Molecular Genetics and Metabolism 121 (2017) 150–156

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism

journal homepage: www.elsevier.com/locate/ymgme

Crystal structure of a mutant glycosylasparaginase shedding light on aspartylglycosaminuria-causing mechanism as well as on hydrolysis of non-chitobiose substrate

Suchita Pande, Damodharan Lakshminarasimhan [1], Hwai-Chen Guo *

Department of Biological Sciences, University of Massachusetts Lowell, 1 University Avenue, Lowell, MA 01854, USA

ARTICLE INFO

Article history:
Received 8 March 2017
Received in revised form 18 April 2017
Accepted 18 April 2017
Available online 19 April 2017

Keywords:
Glycosylasparaginase
Aspartylglycosaminuria
β-AHA
Catalytic mechanism

ABSTRACT

Glycosylasparaginase (GA) is an amidase that cleaves Asn-linked glycoproteins in lysosomes. Deficiency of this enzyme causes accumulation of glycoasparagines in lysosomes of cells, resulting in a genetic condition called aspartylglycosaminuria (AGU). To better understand the mechanism of a disease-causing mutation with a single residue change from a glycine to an aspartic acid, we generated a model mutant enzyme at the corresponding position (named G172D mutant). Here we report a 1.8 Å resolution crystal structure of mature G172D mutant and analyzed the reason behind its low hydrolase activity. Comparison of mature G172D and wildtype GA models reveals that the presence of Asp 172 near the catalytic site affects substrate catabolism in mature G172D, making it less efficient in substrate processing. Also recent studies suggest that GA is capable of processing substrates that lack a chitobiose (Glycan, *N*-acetylchiobios, NAcGlc) moiety, by its exo-hydrolase activity. The mechanism for this type of catalysis is not yet clear. L-Aspartic acid β-hydroxamate (β-AHA) is a non-chitobiose substrate that is known to interact with GA. To study the underlying mechanism of non-chitobiose substrate processing, we built a GA-β-AHA complex structure by comparing to a previously published G172D mutant precursor in complex with a β-AHA molecule. A hydrolysis mechanism of β-AHA by GA is proposed based on this complex model.

1. Introduction

Glycosylasparaginase (GA) belongs to the family of N-terminal nucleophile (Ntn) hydrolases that can hydrolyze various compounds carrying L-asparagine residue with free α-amino and α-carboxylate groups [1,2]. It is a lysosomal amidase which processes L-asparagine linked glycoproteins into smaller units of free amino acids and sugars essential for several metabolic pathways of the body [2,3]. GA is initially synthesized as an inactive single-polypeptide precursor in which α and β-subunits are joined together via a surface loop (called precursor- or P-loop) that blocks the catalytic center of this enzyme [4,5]. A consequent autoproteolysis results in a main-chain cleavage at the P-loop by a self-catalyzed peptide bond rearrangement through an N → O acyl shift, and results in an active form of the hydrolase with α and β-subunits [5–7]. The N-terminal γ-hydroxyl and α-amino group of threonine residue of the newly formed β-subunit acts as an active site nucleophile and the general base, respectively, in the hydrolysis of N-glycosidic bonds of Asn-linked glycoproteins [6–8].

Autoproteolysis of GA precursor could be impaired by a missense mutation. Such a mutation results in a lysosomal storage disease called aspartylglucosaminuria (AGU) which occurs due to misprocessing of asparagine linked glycoproteins. This leads to accumulation of aspartylglucosamine (NAcGlc-Asn) and other glycoconjugates of aspartylglucosamine moiety at the reducing end in body fluids and tissues [9–11]. AGU results in progressive impairment of brain, motor and skeletal development of the patients [11,12].

Catalytic activity of GA is not just restricted to a few substrates but is known to be involved in hydrolysis of various Asn-linked glycoprotein substrates and L-asparagine analogues (Fig. 1). GA is capable of hydrolyzing L-asparagine via β-aspartyl intermediate to form L-aspartic acid and ammonia [13]. It is also known to be involved in the metabolism of β aspartyl peptides. It hydrolyzes β-aspartyl peptides to from L-aspartic acid and other peptides and synthesizes β-aspartyl peptides from β-aspartylglycosylamine. GA utilizes L-asparagine as β-aspartyl donor towards the formation of β-aspartyl peptides [14]. β-Aspartyl enzyme formation occurs after elimination of ammonia during the hydrolysis of L-asparagines by GA [13]. Studies suggest that L-aspartic acid β-hydroxamate (β-AHA) and L-aspartic acid beta methyl ester can also be hydrolyzed by GA even though these substrates lack the di *N*-

Abbreviations: AGU, aspartylglucosaminuria; GA, glycosylasparaginase; NAcGlc-Asn, N^4-(β-*N*-acetylglucosaminyl)-L-asparagine; Ntn, N-terminal nucleophile; β-AHA, L-aspartic acid β-hydroxamate; Asp(pNA)-OH, aspartic acid β-(p-nitroanilide); r.m.s.d., root mean square deviation.

* Corresponding author.
E-mail address: HwaiChen_Guo@uml.edu (H.-C. Guo).
[1] Current Address: Xtal Biostructures Inc., 12 Michigan Drive, Natick, MA 01760, USA.

http://dx.doi.org/10.1016/j.ymgme.2017.04.008
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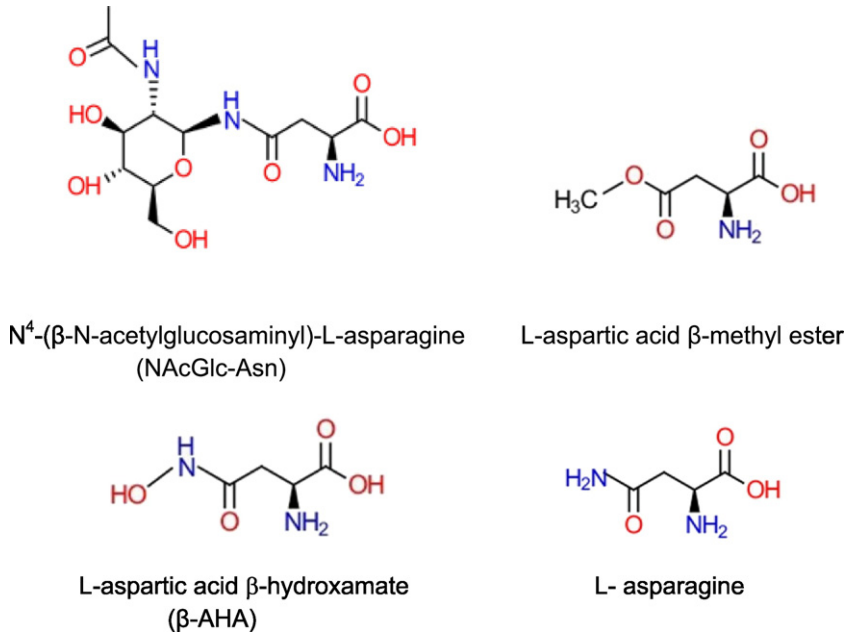

Fig. 1. Chemical structures of representative GA substrates, including glycoasparagines, non-chitobiose β-AHA, and L-asparagine.

acetylchiobios (chitobiose, NAcGlc-NAcGlc) moiety [15]. These results underline that GA shows exo-type hydrolase activity with various glycoasparagines and its reaction mechanism requires a β-aspartyl enzyme intermediate [15]. However a detailed reaction mechanism behind the catalysis of such non-chitobiose substrate remains unclear.

In an AGU allele of a Canadian family, a mutation has been reported that is caused by the change of a nucleotide from G to A in exon 6 [16,17], causing complex clinical presentation [18]. This mutation leads to two alternative splicing forms, resulting in two different lengths of transcripts: one with the normal length, whereas the other with a truncation of about 90 nucleotides due to exon 6 skipping. The truncated transcript is labile and likely to encode a misfolded protein due to a translational frameshift. On the other hand, the normal length transcript translates into a missense mutant that changes residue 203 of human GA from a glycine to an aspartic acid [16]. To study the disease-causing mechanism of this missense mutant, we report here a 1.8 Å resolution structure of a model missense GA corresponding to this Canadian AGU allele. We also propose a plausible explanation for its low activity based on comparison of substrate bound complexes of the mutant model with the wildtype GA model. Since a precursor structure of the same mutant in complex with β-AHA has also been reported previously [17], in this study we further built a GA-β-AHA model complex by superposing a few GA structures: the current model, the precursor-β-AHA complex, and the wild-type GA structures [7,17]. This allows us to analyze catalysis of β-AHA as a non-chitobiose substrate of GA.

2. Material and methods

2.1. Enzyme activity assay

Aspartic acid β-(p-nitroanilide) (Asp(pNA)-OH) was used as a substrate analog for this assay, which forms p-nitroaniline after hydrolysis reaction by glycosylasparaginase (GA). Each reaction was in a volume of 100 μl of 50 mM Tri buffer, pH 7.5, containing appropriate concentration of aspartic acid β-(p-nitroanilide). To monitor enzyme activity, substrate was incubated for 1 h at 37 °C and release of p-nitroaniline is monitored at 405 nm using Spectramax-M2 spectrophotometer.

2.2. Crystallization

GA mutant protein was over-expressed, purified by the published protocol of GA Flavobacterium [19]. Protein crystallizations were undertaken with Hampton Research Index crystal screen conditions. The mature form G172D crystals were obtained in 0.2 M Ammonium Acetate, 0.1 M Bis-Tris pH 5.5, 25% polyethylene glycol 3350 after removal of glycine through 10 kDa cutoff amicon centrifugal filter.

2.3. Data collection and processing

For data collection, crystals were cryoprotected in reservoir solution with 20% glycerol. X-ray data were collected using the beamline X29 at National Synchrotron Light Source at Brookhaven. The data were processed with the iMosflm and scaled and merged using Aimless program in CCP4 suite [20]. The space group of the crystal was *P*1 with two protein molecules in an asymmetric unit.

2.4. Structural refinement

The crystal structure of G172D mutant was solved by molecular replacement method, using the previously published GA D151N mutant protein structure (PDB code 1P4K) [21] as search model. Molecular replacement (MR) was performed with Molrep. Refinement was done using Refmac program by excluding 5% of the total reflection data from the refinement cycles and used to calculate the free R factor (R_{free}) for monitoring refinement progress. This MR model was further refined by rigid body and restrained refinements. Model building was carried out using COOT [22] to obtain the final structure. In the Ramachandran plot, all residues except one (0.4%) of the model are located in the most favorable or allowed regions. The outlier residue is Trp 11 in each molecule of this structure. Several attempts to modify this outlier to a favorable geometry always returned to its original geometry after refinement. Trp 11 is an active site residue the current model fits well into the electron density map. Thus the unusual configuration of Trp 11 appears to be valid and stabilized through interactions