Just add sugar...
Pijning, Tjaard

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Truncated and full-length glucansucrase GTF180 from *Lactobacillus reuteri* adopt an elongated structure in solution

Tjaard Pijning, Andreja Vujičić-Žagar, Slavko Kralj, Lubbert Dijkhuizen and Bauke W. Dijkstra

*Submitted for publication*

A second crystal form of the α(1→6)/α(1→3) specific glucansucrase GTF180-ΔN from *Lactobacillus reuteri* contains a much more compact conformation, in which domain V is hinged by 120°. In solution however, truncated GTF180-ΔN, as well as full-length GTF180 adopt elongated molecular shapes and, despite their intrinsic flexibility, show no large hinging motion, not even when the enzyme is actively processing its substrate into short oligosaccharides.
ABSTRACT

Glucansucrase enzymes synthesize high-molecular-mass extracellular α-glucan polysaccharides from sucrose. Previously, the crystal structure of truncated glucansucrase GTF180-ΔN from Lactobacillus reuteri 180 (lacking the N-terminal domain) revealed an elongated overall structure with two remote domains (IV and V) extending away from the core. In contrast, a new crystal form of the α-1,6/α-1,3 specific glucansucrase GTF180-ΔN showed a ~120° rotation of domain V about a hinge located between domains IV and V, giving a much more compact structure than before. This compact conformation is not found in solution, since small angle X-ray scattering (SAXS) measurements revealed GTF180-ΔN to have a molecular shape that corresponds to the earlier reported elongated crystal structure. In addition, SAXS measurements of GTF180 also provided the first structural data for a full-length glucansucrase, showing that the enzyme has an almost symmetric boomerang-like molecular shape with a bend likely located between domains IV and V. The ~700-residue N-terminal domain, which is not present in the crystal structures, extends away from domain V and the catalytic core of the enzyme; there are no indications of flexibility. In the presence of the substrate sucrose, when early products (oligosaccharides) are being synthesized, only minor changes in the molecular shape are observed. We conclude that, although glucansucrases have an intrinsic flexibility, no large hinging motions occur in solution, not even when the enzyme is actively processing its substrate into short oligosaccharides.

INTRODUCTION

Glucansucrases (EC 2.1.4.5; glucosyltransferases, GTFs) are bacterial enzymes that use sucrose as substrate to synthesize a variety of high molecular mass, extracellular glucose polymers with α-glycosidic linkages (α-glucans) (Kralj et al., 2004a; Leemhuis et al., 2013a; Monchois et al., 1999; Van Hijum et al., 2006). The produced α-glucans form an extracellular biofilm-like layer around the bacteria (Flemming & Wingender, 2010), which is believed to facilitate adhesion of the bacteria to surfaces and to protect them against adverse environmental conditions. Due to variations in linkage type, degree of branching, and size, the α-glucans produced by glucansucrases display greatly varying physicochemical properties (Leemhuis et al., 2013a). For example, the α-glucans produced by Streptococcus mutans glucansucrases in the oral cavity are sticky and are associated with the formation of bacterial plaque on tooth surfaces, which eventually may lead to the development of dental caries (Colby et al., 1999). Natural and modified α-glucans also hold great potential in biotechnology, and in food and health related applications, in particular because of their potential prebiotic properties (Leemhuis et
Truncated and full-length glucansucrase GTF180 from *Lactobacillus reuteri* (Leemhuis *et al.*, 2013a). To further tailor glucansucrases for such applications, it is crucial to understand how glucansucrases synthesize their products, and to comprehend the principles that determine their product specificities (Leemhuis *et al.*, 2013a; Moulis *et al.*, 2006).

Previously, we have reported the crystal structures of N-terminally truncated forms of *Lactobacillus reuteri* 180 glucansucrase GTF180 (which will be referred to as GTF180-ΔN I; (Pijning *et al.*, 2008; Vujičić-Žagar *et al.*, 2010)) and *Lactobacillus reuteri* 121 GTFA (referred to as GTFA-ΔN; Pijning *et al.*, 2012). In addition, crystal structures have been reported of *Streptococcus mutans* GTF-SI (Ito *et al.*, 2011) and *Leuconostoc mesenteroides* NRRL B-1299 ΔN123-GBD-CD2 from DSR-E (Brison *et al.*, 2012). These crystal structures showed that glucansucrases, which are classified in glycoside hydrolase family GH70 (Cantarel *et al.*, 2009), share a common multi-domain organization with three core domains (A, B and C) and two remote domains (IV and V), arranged as C-A-B-IV-V (Figure 1). The core domains structurally resemble the A, B and C domains of GH13 α-amylases (Vujičić-Žagar *et al.*, 2010); domains A (the catalytic domain) and B contribute residues lining the active site cleft, and their possible roles in determining product linkage-type specificity have been discussed in Leemhuis *et al.* (2013a). The two remote domains IV and V extend from the core domains; these domains are only found in glucansucrase enzymes. Notably, GTF180-ΔN I and GTFA-ΔN are elongated structures; domain V is connected loosely to domain IV via two loop regions, and takes up slightly different positions in these enzymes. In contrast, in ΔN123-GBD-CD2 from DSR-E, domain V has a completely different position, resulting in a much more compact structure (Brison *et al.*, 2012).

![Figure 1. Schematic domain organization of glucansucrases.](image)

The construct GTF180-ΔN contains 5 domains, of which domains A, B, IV and V consist of both an N- and a C-terminal polypeptide segment (colored dark and light, respectively). The full-length GTF180 has an additional ~700 residues preceding domain V. Possible flexibility of domain V is indicated by arrows.
Together, these observations suggest that domain V may be flexible and that a hinge region may exist between domain V and the rest of the structure. It has been proposed that domain V of glucansucrases plays a role during α-glucan extension, possibly by ‘swinging’ toward and away from the catalytic domain, while binding intermediate α-glucan products and/or acceptor molecules (Ito et al., 2011). However, direct experimental evidence for such motions or for binding of oligosaccharides or α-glucans by domain V is lacking to date, and the question remains whether positional variability of domain V is of functional relevance.

Importantly, all so far reported glucansucrase crystal structures are of N-terminally truncated proteins, lacking between ~240 and 840 residues preceding the N-terminal end of domain V (or IV in the case of GTF-SI) (Figure 1). These N-terminal glucansucrase segments vary greatly in length and composition; although the presence of sequence repeats in these domains suggests that they may contain the same modular β-solenoid fold as observed in domain V (Vujčić-Žagar et al., 2010), detailed 3D structural information is unknown to date.

Here, we report the structure of truncated L. reuteri 180 glucansucrase GTF180 crystallized in a second crystal form (GTF180-ΔN II) with domain V hinged by ~120°, resulting in a much more compact conformation than previously determined. However, small angle X-ray scattering (SAXS) experiments reveal that in solution GTF180-ΔN adopts an elongated conformation. In addition, we provide the first structural data for a full-length glucansucrase (GTF180); the N-terminal domain of GTF180, being one of the largest variable N-terminal domains reported for glucansucrases, extends away from the remote domains IV and V, resulting in an almost symmetric boomerang-like shape. Like for the truncated enzyme, there are no indications of large conformational changes in full-length GTF180, and only minor changes occur to the structure when the enzyme is synthesizing oligosaccharides. Thus, although different glucansucrase structures seem to suggest that glucansucrases are intrinsically flexible, in solution under the conditions tested this property does not appear to play a role during the glucansucrase catalytic reaction.

**MATERIALS AND METHODS**

**Expression, isolation and purification**

N-terminally truncated GTF180 (GTF180-ΔN) from L. reuteri 180 comprising residues Met-Gly-742-1772-His$_6$ (GTF180-ΔN) was expressed in E. coli strain BL21 (DE3) and purified as described previously (Kralj et al., 2004a).

Full-length mature GTF180 from L. reuteri 180 comprising residues Met-Asp-39-1772-His$_6$ (GTF180) was expressed and purified as described previously (Kralj et al., 2004a).
However, the Ni-NTA eluate containing GTF180 was subjected to two additional purification steps. First, anion-exchange chromatography was done using a Resource Q column (GE Healthcare); the protein was eluted using a gradient of 0-1 M NaCl in 25 mM Tris-HCl buffer, pH 7.5, 1 mM CaCl₂, 1 mM DTT. Fractions containing GTF180 were pooled, concentrated with an Amicon Ultra 4 (Millipore; molecular weight cut-off 50 kDa) and loaded onto a Superdex 200 GL gel filtration column with 25 mM sodium acetate buffer, pH 5.5, 150 mM NaCl, 1 mM CaCl₂, 1 mM DTT as the running buffer. Fractions containing GTF180 were pooled and concentrated to a final protein concentration of ~6 mg ml⁻¹.

The purity and homogeneity of both GTF180-ΔN and GTF180 were analyzed by SDS-PAGE with Coomassie staining, by measuring absorption spectra (NanoDrop ND-1000, Thermo Scientific, Wilmington, USA) and by DLS (see section DLS below).

**Crystallization, data collection and processing**

The crystallization of 3 different crystal forms of GTF180-ΔN has been reported by Pijning et al. (2008). Among these, the GTF180-ΔN II crystal form was used in the current study. Briefly, the GTF180-ΔN II crystal form was obtained initially from condition I-44 of the Structure Screen (Molecular Dimensions Ltd.) containing 2.0 M (NH₄)₂SO₄ and 5% (v/v) 2-propanol in the reservoir. Attempts to reproduce and optimize the crystals proved to be extremely challenging, and were only successful using a streak-seeding protocol (Stura & Wilson, 1991).

The GTF180-ΔN II crystals were cryoprotected in 2.2 M (NH₄)₂SO₄, 5% (v/v) 2-propanol, 25% (v/v) glycerol, and flash-cooled in liquid nitrogen; a native dataset of GTF180-ΔN was collected at beamline X12 at the EMBL Outstation at DESY (Hamburg, Germany) and processed using the HKL2000 suite (Otwinowski & Minor, 1997). Details of data collection and processing statistics are given in Table 1.

**Structure determination and refinement**

The GTF180-ΔN II crystal structure was solved by molecular replacement with PHASER in the CCP4 package (McCoy et al., 2008; Winn et al., 2011). As a search model, the triclinic crystal structure of GTF180-ΔN I (Pijning et al., 2008; Vujičić-Žagar et al., 2010) was used. A correct solution could only be found by dividing the search model in two entities, comprising domains A + B + C + IV (residues 793-1637, search model 1) and domain V (residues 746-792 and 1638-1751, search model 2). Rigid-body and restrained refinement of the two molecules in the asymmetric unit was done with REFMAC5 (Murshudov et al., 1997) within the CCP4 package (Winn et al., 2011). Cycles of refinement were alternated with manual building in COOT (Emsley & Cowtan, 2004).
the final stages, refinement of TLS parameters was included, using four TLS groups corresponding to domains A + B, domain C, domain IV and domain V. The quality of the crystallographic model was assessed using MolProbity (Chen et al., 2010). Structural superpositions were done with the SSM algorithm (Krissinel & Henrick, 2004) within Coot. The program CRYSOC (Svergun et al., 1995) was used to calculate longest dimensions ($D_{\text{max}}$) and radii of gyration ($R_g$) from the crystal structures of GTF180-ΔN I and GTF180-ΔN II. Structural figures were prepared with PyMOL (Schrödinger, LLC).

**Table 1.** X-ray diffraction data collection and refinement statistics. Values in parentheses are for the highest resolution shell.

<table>
<thead>
<tr>
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<tr>
<td>Space group</td>
<td>$P2_12_12_1$</td>
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<tr>
<td>Unit cell (Å, o)</td>
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<tr>
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</tr>
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<tr>
<td>Reflections (unique)</td>
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<tr>
<td>Multiplicity</td>
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<tr>
<td>$R_{\text{merge}}$ (%)</td>
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<tr>
<td>$&lt;I/\sigma(I)&gt;$</td>
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<tr>
<td>Completeness (%)</td>
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</tr>
<tr>
<td><strong>Final model</strong></td>
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</tr>
<tr>
<td>$R_{\text{cryst}} / R_{\text{free}}$ (%)</td>
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<tr>
<td>R.m.s. deviation from ideality</td>
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</tr>
<tr>
<td>Bond lengths (Å)</td>
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</tr>
<tr>
<td>Bond angles (o)</td>
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<tr>
<td><strong>Contents of the asymmetric unit (molecules A/B)</strong></td>
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<tr>
<td>Protein non-H atoms¹</td>
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</tr>
<tr>
<td>No. of Ca²⁺ ions</td>
<td>1 / 1</td>
</tr>
<tr>
<td>No. of glycerol molecules</td>
<td>33 / 23</td>
</tr>
<tr>
<td>No. of sulfate ions</td>
<td>10 / 11</td>
</tr>
<tr>
<td>No. of acetate ions</td>
<td>7 / 5</td>
</tr>
<tr>
<td>No. of 2-propanol molecules</td>
<td>1 / 1</td>
</tr>
<tr>
<td>No. of water molecules</td>
<td>1099 / 1089</td>
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</tbody>
</table>
(Table 1, continued)

Average B-factor (protein atoms) chain A/B
- all domains 15.2 / 19.2
- domains A+B+C+IV 12.9 / 17.3
- domain V 25.7 / 27.2
B-factor Ca^{2+} 8.7 / 13.1

Ramachandran plot
Favored regions (%) 98.0
Additionally allowed regions (%) 1.9
Outliers (%) 0.1

MolProbity
Clash score / percentile 6.8 / 94th
MolProbity score / percentile 1.37 / 98th

1Molecule A contains residues 742-1771; molecule B contains residues 742-1691 and 1695-1771 (residues 1692-1694 were not modeled because of poor electron density).

**Small Angle X-ray Scattering (SAXS) experiments**

Details about SAXS data collection, samples and measurements are given in Table 2. Protein samples for SAXS were prepared by concentrating/washing them extensively in buffer solution (see below for composition); in each case the same corresponding buffer was used to determine the buffer scattering profiles for SAXS measurements. Protein concentrations were determined on-site using a NanoDrop ND-1000 spectrophotometer (ThermoScientific), using specific absorption coefficients at 280 nm of 1.752 ml mg^{-1} cm^{-1} for GTF180-ΔN and 1.584 ml mg^{-1} cm^{-1} for GTF180. All protein and buffer samples were centrifuged 10 min at 13,000 g before data collection.

**GTF180-ΔN measurements.** SAXS experiments with GTF180-ΔN were performed at beamline ID14-3 at the ESRF (Grenoble, France), using 3 different protein concentrations (1.2 - 4.6 mg ml^{-1} GTF180-ΔN in 25 mM sodium acetate buffer, pH 5.5, 62 mM NaCl, 1 mM CaCl\_2). Scattering data was collected to $s = 6$ nm^{-1}. Of the 10 individual exposures of each sample, the last 1-3 showed signs of radiation damage (most likely due to a halted sample flow) and were not used in further data processing.

**GTF180 measurements.** SAXS experiments with GTF180 (full-length) were performed at beamline X33 at the EMBL Outstation at DESY (Hamburg, Germany), using 4 different protein concentrations (1.1 - 3.7 mg ml^{-1} GTF180 in 25 mM sodium acetate buffer, pH 5.5, 150 mM NaCl, 1 mM CaCl\_2). Scattering data was collected to $s = 5$ nm^{-1}. No signs of radiation damage were observed during the 8 individual exposures of each sample. For
the experiment with sucrose, enzyme and substrate were mixed to final concentrations of 3.7 mg ml\(^{-1}\) GTF180, 25 mM sodium acetate buffer, pH 5.5, 150 mM NaCl, 1 mM CaCl\(_2\), 12.5 mM sucrose (~650x excess with respect to the enzyme), immediately stored on ice, and transferred to the sample changer (thermostated at 288 K). The time between mixing and first measurement was ~11 min; the 8 individual exposures (each 15 s) showed no significant change over time, and all 8 acquisitions were averaged and used in

<table>
<thead>
<tr>
<th>Table 2. SAXS data collection and scattering-derived parameters.</th>
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<tbody>
<tr>
<td><strong>Sample</strong></td>
</tr>
<tr>
<td><strong>Data collection parameters</strong></td>
</tr>
<tr>
<td>Beamline</td>
</tr>
<tr>
<td>Wavelength (Å)</td>
</tr>
<tr>
<td>Sample cell type, volume</td>
</tr>
<tr>
<td>Temperature (K)</td>
</tr>
<tr>
<td>Exposure time (s)</td>
</tr>
<tr>
<td>Detector distance (m)</td>
</tr>
<tr>
<td>Detector</td>
</tr>
<tr>
<td><strong>Structural parameters</strong></td>
</tr>
<tr>
<td>(I_0) (cm(^{-1})) from (P(r))</td>
</tr>
<tr>
<td>(R_g) (Å) from (P(r))</td>
</tr>
<tr>
<td>(I_0) (cm(^{-1})) from Guinier</td>
</tr>
<tr>
<td>(R_g) (Å) from Guinier</td>
</tr>
<tr>
<td>(D_{max}) (Å) from Autognom</td>
</tr>
<tr>
<td><strong>Molecular mass determination</strong></td>
</tr>
<tr>
<td>Excluded volume (nm(^3))</td>
</tr>
<tr>
<td>(M_r) (kDa) from Autoporod</td>
</tr>
<tr>
<td>Monomeric (M_r) (kDa) from sequence</td>
</tr>
</tbody>
</table>

further data processing. In the accompanying blank measurement, the buffer contained the same amount of sucrose (12.5 mM). We verified that the enzyme is active at 288 K, by assaying glucansucrase activity by the dinitrosalicylic acid (DNS) method (Miller, 1959). In addition, the products formed under the same conditions as those used in the SAXS experiment were analyzed using thin-layer chromatography (TLC); 1 μl of reaction mixture was spotted on a TLC plate (Merck silica gel 60 F254, 20 ×20 cm) which was
developed with 2-butanol : acetic acid : water (2 : 1 : 1) and stained with orcinol/sulfuric acid (See Supporting Material).

**Data processing.** All scattering curves were inspected, and processed using PRIMUS software (Konarev et al., 2003) to generate Guinier plots, P(r) distributions and Porod-Debye plots. Guinier plots were evaluated to the suggested limit for the Guinier approximation \((s = 1.3/R_g)\). After normalization to the intensity of the incident beam and subtraction of the buffer scattering curve, the scattering curves for the different protein concentrations were merged, and \(R_g\), \(I_0\) and \(D_{max}\) were determined with AUTOGNOM (Svergun, 1992). Excluded volume (Porod volume) and molecular mass were calculated using AUTOPOD (Petoukhov et al., 2007).

**Modeling.** *Ab initio* models of GTF180-ΔN and GTF180 were constructed using GASBOR (Svergun et al., 2001); no symmetry was applied, and the results of ten runs were merged with DAMAVER (Volkov & Svergun, 2003). Additionally, for GTF180-ΔN, a rigid-body modeling approach was done with SASREF (Petoukhov & Svergun, 2005), using BUNCH to first construct the 4 N-terminal and 26 C-terminal residues which are absent in the GTF180-ΔN I crystal structure (PDB ID: 3KLK (Vujičić-Žagar et al., 2010)). The starting model was separated into 3 entities consisting of domains A + B + C, domain IV and domain V, and refinement was constrained by imposing preservation of the connections between the entities. The resulting GTF180-ΔN models derived from the SAXS measurements were superimposed on crystal structures (PDB IDs: 3KLK (Vujičić-Žagar et al., 2010) and 4AYG, this study) with SUPCOMB (Kozin & Svergun, 2001). Structural figures were prepared with PyMOL (Schrödinger, LLC).

To calculate theoretical scattering curves from the GTF180-ΔN I and II crystal structures, and to fit these to the experimental SAXS scattering data, the FoXS webserver (Schneidman-Duhovny et al., 2010) was used. Prior to these calculations, the N- and C-terminal residues missing in the crystal structures were modeled with BUNCH.

**Dynamic Light Scattering (DLS)**

Samples for DLS, containing 3.0 mg ml\(^{-1}\) GTF180-ΔN or 4.2 mg ml\(^{-1}\) GTF180, 20 mM HAc/NaAc, pH 5.5, 120 mM NaCl, 0.8 mM CaCl\(_2\), were centrifuged 10 min at 13000 g at 293 K before measurement. All measurements were done in triplicate.

Measurements were performed using a Wyatt DynaPro NanoStar instrument thermostated at 288 K (samples were allowed to equilibrate to the measuring cell temperature for at least 10 min), and results were analyzed with Dynamics software (version 7). Solvent parameters were set as follows: dynamic viscosity (\(\eta\)) = 1.032 cp; refractive index = 1.335. Since the polydispersity of all samples was below 15%, the cumulants analysis was used.
Accession numbers

The crystal structure and structure factor amplitudes of GTF180-ΔN II have been deposited in the Protein Data Bank with accession number 4AYG.

RESULTS

Purity and homogeneity of the samples

Both GTF180-ΔN and GTF180 were purified to homogeneity. SDS-PAGE gels of both enzymes after the last purification step showed a single band close to the expected molecular weights (Figure 2a). Furthermore, the absorption spectra have an $A_{280}/A_{250}$ ratio of 3.4 and 3.0, respectively, and no significant absorption at 330 nm (Figure 2b). Finally, a dynamic light scattering (DLS) analysis showed that the overall polydispersity is low (3.6 and 8.1% respectively, Figure 3 and Table 3) with no high molecular weight aggregates detected. The estimated molecular weights of 116 ± 3 kDa (GTF180-ΔN) and 223 ± 6 kDa (GTF180) are in agreement with a monomeric state of the proteins.

![Figure 2](image)

**Figure 2.** (a) Coomassie-stained SDS-PAGE gel of purified GTF180 and GTF180-ΔN samples (both 1.0 mg ml$^{-1}$). Lane M, markers (molecular weights are indicated in kDa); lane 1, GTF180; lane 2, GTF180-ΔN. (b) Absorption spectra of 3.7 mg ml$^{-1}$ GTF180-ΔN and 5.0 mg ml$^{-1}$ GTF180 measured against buffer (25 mM HAc/NaAc, pH 5.5, 150 mM NaCl, 1 mM CaCl$_2$); path length = 1 mm.

<table>
<thead>
<tr>
<th>Sample</th>
<th>$R_h$ (nm)</th>
<th>Polydispersity (%)</th>
<th>Estimated MW (kDa)</th>
</tr>
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<tbody>
<tr>
<td>GTF180-ΔN</td>
<td>4.47 ± 0.05</td>
<td>3.6 ± 0.9</td>
<td>116 ± 3</td>
</tr>
<tr>
<td>GTF180</td>
<td>5.95 ± 0.03</td>
<td>8.1 ± 0.05</td>
<td>223 ± 6</td>
</tr>
</tbody>
</table>

Table 3. DLS analysis of GTF180-ΔN and GTF180; average values and standard deviations are given from triplicate measurements of each sample.
Figure 3. DLS analysis of (a) GTF180-ΔN and (b) GTF180 showing the % mass distribution (from regularization analysis).

Crystal structure of ΔN-GTF180 II

A well-diffracting crystal form of GTF180-ΔN (GTF180-ΔN II) with a different space group (P2₁2₁2₁) was obtained at much higher ionic strength compared to the previous triclinic crystal form. The structure was determined by molecular replacement at 2.0 Å resolution to a final R<sub>cryst</sub> / R<sub>free</sub> of 15.5 / 18.3%, with good stereochemical quality. The orthorhombic crystals contain two molecules in the asymmetric unit (Figure 4). In both molecules all residues of the protein are visible, except for the N-terminal methionine and the C-terminal lysine and histidine-tag; one of the two molecules (B) also lacks residues 1692-1694. Details of the model and its stereochemical quality are listed in Table 1.

The individual structures of the five domains (A, B, C, IV and V) observed in the previously reported elongated crystal structure of GTF180-ΔN I (Pijning et al., 2008; Vujičić-Žagar et al., 2010) are essentially unchanged in GTF180-ΔN II. However, a superposition of GTF180-ΔN II and GTF180-ΔN I (Figure 5) revealed a dramatic difference in the location of domain V. While domains A, B, C and IV (residues 794-1633) can be superimposed with root mean square deviation (r.m.s.d.) values of 0.63 and 0.43 Å (for the two molecules in the P2₁2₁2₁ asymmetric unit), domain V of GTF180-ΔN II is rotated by ~120° such that it is ‘folded back’ and interacts with domains IV and B. This relocation of domain V results in a much more compact structure with dimensions of ~115 x 80 x 60 Å (compared to ~150 x 60 x 60 Å for GTF180-ΔN I). One hinge point is observed around residues 794-795 in the peptide that connects the N-terminal parts of domains V and IV (Figure 5, 6). A second hinge point is located at residues 1634-1635, in the long loop that connects the C-terminal parts of domains IV and V. A global hinge between domains IV and V is also detected in a HingeProt analysis (Emekli et al., 2008) of the crystal structures of GTF180-ΔN I, GTFA-ΔN and DSR-E ΔN₁₂₃-GBD-CD2. Notably, domain V in GTF180-ΔN II has an unaltered tertiary structure; superposition with
domain V in GTF180-ΔN I gives r.m.s.d. values of 1.27 and 1.24 Å. The relative position of domain V in GTF180-ΔN II is similar to that of domain V of ΔN123-GBD-CD2 (Brison et al., 2012). In this compact conformation, domain V interacts with residues from domains IV and B (Figure 5) mainly through solvent-mediated hydrogen bonds; not many specific interactions between side chains are observed. Finally, in GTF180-ΔN II, as opposed to the GTF180-ΔN I structure, most of the C-terminal residues are visible in the electron density map. These residues (1753-1771) form a long extended loop protruding away from the core of domain V; residues 1755-1759 form a short $3_{10}$ helix. The C-terminal $\sim$10 residues are near the active site of the enzyme in domain A, but make only a few direct hydrogen bonding interactions. The closest approach to the active site is by residue L1763; its side chain is at about 5-6 Å from where a sugar moiety in subsite +1 would be bound.

**Figure 4.** The crystal structure of GTF180-ΔN II containing 2 molecules in the asymmetric unit (left = A, right = B) in ribbon representation. Domains are colored as follows: domain C, purple; domain A, blue; domain B, green; domain IV, orange; domain V, red. Glycerol, acetate and sulfate molecules are shown in stick representation; Ca$^{2+}$ ions are shown as a green sphere. The first and last visible residues of the structure are indicated with 'N' (residue 742) and 'C' (residue 1771), respectively. The missing residues 1692-1694 in molecule B are indicated with a dotted line.
Truncated and full-length glucansucrase GTF180 from *Lactobacillus reuteri*.

**Figure 5.** Stereo figure of the superposition of the crystal structures of GTF180-ΔN I (grey, only domains IV and V are shown) and GTF180-ΔN II (molecule A; domains are colored the same as in Figure 4). Domain V makes a ~120° rotation about a hinge located between domains IV and V, near residues 794 and 1634 (indicated with a ⋆). Consequently, domain V is now near domains IV and B, while the C-terminal residues (visible up to residue 1771) bind at the protein surface near the active site cleft.

**Figure 6.** Stereo figure showing the hinge region between domains IV (orange) and V (grey: elongated GTF180-ΔN I, red: compact GTF180-ΔN II structure). In the N-terminal segment of the connection between domains IV and V, α-helix 792-801 is 2 residues shorter in the compact conformation. In the C-terminal segment of the connection between domain IV and V, a very long loop, comprising residues 1605-1640 hinges at residue 1634. The stars (⋆) indicate the approximate position of the hinges.
Small Angle X-ray Scattering experiments

To analyze the conformation of truncated and full-length GTF180 in solution, SAXS experiments were done (details are given in Table 2). As evident from Guinier plots and the P(r) distributions (Figure 8) none of the samples showed signs of aggregation (GTF180-ΔN did show a minor uptrend in low-angle scattering data; however the PRIMUS software did not label this data as aggregated).

**GTF180-ΔN.** Processing of the SAXS data from GTF180-ΔN (Table 2, Figure 7a) revealed that the experimental values of the radius of gyration $R_g$ (41 Å) and the maximum distance $D_{max}$ (143 Å) are close to the values calculated from the crystal structure of GTF180-ΔN I (44 and 156 Å, respectively), even though 4 N-terminal and 27 C-terminal residues could not be modeled in this structure. In contrast, the experimental $R_g$ and $D_{max}$ values deviate considerably from the 34 and 117 Å calculated for the GTF180-ΔN II structure. Moreover, the $\chi$ value (6.8) of the fit between the experimental SAXS data and calculated data from the GTF180-ΔN I crystal structure is much better than the $\chi$ value (16.9) obtained with the GTF180-ΔN II crystal structure (Figure 9a).

The Porod-Debye plot of GTF180-ΔN (Figure 9b) shows a plateau reached at values of $s \sim 1.0$ nm$^{-1}$. The estimated molecular mass of 93.4 kDa is somewhat lower than the theoretical molecular mass (116.7 kDa) but within the $\sim 20\%$ accuracy of the AUTOPOROD estimation (Petoukhov et al., 2012).

**Figure 7.** (a) SAXS scattering data for GTF180-ΔN, log(I) vs. s after merging the data from three different concentrations and buffer subtraction. (b) SAXS scattering data for GTF180 (red) and GTF180 + sucrose (green), log(I) vs. s. Data was recorded to $s = 6$ nm$^{-1}$ but is shown here to $\sim 2.3$ nm$^{-1}$ because of noise at high resolution.
Truncated and full-length glucansucrase GTF180 from *Lactobacillus reuteri*.

Figure 8. Guinier plots (left) and distance distributions (P(r), right) for (a,b) GTF180-ΔN, (c,d) GTF180 and (e,f) GTF180 + sucrose. In the Guinier plots, the red lines represent the Guinier fit; the green lines are the residuals.
Figure 9. (a) Results from the FoXS server; experimental SAXS scattering for GTF180-ΔN, fit with the calculated scattering curves for the elongated (blue) and compact (red) crystal structures. The calculated scattering for the elongated structure fits better and has lower errors (lower curves) than the compact structure. (b) Porod-Debye plots of the SAXS scattering data for GTF180-ΔN (blue), GTF180 (red) and GTF180 with sucrose (green). For all samples, the data reach a plateau, indicating that the molecules under study are well-folded.

The ab initio constructed low-resolution molecular shape of GTF180-ΔN in solution (using GASBOR [Svergun et al., 2001] shown in Figure 10a, b) shows a much better agreement with the elongated structure of GTF180-ΔN I than the compact GTF180-ΔN II structure. A rigid-body approach using three independent entities (domains ABC, IV, and V, respectively) to fit the SAXS data using SASREF (Petoukhov & Svergun, 2005) also resulted in an extended conformation, with a slight rotation of domains IV and V compared to their positions in the crystal structure.

**GTF180 (full-length).** Processing of the SAXS data of full-length GTF180 (Table 2, Figure 7b) gave values of $R_g$, $D_{max}$ excluded volumes and molecular mass of GTF180 that are larger than the values obtained for the truncated sample (Table 2). The estimated molecular mass of GTF180 (151 kDa) is ~22% lower than the predicted value. The Porod-Debye plot (Figure 9b) reaches a plateau at values of $s \sim 1.2$ nm$^{-1}$. The *ab initio* molecular shape of full-length GTF180 generated by GASBOR (Figure 11) is more extended compared to the truncated protein. With a bend about halfway the molecule, it has an almost symmetrical shape.
Figure 10. Ab initio constructed low-resolution molecular SAXS shape of GTF180-ΔN (grey molecular surface). (a) Superposition with the GTF180-ΔN I crystal structure (PDB ID: 3KLK; Vujičić-Žagar et al., 2010) shows a good fit. (b) Superposition with the GTF180-ΔN II crystal structure (PDB ID: 4AYG; this study); the reoriented domain V sticks out and a large part of the molecular shape is unaccounted for. (c) Superposition with the model calculated by SASREF; the three rigid bodies are colored as follows: domain V, red; domain IV, yellow; domains A + B + C, cyan. The extra C-terminal residues modeled by BUNCH prior to SASREF are shown as red spheres. With respect to the crystal structure of GTF180-ΔN I, the SASREF model shows a slight reorientation of the domains.

GTF180 (full-length) + sucrose. To assess whether any large-scale conformational changes occur upon product formation, we also determined the solution structure of active full-length GTF180 after incubation with 12.5 mM sucrose for ~12 min at 288 K. A thin-layer chromatography analysis (Figure 12) showed that under these conditions, GTF180 processed sucrose to form oligosaccharides up to a degree of polymerization (DP) of ~7. The calculated values of $R_g$, $D_{max}$, excluded volumes, and molecular mass, obtained from the SAXS measurements, are slightly smaller than those of the GTF180 sample without sucrose (Table 2). The Porod-Debye plot (Figure 9b) shows a plateau reached at $s \sim 1.2$ nm$^{-1}$. The ab initio model from GASBOR (Figure 11) is extended like the GTF180 model without sucrose, but the two ‘arms’ appear somewhat more curved.
Figure 11. Ab initio constructed molecular shapes of full-length GTF180 (a) without sucrose and (b) with sucrose. (c) Ab initio molecular shape of GTF180-ΔN, for comparison.

Figure 12. Thin-layer chromatography (TLC) analysis of the reaction products after incubating GTF180 with 12.5 mM sucrose for 12 and 30 min at 15 °C. From left to right: M, G1 - G7 malto-oligosaccharides; 1 and 2, reaction products after 12 and 30 min, respectively. During incubation, sucrose is consumed to form oligosaccharides up to a degree of polymerization of ~7.

DISCUSSION

Crystal structures have shown that domain V of glucansucrases adopt different positions with respect to the rest of the enzyme. For instance, small positional differences of domain V were seen in the elongated glucansucrase structures of GTF180-ΔN I (Vujičić-Žagar et al., 2010) and GTFA-ΔN (Pijning et al., 2012). A much larger positional
difference for domain V was observed in ΔN<sub>123</sub>-GBD-CD2, yielding a much more compact structure (Brison et al., 2012). The crystal structure of GTF180-ΔN in a different crystal form presented here revealed a compact structure similar to ΔN<sub>123</sub>-GBD-CD2; thus, in GTF180-ΔN domain V can pivot between two extreme conformations, ~120° apart (Figure 2). Given the high sequence identity of GH70 glucansucrases (30-80%) (Henrissat & Davies, 1997) and their similar makeup of core and remote domains, such intrinsic conformational flexibility may be a general feature of these enzymes. Indeed, flexibility of domain V has been proposed to facilitate transfer of growing α-glucan chains towards and away from the active site (Ito et al., 2011). However, although deletion of C-terminal sequence repeats in these domains affected enzyme activity, it did not alter product size or linkage type (Kralj et al., 2004a) and deletion of the complete domain V (both N- and C-terminal segments) did not slow down α-glucan synthesis (X. Meng, personal communication). Moreover, no sugar binding in domain V was observed using crystal soaking studies (Vujičić-Žagar et al., 2010). Thus, direct evidence for the involvement and movement of domain V during α-glucan synthesis is lacking. The two conformations of domain V observed for GTF180-ΔN offered a unique opportunity to study conformational flexibility in a single glucansucrase.

In general, domains of multi-domain proteins may adopt different conformations during crystallization depending on the precise crystallization conditions. Because of conformational freedom, flexible domains often have a higher average B-factor in crystal structures, and this is indeed the case for domain V in the GTF180-ΔN crystal structures (data not shown). Since the GTF180-ΔN I and II crystal forms were obtained from low and high ionic strength crystallization conditions (Pijning et al., 2008), respectively, we cannot exclude that the different crystal lattices of GTF180-ΔN (and thus the different conformations) result from these different conditions. Therefore, to investigate whether the different conformations of domain V also occur in a physiologically more relevant environment, we carried out SAXS experiments to determine the solution structure of GTF180-ΔN. These experiments clearly showed that in solution, GTF180-ΔN adopts only an elongated molecular shape that is very similar to GTF180-ΔN I (Figure 10); the solution structure of GTF180 is incompatible with the compact GTF180-ΔN II structure. Domains IV and V may be slightly rotated with respect to the elongated crystal structure, as suggested by the rigid-body modeling approach and in agreement with the hinges detected by HingeProt.

In full-length GTF180, the truncated ~700 N-terminal domain precedes the first residue of the crystallized construct. Considering the positions of the first visible residues (746 in GTF180-ΔN I, 742 in GTF180-ΔN II, see Figure 4), the full-length enzyme can adopt a conformation where domain V is either in an ‘extended’ or in a ‘folded’ position (corresponding to GTF180-ΔN I or GTF180-ΔN II, respectively). The ab initio model of GTF180 derived from the SAXS data (Figure 11) reveals for the first time a full-length
glucansucrase structure. In this elongated structure, the large ~700-residue N-terminal domain extends away from domain V, with which it likely forms one ‘arm’ of the boomerang-like molecular shape (~900 residues in total). The other arm then would harbor domains C, A, B and IV (~840 residues in total). A relatively small rotation of domain V (with respect to the solution structure of the truncated enzyme) about the hinge region seems to be needed to account for the observed bend between the two arms in full-length GTF180; this is compatible with our conclusions about the intrinsic flexibility of glucansucrases. In contrast, a compact, folded back conformation (like in the GTF180-ΔN II crystal structure) does not fit with the observed solution structure of full-length GTF180.

In the presence of the substrate sucrose, GTF180 has a similar conformation as observed in the absence of substrate. We chose a relatively short reaction time (12 min), low temperature (288 K) and low sucrose concentration (~650-fold excess); under these conditions, short oligosaccharides (up to DP ~7) are synthesized, but no α-glucan polymers (Figure 12). The ab initio calculated molecular shape of the enzyme (Figure 11b) shows that the ‘arms’ are somewhat more curved; the slightly decreased values of $R_g$, $D_{max}$ and macromolecular volume suggest that the enzyme adopts a slightly more compact conformation. However, a large conformational change (such as a ~120° rotation about a hinge between domains IV and V) is not observed.

Porod-Debye plots, together with calculated macromolecular volume and density can be used to distinguish between rigid and flexible proteins (Rambo & Tainer, 2011). In the Porod-Debye plots of GTF180-ΔN and GTF180 (Figure 9b), a clear plateau is visible, indicating a sharp homogenous electron density contrast between protein and solvent. In the presence of sucrose, GTF180 still displays a plateau, indicating that there is no large change in flexibility of the enzyme. This is in sharp contrast to very recent SAXS results obtained for the N-terminal domains of the cellulosome, which showed that these domains exhibit a high degree of structural dynamics in solution (Currie et al., 2013).

Possibly, GTF180 enzyme flexibility becomes important when, instead of oligosaccharides, which are the intermediate products of α-glucan synthesis, α-glucan polymers are synthesized, which may remain bound to the enzyme. Analysis of the GTF180 molecular shape at different time points during the reaction, accompanied by analysis of the product size may give more insight in the significance of glucansucrase flexibility during the synthesis of α-glucan polymers. However, since long carbohydrate chains (such as α-glucans) will contribute significantly to the scattering, a careful validation of their compatibility with SAXS measurements should be taken into account in such experiments.

In conclusion, our experiments reveal the first structural data on flexibility of domain V in a single glucansucrase. Crystal structures show that this remote domain may take up completely different positions. In solution, GTF180-ΔN adopts only an elongated...
conformation, with domain V extending away from the rest of the structure. In addition, our SAXS experiments provide the first insights into the molecular shape of a full-length glucansucrase. The ~700-residue N-terminal domain of the α-1,6/α-1,3 specific GTF180 from *L. reuteri* 180 extends the previously determined elongated crystal structure, resulting in a boomerang-like molecular shape. At this stage a precise assignment of the location of the domains in the molecular shape was not possible, requiring a crystal structure of a full-length glucansucrase; experiments towards this goal are currently in progress in our laboratories. We did not detect a large hinging motion of domain V (and the N-terminal domain connected to it); in fact, the compact conformation of GTF180-ΔN II was not observed in solution. Thus, although intrinsic flexibility may be a general feature of GH70 glucansucrases, under the experimental conditions used here (leading to the synthesis of short oligosaccharides), large conformational changes do not appear to play a significant role during catalysis.

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SUPPORTING MATERIAL

Construction of GTF180-ΔNΔV

The gtf180ΔNΔV gene fragment (encoding GTF180 amino acids 794-1636) was amplified from p15GTF180-ΔN (Vujičić-Žagar *et al*., 2010) using primers FwdV 5’-GATGCATGAGCTCCCATGGATGAGCAATATCGAGGAAATGAAGCC-3' and RdV 5’-ATATCGAGGGCCCCGGATCCTATTAGTGATGGTGATGGTGATGATCTTGAACTGATAATTGAGACGGTAAGAA-3’ and cloned in pET15b for expression. GTF180-ΔNΔV contains a C-terminal fused (His)_6 tag. The removal of domain V is based on the 3D structure of GTF180-ΔN (Vujičić-Žagar *et al*., 2010).

Expression and purification of GTF180-ΔN and GTF180-ΔNΔV

The recombinant GTF180-ΔN and GTF180-ΔNΔV enzymes were expressed and purified as previously described (Kralj *et al*., 2004a).
Enzymatic assays

Briefly, 300 nM of GTF180-ΔN or GTF180-ΔNΔV enzyme was incubated with 0.1 M and 1.0 M sucrose for 120 h at 37 °C in 25 mM sodium acetate buffer, pH 4.7, 1 mM CaCl₂. Aliquots were taken at different time points for thin-layer chromatography (TLC) analysis of products formed.

Thin layer chromatography (TLC)

Samples of 1 μl were spotted on TLC sheets (Merck silica gel 60 F254, 20 ×20 cm). A mixture of gluco- and malto-oligosaccharides (G1-G7, maltose to maltoheptaose) was used as standard. The TLC plates were developed with 2-butanol : acetic acid : water (2 : 1 : 1) and stained with orcinol/sulfuric acid (Figure 13).

![Figure 13](image-url)
Incubation of 300 nM GTF180-ΔN and GTF180-ΔNΔV with 0.1 M and 1.0 M sucrose clearly showed that both enzymes were active and produced oligosaccharides and polysaccharides. With 0.1 M sucrose, both enzymes quickly consumed sucrose and produced oligosaccharides and polysaccharides within 2 hours. With 1.0 M sucrose, both enzymes clearly produced lower amounts of polysaccharides than oligosaccharides. Only after 4 days of incubation, 1.0 M sucrose was depleted by both enzymes. The exact amounts and structures of oligosaccharides and polysaccharides formed by both enzymes under these conditions still need to be determined.