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Crystal structure and substrate-binding mode of GH63 mannosylglycerate hydrolase from *Thermus thermophilus* HB8

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ABSTRACT

Glycoside hydrolase family 63 (GH63) proteins are found in eukaryotes such as processing α-glucosidase I and also many bacteria and archaea. Recent studies have identified two bacterial and one plant GH63 mannosylglycerate hydrolases that act on both glucosylglycerate and mannosylglycerate, which are compatible solutes found in many thermophilic prokaryotes and some plants. Here we report the 1.67-Å crystal structure of one of these GH63 mannosylglycerate hydrolases, *Tt*8MGH from *Thermus thermophilus* HB8, which is 99% homologous to mannosylglycerate hydrolase from *T. thermophilus* HB27. *Tt*8MGH consists of a single $(\alpha/\alpha)_6$-barrel catalytic domain with two additional helices and two long loops which form a homotrimer. The structures of this protein in complexes with glucose or glycerate were also determined at 1.77- or 2.10-Å resolution, respectively. A comparison of these structures revealed that the conformations of three flexible loops were largely different from each other. The conformational changes may be induced by ligand binding and serve to form finger-like structures for holding substrates. These findings represent the first-ever proposed substrate recognition mechanism for GH63 mannosylglycerate hydrolase.

1. Introduction

Extremophiles produce various osmolytes to adapt themselves to high-salt environments. These osmolytes are called "compatible solutes" and include sugars, amino acids, and polyols (Kempf and Bremer, 1998; Empadinhas and da Costa, 2011). 2-*O*-α-D-Mannopyranosyl-D-glycerate (mannosylglycerate, MG) is a compatible solute initially found in red algae (Colin and Augier, 1939) that also accumulates in thermophilic bacteria and archaea such as *Thermus*, *Rubrobacter*, *Rhodothermus*, and *Pyrococcus* (Empadinhas and da Costa, 2011). 2-*O*-α-D-Glucopyranosyl-D-glycerate (glucosylglycerate, GG) is a similar compound produced in many prokaryotes, such as *Persephonella marina* and actinobacteria, and is a precursor of lipopolysaccharide synthesis in mycobacteria (Empadinhas and da Costa, 2011). Many studies of physiological functions and synthesis of MG and GG have been published (Borges et al., 2014), but reports on the catabolism of these solutes are less common.

Recently, glycoside hydrolases (mannosylglycerate hydrolases, MGHs) active on both MG and GG have been identified in two bacteria [*Thermus thermophilus* HB27 (*Tt*27MGH) and *Rubrobacter radiotolerans* RSPS-4 (*Rr*MGH)] and the plant *Selaginella moellendorffii* (*Sm*MGH) (Alarico et al., 2013; Nobre et al., 2013). They are classified in glycoside hydrolase family 63 (GH63) in the carbohydrate-active enzymes (CAZy) database (http://www.cazy.org) (Lombard et al., 2014). GH63 proteins are found in archaea, bacteria, and eukaryotes, and the most studied enzyme among them is processing α-glucosidase I (EC 3.2.1.106) (Kalz-Füller et al., 1995; Palcic et al., 1999; Dhanawansa et al., 2002; Faridmoayer and Scaman, 2004, 2005, 2007; Frade-Pérez et al., 2010; Miyazaki et al., 2011). This enzyme specifically hydrolyzes the α-1,2-glucoside linkage of $Glc_3Man_9GlcNAc_2$, an oligosaccharide precursor of eukaryotic *N*-linked glycoproteins (Herscovics, 1999; Helenius and Aebi, 2004). The apo structure of processing α-glucosidase I from *Saccharomyces cerevisiae* (*Sc*Cwh41p) has been reported (Barker and Rose, 2013). We previously reported the crystal structure of a glycoside hydrolase YgjK (*Ec*YgjK) from *Escherichia coli* K-12 as the first structure among GH63 members (Kurakata et al.,

Abbreviations: GG, 2-*O*-α-D-glucopyranosyl-D-glycerate; GGalase, 2-*O*-α-D-glucopyranosyl-D-galactose hydrolase; GH63, glycoside hydrolase family 63; MG, 2-*O*-α-D-mannopyranosyl-D-glycerate; MGH, mannosylglycerate hydrolase.

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2008), and subsequent substrate screening using the combination of glycosynthase reaction and structural analysis revealed that the enzyme was likely a hydrolase (GGalase) specific for 2-*O*-α-D-glucopyranosyl-D-galactose (Miyazaki et al., 2013).

Additionally, the 2.1 Å-resolution apo structure of an uncharacterized protein (TTHA0978; *Tt*8MGH) from *T. thermophilus* HB8 was determined by the structural genomics project (Protein Data Bank [PDB]: 2Z07). Although there is no report on the biochemical and structural characterization of *Tt*8MGH, due to its 99% sequence identity with *Tt*27MGH, the protein appears to be a glycoside hydrolase active on MG and GG. Although the structure-determined GH63 enzymes share $(\alpha/\alpha)_6$-barrel catalytic domains, their substrate specificities differ, especially in aglycons. To elucidate the relationship between the structure and substrate specificity of MGHs and differences among other GH63 proteins, here we determined the crystal structures of *Tt*8MGH in apo form and in complexes with glucose (Glc) and glycerate. The present study represents the first proposed substrate recognition mechanism for *Tt*8MGH with large conformational changes of loops.

2. Materials and methods

2.1. Protein expression and purification

The expression plasmid for *Tt*8MGH (TTHA0978, GenBank: BAD70801.1) was purchased from RIKEN Bioresource Center (Tsukuba, Japan) and was derived from pET-11a vector (Novagen, Madison, WI, USA) subcloned with a gene for *Tt*8MGH. *E. coli* BL21 (DE3) was used for overexpression of the gene. Cells harboring the plasmids were cultured at 37 °C in 1 L Luria–Bertani medium containing 50 μg mL^{-1} ampicillin. When the culture reached an optical density of 0.6 measured at 600 nm, it was induced with isopropyl-β-D-thiogalactopyranoside at a final concentration of 0.5 mM and cultured for another 6 h at 30 °C. The cells were harvested by centrifugation at 10,000×*g* for 5 min, resuspended in 30 mL 20 mM Tris–HCl buffer (pH 7.5) and then disrupted by sonication. The cell lysate was centrifuged at 10,000×*g* for 20 min to remove insoluble debris, and the soluble fraction was heated at 70 °C for 10 min, followed by centrifugation at 20,000×*g* for 30 min to eliminate the aggregated proteins derived from *E. coli*. The supernate was applied to a HiPrep Butyl FF 16/10 column (GE Healthcare, Little Chalfont, Buckinghamshire, UK) equilibrated with 20 mM Tris–HCl buffer (pH 7.5) containing 0.4 M ammonium sulfate. The protein was eluted using a decreasing linear gradient of 0.4–0 M ammonium sulfate at a flow rate of 3 mL min^{-1}. The fractions containing the protein were collected, dialyzed against 20 mM Tris–HCl (pH 7.5) buffer, and then applied to a HiLoad 16/10 Q-Sepharose HP column (GE Healthcare) equilibrated with the same buffer. The protein was eluted with a linear gradient of 0–0.5 M sodium chloride in the same buffer at a flow rate of 3 mL min^{-1}. The purity of the protein was confirmed by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS–PAGE) (Supplementary Fig. S1).

2.2. Crystallization, data collection, structure determination, and refinement

Prior to crystallization, the purified protein was concentrated to 10 mg mL^{-1} in 10 mM Tris–HCl (pH 7.5) using the ultrafiltration device Amicon Ultra 10 K (Millipore, Bedford, MA, USA). *Tt*8MGH was crystallized at 20 °C using the hanging-drop vapor diffusion method in which 1.0 μL protein solution was mixed with an equal volume of crystallization mother liquor containing 20–35% (vol/vol) 2-methyl-2,4-pentanediol and 100 mM Tris–HCl buffer (pH

Table 1
Data collection and refinement statistics.

	*Tt*8MGH-tris	*Tt*8MGH-Glc	*Tt*8MGH-glycerate
Data collection			
Beamline	PF AR-NW12A	PF AR-NE3A	PF AR-NE3A
Wavelength (Å)	1.0000	1.0000	1.0000
Space group	*R*3	*R*3	$P32_1$
Cell dimensions			
$a = b$ (Å)	92.0[a]	93.4[a]	93.0
c (Å)	267.2[a]	254.1[a]	190.4
Resolution range (Å)	50–1.67 (1.73–1.67)[b]	50–1.77 (1.83–1.77)[b]	50–2.10 (2.21–2.10)[b]
Measured reflections	524,392	447,604	250,934
Unique reflections	96,185	80,188	55,067
Completeness (%)	99.2 (98.2)[b]	99.3 (100)[b]	97.6 (93.6)[b]
$I/\sigma(I)$	37.0 (6.5)[b]	51.3 (10.8)[b]	9.3 (5.5)[b]
R_{merge}	0.059 (0.318)[b]	0.045 (0.208)[b]	0.110 (0.190)[b]
Refinement statistics			
R_{work}	0.146	0.159	0.245
R_{free}	0.173	0.188	0.280
RMSD			
Bond lengths (Å)	0.008	0.010	0.010
Bond angles (°)	1.314	1.422	1.333
Number of atoms			
Protein	6871	6714	6796
Ligands	72	44	30
Chloride ion	2	2	2
Water	674	548	265
Average B (Å^2)			
Protein	17.5	23.7	30.4
Ligands	25.4	31.2	31.7
Chloride ion	13.3	17.1	21.7
Water	29.8	31.2	26.5
Ramachandran plot			
Favored (%)	97.0	97.4	96.4
Outliers (%)	0	0	0.3

[a] The cell parameters are in hexagonal setting.
[b] The values for the highest resolution shells are provided in parentheses.