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Purification and crystallization of precursors and autoprocessed enzymes of *Flavobacterium* glycosylasparaginase: an N-terminal nucleophile hydrolase

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Glycosylasparaginase (GA) represents a novel group of proteins that are activated by self-catalyzed peptide-bond cleavage from a single-chain precursor to yield the two subunits required for hydrolase activity. The wild-type GA precursor autoproteolyzes spontaneously into α and β subunits. Strategies are reported here for purification to homogeneity of GA from *Flavobacterium meningosepticum* in both single-chain precursor and mature (autoprocessed) forms. The recombinant proteins crystallize in different space groups: $P1$ and $P2_1$ for the precursor and mature enzymes, respectively. The precursor crystals diffract to 1.9 Å resolution with laboratory X-ray radiation.

1. Introduction

Lysosomal glycosylasparaginase (GA) hydrolyzes the amide bond joining carbohydrate to protein in Asn-linked glycoproteins. This enzyme belongs to a novel class of hydrolases which use a processed N-terminal threonine, serine or cysteine as both a polarizing base and a nucleophile in catalysis (Brannigan *et al.*, 1995). In humans, a deficiency of GA leads to the disease aspartylglycosaminuria (AGU; Mononen *et al.*, 1993). AGU is the most common genetic disorder of glycoprotein degradation. GA effectively hydrolyzes both complex-type and high-mannose glycoasparagines, including those isolated from the urine of AGU patients (Kaartinen *et al.*, 1992). Lack of GA activity results in accumulation of glycoasparagines in tissue lysosomes, with severe clinical symptoms.

Another intriguing aspect of this enzyme is that a single-chain precursor is processed by intramolecular proteolysis to generate the newly exposed N-terminal threonine and an active hydrolase with α and β subunits (Guan *et al.*, 1996). Cleavage is essential for GA hydrolase activity and occurs at the amide bond in the consensus site between residues Asp151 and Thr152 (Ikonen *et al.*, 1993; Fisher *et al.*, 1993; Tarentino *et al.*, 1995). This conserved threonine is thought to play central roles in both hydrolase activity (Kaartinen *et al.*, 1991; Fisher *et al.*, 1993) and autoproteolysis (Guan *et al.*, 1996). Like other recently discovered protein autoprocessing pathways, GA self-catalyzes peptide-bond rearrangement through an N→O or N→S acyl shift (Perler, 1998*a*; Paulus, 1998). These proteins utilize the side chain of cysteine, serine or threonine as a nucleophile to activate the immediate upstream peptide bond and to substitute the peptide amide bond with a more reactive (thio)ester bond. These N→O or N→S acyl shifts initiate diverse autoprocessing pathways, including autoproteolysis, protein splicing, the addition of prosthetic groups and enzyme activation (Perler, 1998*b*; Paulus, 1998).

The atomic details of autoprocessing reactions *via* an N→O or N→S acyl shift have yet to be determined. The crystal structure of the GA precursor will provide a structural basis for examining the detailed mechanisms of autoprocessing reactions *via* an N→O or N→S acyl shift. In addition, structures of both precursor and autocleaved GA will allow us to study the activation process of the hydrolase activity. To these ends, we have initiated structural studies of GA in both the precursor and autocleaved forms.

2. Experimental methods

2.1. Expression and purification

Point mutations at the active site were generated as described previously (Guan *et al.*, 1996; Liu *et al.*, 1998). With the aid of the structure of the mature enzyme (Guo *et al.*, 1998), residues surrounding Thr152 were chosen for site-directed mutagenesis. The expression of MBP (maltose-binding protein)–GA fusion proteins was induced in TB1 cells by adding 1 m*M* isopropyl-1-thio-β-D-galactopyranoside at 303 K for 4–6 h. Cells were pelleted by centrifugation, resuspended in 20 m*M* Tris buffer pH 7.6, 50 m*M* NaCl, 1 m*M* EDTA and lysed by sonication. Fusion proteins were affinity purified from the crude extracts over an amylose column according to the protocol of the manufacturer (New England Biolabs). All purification procedures

were carried out at 277 K. The purified fusion proteins were then digested with factor Xa at 310 K for 1 h and GA was then separated from MBP and factor Xa with a HiTrap Q (Pharmacia) column. Similar protocols were used to purify autoproteolytically active mutant precursors, except that 10 m*M* glycine was included during the entire course of protein expression and purification in order to inhibit autoproteolysis. Using this protocol, we were able to obtain about 30 mg of GA protein from a litre of bacterial culture (Fig. 1).

2.2. Crystallization and crystallographic analysis

Crystallization was performed with the hanging-drop vapour-diffusion technique using equal volumes of protein and reservoir solutions. Crystallization dishes were kept in the dark. Prior to data collection, crystals were equilibrated with crystallization buffer supplemented with 20%(*v*/*v*) glycerol as cryoprotectant. The crystals were then mounted in a thin film of crystallization buffer plus cryoprotectant, supported by a loop made of dental floss (Teng, 1990) and flash-cooled directly in the cold nitrogen stream with an MSC cryo-system. X-ray diffraction data were collected at 100 K using an R-AXIS II detector. From a rotating-anode generator (Rigaku RU-300, with a nickel foil filter and a set of MSC/Yale Total-Reflection Mirrors) operated at 50 kV and 100 mA, monochromatic Cu $K\alpha$ radiation (λ = 1.5418 Å) was used to collect still photographs and native data sets. Data reduction, space group and unit-cell parameters were determined using the *HKL* suite (Otwinowski & Minor, 1997).

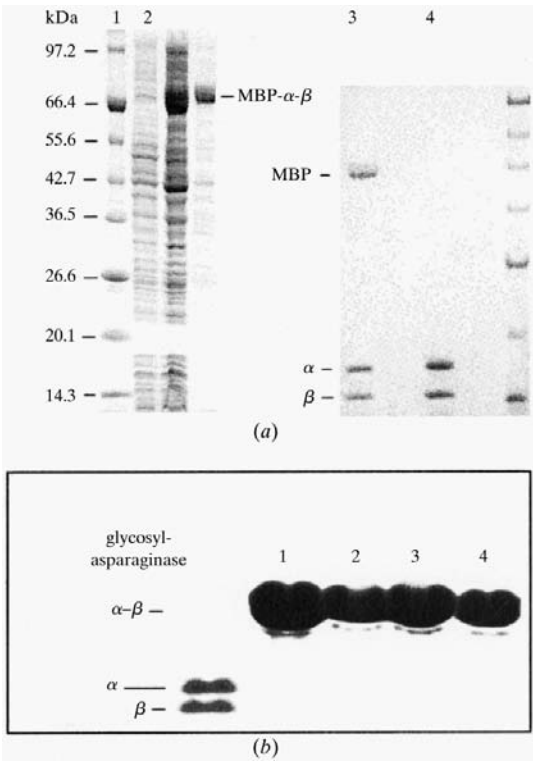

Figure 1
Protein purification of glycosylasparaginase. (*a*) Left panel shows the SDS–PAGE of total cell extract without (lane 1) and with IPTG induction (lane 2) to express the fusion protein of GA precursor with MBP (maltose-binding protein). Right panel shows the SDS–PAGE of the protein preparations of wild-type GA after factor Xa digestion (lane 3) and purification by the HiTrap Q (Pharmacia) column (lane 4). Protein bands for MBP–GA fusion protein (MBP–$\alpha\beta$), maltose-binding protein (MBP), as well as heavy (α) and light (β) subunits of mature GA are marked. (*b*) Protein purification of GA mutant precursors ($\alpha\beta$) of T152A (lane 1), T152C (lane 2), T152S (lane 3) and W11F (lane 4). The autocleaved subunits α and β of the wild-type enzyme are also designated.

3. Results and discussion

To prepare precursor proteins for crystallization, autoproteolysis during the course of protein purification and crystal growth must be stopped or drastically slowed. Given the high efficiency of this reaction in the wild-type protein, we apply two strategies to stabilize the precursor proteins. The first is to isolate point mutants that have much slower rates of autoproteolysis but are still active in the reaction. The structure of a precursor that is still capable of autoproteolysis will reveal more information on the mechanism of the reaction than a mutant with complete loss of function. The second strategy is to search for inhibitors which can stop autoproteolysis indefinitely but reversibly.

Several point mutants that autoproteolyze with a much lower activity have been generated, as well as mutants that are deficient in autoproteolysis. The hydroxyl group of Thr152 is believed to be the nucleophile in both autoproteolysis and hydrolase catalysis (Guan *et al.*, 1996), suggesting an overlap between these two activity centres. We therefore made several point mutations at the functional groups proposed to be involved in the hydrolase activity (Oinonen *et al.*, 1995; Guan *et al.*, 1996; Guo *et al.*, 1998). For example, point mutants were generated at Thr152 (named T152X, where X stands for the substituting residue) and at Trp11 (named W11F, where Trp11 has been substituted with a phenylalanine). Two groups of mutants were found. One group of enzymes is autoproteolytically active with reduced hydrolase activity, *e.g.* the T152S, T152C and W11F mutants. For this group of mutants, the rate reduction of autoproteolysis ranges from tenfold to more than three orders of magnitude (Guan *et al.*, 1998). The other group of mutants is autoproteolytically deficient and cannot produce the α-amino group that is essential for the hydrolase activity, *e.g.* the T152A mutant.

To further slow the autoproteolysis for preparing precursors and crystallization, we searched for inhibitors of autoproteolysis. Since peptide-bond cleavage at Thr152 is necessary to activate the hydrolase activity, we reasoned that in the single-chain precursor the substrate site of the hydrolase might be partially blocked by the uncleaved peptide chain. Aspartate has been found to bind in the substrate site of mature GA and interact with conserved residues (Oinonen *et al.*, 1995). We therefore screened amino acids for their ability to bind and inhibit the precursor proteins. We found that small amino acids such as glycine reversibly inhibit the autoproteolysis reaction. In the presence of 10 m*M* glycine in the bacterial media and purification solutions, we were able to isolate mutant precursor proteins to homogeneity (Fig. 1*b*). After the glycine was removed by dialysis, the autoproteolytically active mutant precursors can autoproteolyze into α and β subunits like the wild-type GA (Guan *et al.*, 1998; Liu *et al.*, 1998). Thus, the GA proteins were prepared as precursors and were converted when necessary into the mature form by removing glycine and activating autoproteolysis at 310 K. Autoproteolysis *in vitro* can be facilitated by the presence of 250 m*M* hydroxylamine (Guan *et al.*, 1996). It is of interest to note that autoproteolysis of the wild-type GA is not inhibited by glycine. One explanation is that the β-methyl group of Thr152 prevents glycine from accessing the partially formed substrate site in the precursor. In contrast, no autoproteolysis was detected for the T152A precursor, either in the presence or absence of glycine or hydroxylamine.

For crystallization, precursor proteins were dialyzed into 10 m*M* Tris pH 7.4, 50 m*M* NaCl, 1 m*M* EDTA, 20 m*M* glycine and concentrated to $\sim$5 mg ml^{-1}. Initial crystallization screens were attempted using the sparse-matrix sampling method (Jancarik & Kim, 1991). Crystals grew at 283 K from both 8% PEG 8000 and 30% PEG 4000 with 0.2 *M* lithium sulfate

Table 1
Protein crystals of glycosylasparaginase in precursor and autoprocessed forms.

Crystal	Space group	Number of molecules per asymmetric unit
Precursor	$P1$, a = 46.3, b = 52.8, c = 62.4 Å, α = 80.8, β = 90.5, γ = 105.1°	2
Autoprocessed	$P2_1$, a = 46.2, b = 97.3, c = 61.8 Å, β = 90.3°	2

Table 2
Data-processing statistics for crystal of the T152C precursor.

	Overall	Outer resolution shell
Resolution (Å)	25.0–1.9	1.97–1.9
R_{merge}	0.056	0.185
Completeness (%)	94.3	89.1
Redundancy	2.2	1.6
Average I/σ_I	9.4	4.0

buffered at pH 8.5 with 0.1 *M* Tris, but these crystals were of poor diffraction quality. Further crystallization trials and refinement produced good diffracting crystals at 277 K from 15% PEG 3300, 0.1 *M* Tris pH 7.5, 0.2 *M* lithium sulfate, 0.1% sodium azide. Seeding techniques were used to improve and ensure the reproducibility of the high quality of the crystals; they grow within two weeks. The dimensions of a typical crystal are about 400 × 100 × 20 µm (Fig. 2*a*). Under the same conditions as above, we have crystallized precursor proteins of T152C (with glycine), T152S (with glycine), W11F (with glycine) and T152A (no inhibitor).

Mature (autocleaved) GA protein was purified either in the absence of glycine or autoproteolyzed from the purified precursor by removing the glycine inhibitor. The protein was then dialyzed into 5 m*M* Tris pH 7.4, 1 m*M* EDTA and concentrated to ∼8 mg ml^{-1}. After some trials and refinement of crystallization conditions, diffraction-quality crystals grew at room temperature within 1 week from 15% PEG 3300, 0.1 *M* HEPES pH 7.5, 0.1% sodium azide. The dimensions of a typical crystal are about 300 × 100 × 50 µm (Fig. 2*b*). We have now crystallized mature enzymes of the wild-type GA as well as T152C and T152S mutants under the same conditions.

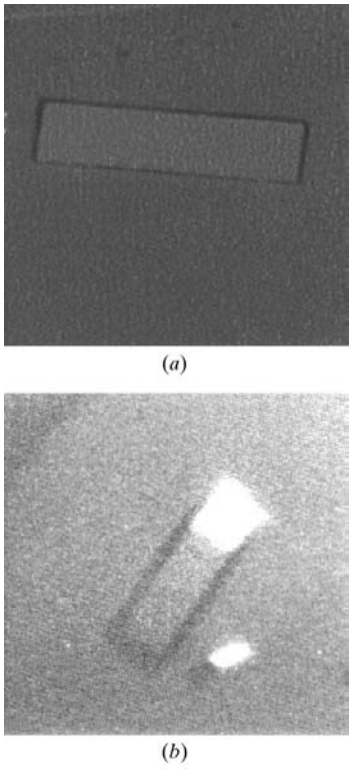

Figure 2
Protein crystals of glycosylasparaginase. (*a*) Single-chain precursor protein. (*b*) Autoprocessed mature enzyme with α and β subunits.

Using X-ray radiation from a rotating-anode generator, reflections could be measured to 1.9 Å resolution for crystals of precursor GA. Data collections and crystal structures of mature enzymes of the wild-type GA and the T152C mutant have been presented elsewhere (Guo *et al.*, 1998). All of the four precursor proteins investigated in this study crystallize in the triclinic space group $P1$, with unit-cell parameters a = 46.3, b = 52.8, c = 62.4 Å, α = 80.8, β = 90.5, γ = 105.1°. The crystals of all three mature GAs investigated belong to the monoclinic space group $P2_1$, with unit-cell parameters a = 46.2, b = 97.3, c = 61.8 Å, β = 90.3°. A comparison of space groups between precursor and mature forms of GA is shown in Table 1. It is evident that these two crystal forms are closely related by axes a and c and by a common angle between them (β = ∼90.4°). V_M calculations (Matthews, 1968) suggest that there are most likely to be two molecules in the asymmetric unit in both crystal forms. The correlation between these two crystal forms indicates that a similar dimeric structure exists in both crystal forms, even though the conditions for crystallization are different. With a dimer of precursor molecules in the $P1$ unit cell, V_M = 2.26 Å^3 Da^{-1}, corresponding to a solvent content of about 45%. In the crystals of mature enzyme, in which there are two $\alpha\beta$ heterodimers in the asymmetric unit, V_M = 2.16 Å^3 Da^{-1}, corresponding to a solvent content of about 43%. Data-processing statistics for a T152C precursor crystal (with glycine) are presented in Table 2. Diffraction data of similar quality have also been collected for precursor crystals of T152S (with glycine), W11F (with glycine) and T152A mutants (no inhibitor).

Solution of the crystal structures of both precursor and mature forms of GA will provide the structural basis for the study of the mechanism of intramolecular proteolysis and for the elucidation of the conformational changes responsible for activating GA hydrolase activity. To our knowledge, the GA precursor crystals represent the first crystallization of a precursor protein with autoprocessing activity. The strategies reported here may also be suitable for purification and crystallization of precursors of other N-terminal nucleophile (Ntn) hydrolase or autoprocessing proteins which react *via* a N→O or N→S acyl shift mechanism.

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