stabilize an oxyanion intermediate during the N-O acyl shift (Xu et al., 1999). Incubation of the



**Figure 2. SDS-PAGE Analysis of Autoproteolysis of Wild-Type GA and the D151N Mutant**

Wild-type GA (WT) is freshly purified and had spontaneously undergone autoproteolysis into two subunits ( $\alpha$  and  $\beta$ ). Purified D151N precursor samples were further incubated in the absence (-) or presence (+) of 10 mM glycine (Gly) or 500 mM hydroxylamine (HA) and then separated on a 15% SDS-polyacrylamide gel. Lane M is a mixture of molecular weight markers. The precursor ( $\alpha$ - $\beta$ ) and autoproteolyzed subunits ( $\alpha$  and  $\beta$ ) are marked on the right.

D151N mutant in the presence of 10 mM glycine does not alter the rate of autoproteolysis (Figure 2). It has also been shown that a strong nucleophile hydroxylamine ( $\text{NH}_2\text{OH}$ ), which is highly reactive with an ester or thioester, can increase the rate of autoproteolysis (Guan et al., 1996). Nonetheless, incubation of the D151N mutant precursor in the presence of 500 mM  $\text{NH}_2\text{OH}$  does not promote autoproteolysis (Figure 2). These results indicate that the N-O acyl shift does not occur in the D151N mutant precursor, and, thus, there is no accumulation of ester intermediate to be resolved by hydroxylamine.

### Structural Effects of the D151N Mutation

To investigate the structural effects of the D151N mutation, we have determined two crystal structures of the D151N precursor at 1.9 Å resolution, in the absence or presence of a glycine inhibitor (Table 1). Apart from differences in the autoproteolytic centers, where a single residue was changed (see below), the D151N precursor structures are essentially identical to other autoproteolytically active precursors, such as the T152C precursor (Xu et al., 1999). The root-mean-square deviation (rmsd) between the D151N and T152C precursors is 0.25 Å for all main chain atoms of 275 residues. Like other GA structures, the D151N precursor forms a four-layer  $\alpha$ - $\beta$ - $\beta$ - $\alpha$  structure (Oinonen et al., 1995; Guo et al., 1998; Xuan et al., 1998; Xu et al., 1999; Oinonen and Rouvinen, 2000). In addition, dimerization has been previously demonstrated to be a prerequisite to trigger autoproteolysis, presumably by creating structural constraints near the scissile peptide bond (Wang and Guo, 2003). In the crystals, the D151N precursor also forms a dimer that is similar to the dimer structure of other active precursors and autoproteolyzed enzyme (Guo et al., 1998; Xu et al., 1999).

Table 1. Crystallographic Statistics of the D151N Precursor Protein

	Without Glycine	With Glycine
<b>Data Collection</b>		
Space group	P1	P1
Cell axes (Å)	46.2, 52.7, 61.9	45.9, 52.4, 61.7
Cell angles (°)	80.9, 90.2, 105.1	80.9, 90.4, 105.1
Resolution range (Å)	20–1.9	20–1.9
Reflection (total/unique)	80,492/43,700	81,010/41,067
R <sub>sym</sub> <sup>a</sup> (final shell)	0.02 (0.04)	0.03 (0.08)
Completeness (%), overall (final shell)	95.1 (95.2)	95.3 (95.3)
I/σ <sub>1</sub>	28.8	24.9
<b>Refinement</b>		
<b>Nonhydrogen atoms</b>		
Protein <sup>b</sup>	4480	4479
Water	216	212
R <sub>cryst</sub> (%) <sup>c</sup>	18.19	19.28
R <sub>free</sub> (%) <sup>d</sup>	21.98	22.54
<b>Rms deviations<sup>e</sup></b>		
Bond length (Å)	0.008	0.006
Bond angles (°)	1.4	1.4
NCS (Å)	0.37	0.44
<b>Average B value (Å<sup>2</sup>)</b>		
Main chain	11.0	11.1
Side chain	15.3	16.1
Water	15.7	16.1
<b>Ramachandran plot (%)</b>		
Most favored	91.9	91.3
Additional	8.1	8.7
Disallowed	0.0	0.0

<sup>a</sup>  $R_{sym} = \sum_h \sum_i |I_{hi} - \bar{I}_h| / \sum_h \sum_i I_{hi}$  for the intensity (I) of i observations of reflection h.

<sup>b</sup> The number of atoms includes both molecules in the asymmetric unit.

<sup>c</sup>  $R_{cryst} = \sum |F_{obs} - F_{calc}| / \sum |F_{obs}|$ , where  $F_{obs}$  and  $F_{calc}$  are the observed and calculated structure factor amplitudes, respectively.

<sup>d</sup> R<sub>free</sub> was calculated as R<sub>cryst</sub>, but with 10% of the amplitudes chosen randomly and omitted from the start of refinement.

<sup>e</sup> Rms deviations are deviations from ideal geometry, or deviations for all main chain atoms between the two molecules in the asymmetric unit.

However, unlike in other active precursors, no backbone distortion was found in the D151N precursor near the defective scissile peptide bond that would have been autoproteolyzed in an active precursor. In the autoproteolytic site of a single-chain precursor, the scissile peptide bond is located in a surface loop (P loop for precursor) of 15 residues. Figure 3A shows the electron density map at the autoproteolytic site of the D151N precursor in the absence of glycine inhibitor. Interestingly, the small pocket that binds the glycine inhibitor in other precursor plus glycine complexes is occupied by a glycerol molecule (used as cryoprotectant for cryocrystallographic data collection) through hydrogen bond interactions with Arg180 and Gly206. While the surrounding active site residues of the inactive D151N precursor closely superimposed on those of the active T152C precursor (rmsd of 0.26 Å for main chain atoms of six active site residues; see Experimental Procedures), the P loop that comprises the defective scissile peptide bond has undergone significant conformational rearrangements (Figure 4A). The most significant differences between these two structures are in Asp/Asn151 and His150, where main chain atoms have shifted by more than 4 Å and side chain atoms have moved by as much as 12 Å at His150. These rearrangements apparently relieve the backbone distortion observed in other active precursors. The planarity of the peptide bond ( $\omega$

value) between residues 152 and 153 is near ideal (176°) in the D151N precursor, compared to 159° (deviated more than 3 standard deviations from ideality of 180°) in the T152C precursor. Similarly, the backbone angle N-C $\alpha$ -C' ( $\tau$ ) of residue 151 is near ideality of 110° (109°) in the D151N precursor, whereas the same backbone angle deviates from ideality by more than 2 standard deviations ( $\sim$ 121°) in the T152C structure. As a result, in the D151N precursor, the backbone near the defective scissile peptide bond has become a regular *trans* peptide bond. Thus, in addition to the loss of the critical base, elimination of such backbone distortion in the D151N precursor is likely to be another factor for the loss of the autoproteolytic activity.

In addition to the D151N substitution and backbone relaxation near the defective scissile peptide bond, the geometry and chemistry of other functional groups involved in initiating the nucleophilic attack of the N-O acyl shift is also altered significantly. Coincident with the backbone rearrangements, a hydrogen bond between the side chains of residues 151 and 203 is lost in the D151N precursor. Similarly, the side chain contact between Asp151 and Thr152, which has been proposed to deprotonate nucleophile Thr152, is also lost. As a result, the hydroxyl group of Thr152 moves away from the target of the presumed nucleophilic attack, the immediate upstream backbone carbonyl of the defective

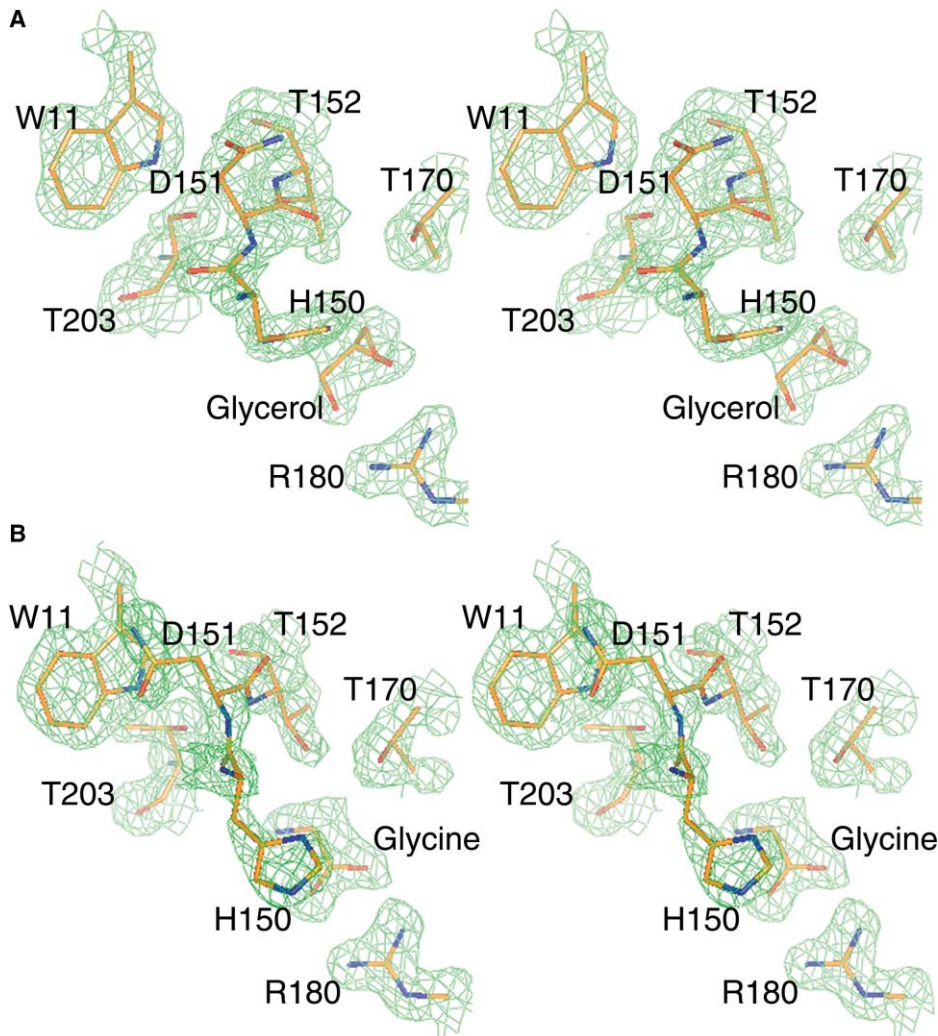


Figure 3. Electron Density Maps at the Autoproteolytic Sites of the D151N Precursor

(A) Stereo view of the electron density map of the D151N precursor in the absence of free glycine inhibitor. The cyan maps are of the  $2F_o - F_c$  type at 1.9 Å resolution, within 2.3 Å of the atomic model, and contoured at 1.2  $\sigma$ . The active site residues in the final model are colored by atom type: orange, carbons; blue, nitrogens; red, oxygens. Also shown is a glycerol (one of two alternative conformations) bound in the active site.

(B) Stereo view of the electron density map of the D151N precursor in the presence of free glycine inhibitor. Also shown is a glycine inhibitor bound in the active site. Color-coding is the same as in (A). These figures were made with the program Pymol (Delano Scientific) and rendered with Adobe Photoshop.

scissile peptide bond, to a distance of 4.3 Å (compared to 3.1 Å in other active precursors) and an orientation not suitable for a nucleophilic attack. Furthermore, the contact to stabilize the oxyanion intermediate of the N-O acyl shift, a hydrogen bond between the carbonyl oxygen of Asp151 and the hydroxyl oxygen of Thr170 (Figure 1), is also broken in the D151N precursor. Thus, in the D151N precursor, both the loss of the nucleophile activation by the base Asp151 and the loss of the unusual, but precise, geometries to initiate a nucleophilic attack of the N-O acyl shift, contributes to the loss of autoproteolytic activity. One interesting question is the role of the conserved His150 in GA autoproteolysis. This conserved histidine was previously proposed to act as the base (Guan et al., 1998), through a mechanism similar to serine proteases (Barrett and Rawlings, 1995).

Interestingly, in the D151N precursor, His150 has flipped to the other side of the scissile peptide bond, with the side chain atoms displaced by as much as 12 Å (Figure 4A). Nonetheless, like in other GA precursor structures, it is not positioned with appropriate geometry to be the general base. Consistent with these structural analyses, mutational studies of the conserved His150 demonstrate that this histidine is not the base to activate the nucleophile and plays only a structural role to stabilize the backbone distortion in GA.

#### Effects of Glycine on the Structure of the D151N Mutant

To address the question of whether the glycine inhibitor is the cause of the backbone distortion previously observed in active precursor plus glycine complexes, we

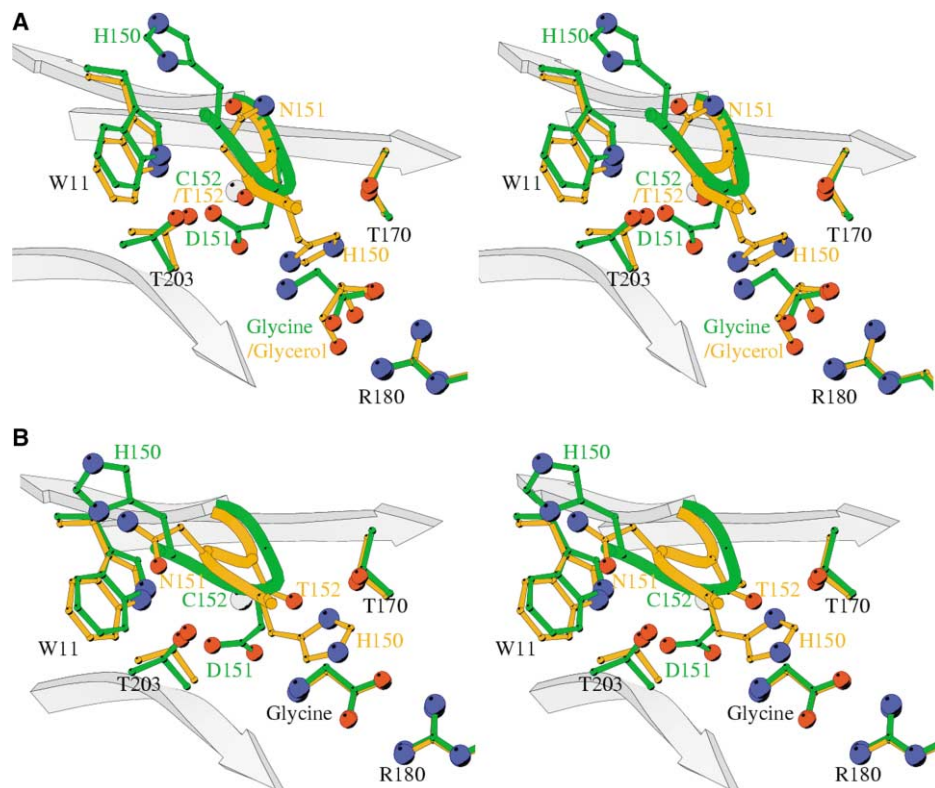


Figure 4. Comparison of the Active Site Residues between the D151N and T152C Precursors

(A) Stereo view of the superimposition of the active site residues in the D151N (orange; without glycine) and the T152C (green; with glycine) precursors. Side chain atoms of GA are shown as ball and stick models, and main chain traces between His150 and Thr/Cys152 are shown as tubes. Also shown are a glycerol and a glycine bound to the D151N (orange) and the T152C (green) precursors, respectively. Atoms are colored by type: orange and green, carbons of D151N and T152C, respectively; blue, nitrogens; red, oxygens; gray, sulfur.

(B) Stereo view of the superimposition of the active site residues in the D151N (orange; with glycine) and T152C (green; with glycine) precursors. Color-coding is the same as in (A). These figures were made with the program MOLSCRIPT (Kraulis, 1991).

have determined and analyzed a second crystal structure of the D151N precursor in complex with a glycine molecule. In the D151N plus glycine structure, a free glycine binds to the small pocket in the autoproteolytic site through the same interactions as in other precursor plus glycine structures (Xu et al., 1999). The electron density map showing the glycine molecule and surrounding residues at the autoproteolytic center of the D151N precursor is displayed in Figure 3B. Like in other precursor plus glycine complexes, the  $\alpha$ -amino group of glycine makes hydrogen bonds with Asp183 and Gly204, and the  $\alpha$ -carboxylate of glycine forms salt bridges with Arg180 (Figure 4B). But, unlike in other precursor plus glycine complexes, no backbone distortion was found near the defective scissile peptide bond of the D151N precursor. Thus, binding of glycine at the autoproteolytic site does not cause a backbone distortion. Instead, the distorted *trans* peptide configuration is an intrinsic property of an autoproteolytically active GA precursor.

Binding of glycine does, however, result in small adjustments of side chain conformations at the active site. Both His150 and Asn151 change their side chain rotamer conformations upon binding of glycine (Figure 4). Furthermore, the hydroxyl group of Thr152 is rotated back to an orientation similar to the nucleophile in other active precursors, forming a hydrogen bond with the  $O_{\gamma}$  of

Thr170. However, in the D151N plus glycine precursor, the hydroxyl group of Thr152 is still not in a precise geometry suitable for a nucleophilic attack on the carbonyl carbon of the defective scissile peptide bond because of the backbone relaxation. Therefore, a combination of the base-eliminating D151N mutation and the loss of backbone distortion prevent a productive nucleophilic attack for the N-O acyl shift. Similar to the D151N mutation, a D151G mutation leads to dramatic reduction in the rate of autoproteolysis, with a rate estimated to be  $3 \times 10^{-5}$  of that of the wild-type. Interestingly, this low autoprocessing rate of the D151G mutant can be stimulated slightly (about 2-fold) in the presence of 10 mM glycine (Guan et al., 1998). One intriguing explanation is that, like in the D151N plus glycine complex, glycine helps the hydroxyl group of Thr152 achieve a better orientation for the residual nucleophilic attack.

#### A Dual Role for the Asp151 in GA Autoproteolysis

The significance of Asp151 in GA autoproteolysis is 2-fold. First, it acts as the general base to deprotonate the hydroxyl oxygen of Thr152 to enhance its nucleophilicity for an N-O acyl shift. Of thirteen different substitutions at the Asp151 (D151G/A/V/L/M/P/N/Q/S/T/H/R/E), all but two mutants (D151E and D151G) are inactive in autoproteolysis (Guan et al., 1998). The autoprocessing

rate of the D151E mutant is reduced by about 1000-fold, whereas the D151G mutant had its rate reduced by five orders of magnitude. Incubation of these mutant precursors with  $\text{NH}_2\text{OH}$  does not significantly promote autoproteolysis, indicating that the loss of autoproteolytic activity is due to the lack of the initial step of an N-O acyl shift to produce an ester intermediate. Taken together, these results indicate that Asp151 is essential to initiate the N-O acyl shift for autoproteolysis. Second, Asp151 holds the backbone near the scissile peptide bond in a highly distorted *trans* configuration, by interacting and forming two hydrogen bonds with Thr152 and Thr203, as well as looping back via a six-member ring to form a third hydrogen bond with the amide nitrogen of the scissile peptide bond (Figure 1). Point mutations that selectively eliminate one of these hydrogen bonds (such as the W11F or T152C precursor described in Xu et al. [1999]) still can retain a backbone distortion near the scissile peptide bonds. However, the D151N mutation results in the loss of all three hydrogen bonds and causes the relaxation of the backbone near the defective scissile peptide bond into regular *trans* peptide bond configurations. In the W11F precursor structure, a backbone distortion is held by one carboxylate oxygen of Asp151 that forms two hydrogen bonds with the amide nitrogen of the scissile peptide bond and the hydroxyl oxygen of Thr152. Molecular modeling suggests that, in the D151N precursor, it is plausible to form such a hydrogen bond pattern via the carbonyl oxygen of the side chain of Asn151. Furthermore, with some adjustments of proton orientation, it also appears plausible to form a third hydrogen bond between the amine nitrogen of Asn151 and the hydroxyl oxygen of Thr203. However, in the crystal structures of the D151N precursor, none of these speculative hydrogen bond interactions occurs, suggesting that the precise chemistry and geometry provided by the side chain of Asp151 are necessary to form these critical hydrogen bonds and hold the backbone near the scissile peptide bond in the distorted *trans* conformation. It is thus interesting to note that such an aspartic acid immediately upstream of the scissile peptide bond, conserved in GA, is not found in other autoproteolytic proteins, such as proteasomes and nucleoporin Nup98 homologs (Schmidtke et al., 1996; Ditzel et al., 1998; Rosenblum and Blobel, 1999; Teixeira et al., 1999). A conserved glycine and phenylalanine are found at the corresponding position of the  $\beta$  subunit of the 20S proteasome and Nup98, respectively. Further studies are necessary to reveal the mechanistic similarities and differences in autoproducting of these autoproteolytic proteins.

### Conclusions

Asp151 is critical in GA autoproteolysis, by playing both catalytic and structural roles. It acts as the general base to activate the nucleophile and holds the backbone near the scissile peptide bond in the distorted *trans* conformation to drive the N-O acyl shift toward an ester linkage. In the D151N mutant precursor, both the lack of a general base and the relaxation of the backbone account for the complete elimination of autoproteolytic activity. Binding of glycine to the D151N precursor does

not restore the distorted backbone conformation. Thus, the structural constraints observed in other autoproteolytically active precursor plus glycine complexes are intrinsic properties maintained by the Asp151.

### Experimental Procedures

#### Autoproteolytic Reactions

Protein expression and purification for autoproteolytic reactions and crystallization were carried out as described previously (Cui et al., 1999). Autoproteolysis reactions of the D151N precursor were performed in a buffer of 40 mM potassium-phosphate (pH 7.4), 50 mM NaCl, and 1 mM EDTA, incubated for 2 hr at 37°C. When appropriate, 10 mM glycine or 500 mM hydroxylamine ( $\text{NH}_2\text{OH}$ ) was added into the reaction buffer. Reaction samples were denatured in 2.5% SDS and 1%  $\beta$ -mercaptoethanol at 90°C for 10 min and then analyzed by SDS-PAGE. The gels were stained with Coomassie blue.

#### Crystallization

Protein crystals of the D151N mutant were grown as described previously for GA precursors (Cui et al., 1999), in the absence or the presence of 50 mM glycine.

#### Data Collection and Processing

Oscillation data were collected with crystals flash-cooled at 100 K with synchrotron X-radiation (beamlines X-4a and X-12c at the National Synchrotron Light Source at the Brookhaven National Laboratory). All intensity data were processed and scaled with the HKL suite (Otwinowski and Minor, 1997) and converted to structure factors with TRUNCATE from the CCP4 software package (CCP4, 1994). The statistics of diffraction data are shown in Table 1.

#### Structure Determination and Refinement

The structures were determined by molecular replacement (MR) methods. To avoid model bias, we used the starting model of the T152C precursor structure (Xu et al., 1999) with a small part omitted at a time (in both noncrystallographic symmetry [NCS]-related molecules; 5%–10% of the structure) in the MR phasing. These partial models were further subjected to simulated annealing before being used to calculate MR phases. Real-space averaging yielded high-quality electron density maps, and the model for the omitted portion was then built into the density. This procedure, with one region omitted at a time, was repeated throughout the entire structure.

In the first round, 173 out of 295 residues were built on the basis of DM (density modification)-modified omit maps. Automated refinement included rigid body, overall temperature factor, positional, and restrained atomic temperature factor, as well as simulated annealing with a slow-cooling protocol in CNS (Brunger et al., 1998). After the first round of manual rebuilding, the structure was used for independent model rebuilding and refinement of the two structures (without glycine and with glycine). During refinements, tight NCS restraints were applied, exclusive of residues that were involved in crystal contacts or in nonstructured loops. After a few rounds of model rebuilding and automated refinement, clear electron density could be seen for all residues in the final models. Continuous electron density for the main chain and most of the side chains of the P loop was evident, despite the exposed nature of the loop and the relatively high B factors (main chain average from residue 141 to 151 is 33.7 Å<sup>2</sup>; overall main chain average is 11.1 Å<sup>2</sup>). In the structure without glycine, two alternative conformations of glycerol were built in the active site, as well as two slightly different P loop traces for the two NCS-related molecules, but only one conformation is shown in Figures 3A and 4A. When the  $R_{\text{free}}$  appeared to have reached a minimum, water molecules were added and the structure was subjected to another round of temperature factor and positional refinement and manual rebuilding. The statistics of the final structures are shown in Table 1.

All superimpositions of different structures were performed with LSQKAB (CCP4, 1994). In Figure 4, main chain atoms of Trp11, Thr170, Arg180, Asp183, Thr203, and Gly204 are superimposed.

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## Accession Numbers

Atomic coordinates have been deposited in the Protein Data Bank with accession codes 1p4k (without glycine) and 1p4v (with glycine).