**Thermus thermophilus** Glycoside Hydrolase Family 57 Branching Enzyme

**CRYSTAL STRUCTURE, MECHANISM OF ACTION, AND PRODUCTS FORMED**

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Branching enzyme (EC 2.4.1.18; glycogen branching enzyme; GBE) catalyzes the formation of α,1,6-branching points in glycogen. Until recently it was believed that all GBEs belong to glycogen hydrolase family 13 (GH13). Here we describe the cloning and expression of the *Thermus thermophilus* family GH57-type GBE and report its biochemical properties and crystal structure at 1.35-Å resolution. The enzyme has a central (β/α)7-fold catalytic domain A with an inserted domain B between β2 and α5 and an α-helix-rich C-terminal domain, which is shown to be essential for substrate binding and catalysis. A maltotriose was modeled in the active site of the enzyme which suggests that there is insufficient space for simultaneously binding of donor and acceptor substrates, and that the donor substrate must be cleaved before acceptor substrate can bind. The biochemical assessment showed that the *Thermus* GBE possesses about 4% hydrolytic activity with amylose and in vitro forms a glucan product with a novel fine structure, demonstrating that the GH57 GBE is clearly different from the GH13 GBEs characterized to date.

Glycogen is the major energy reserve polymer of many animals and microorganisms. It consists of a backbone of glucose residues linked by α1,4-bonds with α1,6-side chains (1, 2). In bacteria, at least three enzymes are required for glycogen biosynthesis: ADP-pyrophosphorylase, which catalyzes ADP-Glc synthesis (the main regulatory step of the biosynthesis), glycogen synthase, which is involved both in initiation and elongation of the α1,4-linear chain (3), and glycogen branching enzyme (GBE), which introduces the side chains. GBE or 1,4-α-glucan branching enzyme (EC 2.4.1.18) catalyzes the formation of α1,6-branching points, by cleaving an α1,4-glycosidic bond and transferring the cleaved-off oligosaccharide to a glucan chain via an α1,6-bond. Until recently it was believed that all GBEs belong to glycose hydrolase family 13 (GH13) subfamilies 8 (*Eukaryota*) or 9 (*Bacteria*) (4), until Imanaka and co-workers (5) identified a GBE in *Thermococcus kodakarenensis* KOD1 (TkGBE) that was not a GH13 enzyme but was classified as a GH57 family.

The GH family 57 was established as a new family in 1996 (6) on the basis of two α-amylase sequences that lacked the conserved sequence regions characteristic of GH13 enzymes. Members of GH57 display high sequence diversity, their lengths vary from 400 to 1500 amino acid residues, and they contain five conserved amino acid regions including the two proposed catalytic residues (7). They are believed to be α-retaining enzymes (7), i.e. acting on α-glycosidic linkages and generating α-linked products, via a double-displacement reaction mechanism in which catalysis proceeds via a glycosyl-enzyme intermediate. The enzyme specificities found in family GH57 are α-amylase (EC 3.2.1.1), 4-α-glucanotransferase (EC 2.4.1.25), α-galactosidase (EC 3.2.1.22), amylomaltase (EC 3.2.1.41), and branching enzyme (EC 2.4.1.18). The latter reaction specificity was only added in 2006 (5). Subsequently, several genes were identified in other microorganisms with similarity to the GH57 TkGBE. These putative GH57 GBE enzymes are present in many members of the domain Bacteria, whereas in the domain Archaea they only have been found in the *Thermococcales*.

A putative GH57 GBE was also identified in the thermophilic Gram-negative bacterium *Thermus thermophilus* HB8, one of the few microorganisms from which glycogen has been isolated and characterized (8). With an average chain length of seven glucose units, the glycogen from *T. thermophilus* is one of the most highly branched glycogens known. However, no GBE has been annotated in its genome sequence.

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Characterization of T. thermophilus Family GH57 GBE

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The T. thermophilus HB8 gene TTHA1902 encodes a 520-amino acid protein (TtGBE) that is 43% identical to the T. kodakaraensis KOD1 GBE (TkGBE) and contains the five conserved family GH57 sequence motifs including the putative catalytic nucleophile Glu-184 and acid/base Asp-353. Moreover, it possesses the D region thought to be typical for the GH57 GBEs subfamily (5).

Here we describe the cloning and expression of the T. thermophilus GH57-type GBE and report its biochemical properties and crystal structure. The enzyme consists of a central catalytic domain A with an inserted domain B and an α-helix-rich C-terminal domain, which is shown to be essential for substrate binding and catalysis. The biochemical assessment showed that the GH57 GBE possesses about 4% hydrolytic activity and forms a glucan product with relatively short branches.

EXPERIMENTAL PROCEDURES

Molecular Techniques

General procedures for DNA manipulation and cloning were done as described (9). Restriction endonucleases and T4 DNA ligase were from Fermentas GMBH and were used as recommended by the supplier. Primers were obtained from Eurogentec and sequencing was performed by GATC Biotech (Germany).

Cloning of T. thermophilus GBE Gene

T. thermophilus HB8 was supplied by JMC RIKEN BRC (Japan). It was grown in TM medium at 70 °C and its chromosomal DNA was isolated. The gene TTHA1902 was amplified from genomic DNA by PCR using Expand High Fidelity polymerase (Fermentas) and oligonucleotides Tt-FP/Tt-RP (supplemental Table S1) and the product was cloned into pCR-XL-TOPO (Invitrogen) resulting in pTt-TOPO plasmid. For expression of the protein, the gene was cloned into pET15b (Novagen) using Ndel and BgII restriction sites, leading to the pTt plasmid, which encodes TtGBE with an N-terminal His tag.

Construction of Truncated Genes and Mutants

The gene fragment encoding the catalytic domain of TtGBE (amino acids 1–420) was amplified using Expand High Fidelity polymerase (Fermentas) with pTt as template and primers Tt-FP and TtC-5′-RP (supplemental Table S1) and cloned into the Ndel and BgII sites of pET15b (Novagen) yielding plasmid pTtC. Similarly, the gene fragment encoding the DUF1957 domain (the C-terminal amino acids 420–520) was amplified from pTt using Pfu DNA polymerase (Fermentas) and primers Tt-FP and TtC-5′-RP (supplemental Table S1) and cloned into BamHI and Sall sites of pGEX-4T-3 (GE Healthcare) yielding pGEX-DUF1957. In this vector the DUF1957 gene fragment is fused downstream of a gst gene as a single open reading frame.

Mutations were introduced in pTt-TOPO by the QuickChange site-directed mutagenesis kit (Stratagene) using appropriate primer pairs (supplemental Table S1). After confirmation of the mutations by DNA sequencing the mutated genes were cloned into the Ndel and BglII sites of pET15b (Novagen).

Expression and Purification

Overexpression of all proteins, except for the TtGBE-C domain, was achieved by inoculation of Escherichia coli BL21(DE3) Star cells containing the corresponding plasmid at 37 °C and 210 rpm in Luria-Bertani (LB) medium supplemented with 50 μg/ml of ampicillin. The cultures were induced with 1 mM isopropyl-β-D-galactopyranoside at 37 °C for 5 h.

TtGBE-C domain was expressed as a C-terminal fusion to the glutathione S-transferase (GST) protein in E. coli C41 cells cultivated in LB medium. Protein expression was induced at an A600 of 0.4 with 0.1 mM isopropyl-β-D-galactopyranoside for 25 h at 18 °C. Cells were harvested by centrifugation, resuspended in 50 mM sodium phosphate buffer (pH 8.0), and disrupted by sonication. The proteins were purified by His tag affinity chromatography using a HiTrap Chelating column (GE Healthcare) charged with nickel sulfate and eluted with a linear gradient of 0–500 mM imidazole on an Äkta prime purification system (Amersham Biosciences). Fractions containing the protein of interest were pooled and dialyzed against sodium phosphate buffer (25 mM, pH 6.5).

Protein Determination and PAGE

Protein concentrations were determined by measuring the absorbance at 280 nm on a Nanodrop ND-1000 spectrophotometer (Isogen Life Science) and their extinction coefficients were estimated according to the method of Gill and von Hippel (11).

Protein purity and molecular mass were analyzed by SDS-PAGE, using the PageRuler Prestained Protein Ladder (Fermentas) as a standard and Bio-Safe Coomassie stain (Sigma) affinity chromatography following the recommendations of the manufacturer. The Deinococcus geothermalis DgGBE and DgGBE3D22A proteins were expressed and purified as previously reported (10).

Thermal Unfolding

Thermal unfolding of wild type TtGBE and mutants was monitored using the environmentally sensitive fluorescent dye Sypro Orange (Invitrogen) (12). The assay volume was 25 μl and the protein was at a final concentration of 0.5 mg/ml in sodium phosphate buffer (25 mM, pH 6.5) and 500 times diluted Sypro Orange. Samples were assayed using an iQ5 real time PCR detection system (Bio-Rad) at a temperature gradient of 20 to 95 °C with 0.5 °C steps every 20 s, whereas continuously monitoring the fluorescence with excitation/emission wavelengths at 490/530 nm.

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Circular Dichroism

Multiple far UV spectra (195–260 nm) were recorded in sodium phosphate buffer at pH 6.5 and 20 °C using a concentration of 0.1 mg/ml on a Jasco J-715 spectropolarimeter (Jasco, Japan) in quartz cells with an optical path length of 1 mm; data interval 0.2 nm; bandwidth 1.0 nm; sensitivity 20 mdeg; and response time 0.125 s. All recorded spectra were corrected by subtraction of the spectrum of the buffer.

Enzyme Activity Assays

Iodide Assay—The total activity of the enzymes was measured using the iodide assay (13), which is based on formation of a blue complex between iodine-iodide and a linear α,1,4-glucan of a certain length. The sum of the transglycosylation and hydrolytic activities of the enzymes was measured as previously described (10) using 0.0031–0.125% (w/v) amylose V (AVEBE) or 0.25% potato amylpectin type III (Sigma) in 25 mM sodium phosphate buffer at pH 6.5 and 65 °C with 15–25 μg/ml (mutant) TtGBE enzyme. One unit of enzyme activity is defined as the decrease in absorbance of 1.0 per min at 660 nm for amylose and at 530 nm for amylpectin.

To determine the optimal assay conditions for TtGBE activity, the assay was performed at temperatures ranging from 30 to 80 °C and pH 4 to 8 in phosphate-citrate buffer. For thermostability studies, the enzyme (0.5 mg/ml) was incubated in the absence of substrate for 1 h at different temperatures from 50 to 95 °C, and the residual activity in the supernatant (after centrifugation at 13,000 × g for 1 min) was measured at 65 °C.

Branching Assay—This method allows determination of the amount of α,1,6-branch points introduced by measuring the difference in the amount of reducing ends before (hydrolytic activity) and after debranching (total activity) of the product by isoamylase (14). It was performed as previously described (10) by incubating 0.5–2 μg/ml of enzyme (TtGBE or DgGBE) with 0.125% amylose V (AVEBE) or 0.25% amylpectin type III (Sigma). One unit of GBE branching activity is defined as 1 μmol of α,1,6-linkages synthesized per min.

Substrate Utilization by TtGBE

TtGBE (10 μg/ml) was separately incubated with maltoligosaccharides of degrees of polymerization (DP) 2–7, β-cyclodextrin (β-CD), 6-O-glucosyl-β-cyclodextrin (6-O-Glc-β-CD) or 6-O-maltosyl-β-cyclodextrin (6-O-Mal-β-CD), all at a concentration of 1 mM, in 25 mM phosphate buffer (pH 6.5) at 65 °C for 16 h. The incubations were repeated in the presence of 0.125% amylose V (AVEBE).

Analysis of Chain Length Distribution

TtGBE action on polysaccharides was investigated by a 72-h incubation at 65 °C of the enzyme (20 μg/ml) in 25 mM phosphate buffer (pH 6.5) with 10 ml of 0.125% amylose V (AVEBE) or 0.25% amylpectin type III (Sigma). Samples (2 ml) were taken at different time points and the reaction was terminated by boiling the mixture for 10 min. The reaction mixture was divided in two equal parts; one part was treated with 10 units of isoamylase from Pseudomonas sp. (Megazyme) (involving addition of 750 μl of 1 m citrate buffer, pH 4.0, and incubation for 20 h at 40 °C) to hydrolyze α,1,6-glycosidic linkages.

The chain length distribution of glucans before and after debranching was analyzed by high performance anion-exchange chromatography (HPAEC) with a pulse amperometric detector ( Dionex) as previously described (10).

1H NMR and Two-dimensional NMR Spectroscopy

Resolution-enhanced one-dimensional/two-dimensional 500-MHz 1H NMR spectra were recorded in D2O on a Bruker DRX-500 spectrometer (Bijvoet Center, Department of NMR Spectroscopy, Utrecht University) at probe temperatures of 300 K. Prior to analysis, samples were exchanged twice in D2O (99.9% atom D, Cambridge Isotope Laboratories, Inc., Andover, MA) with intermediate lyophilization, and then dissolved in 0.6 ml of D2O. Suppression of the HOD signal was achieved by applying a WEP pulse sequence for one-dimensional experiments and by a 1-s pre-saturation during the relaxation delay in two-dimensional experiments. The two-dimensional TOCSY spectra were recorded using an MLEV-17 mixing sequence with spin-lock times of 20–200 ms. The two-dimensional NOESY spectra were recorded using standard Bruker XWINNMR software with a mixing time of 200 ms. The carrier frequency was set at the downtown edge of the spectrum to minimize TOCSY transfer during spin locking. Resolution enhancement of the spectra was performed by a Lorentzian to Gaussian transformation for one-dimensional spectra or by multiplication with a squared-bell function phase shifted by π/(2.3) for two-dimensional spectra, and when necessary, a fifth order polynomial baseline correction was applied. 1H chemical shifts (δ) are expressed in ppm by reference to internal acetone (δ 2.225).

Ultracentrifugation Protein Binding Assay

Proteins (1 mg/ml) were incubated with different concentrations (0.078–2.5 mg/ml) of amylose or glycogen at 4 °C for 30 min followed by ultracentrifugation 200,000 × g at 4 °C for 45 min. In case of binding, the amount of protein present in the supernatant decreased proportionally to the concentration of polysaccharide.

Isothermal Titration Calorimetry (ITC)

Measurements were done on an iTC200 Microcalorimeter (MicroCal) at 20 °C. The proteins were dialyzed overnight in 25 mM phosphate buffer at pH 6.5 (TtGBE_E184A, TtGBE-ΔC, and TtGBE-C domain) or 8.0 (DgGBE_D322A). 50–100 μM protein was introduced into the sample cell and titrated with aliquots of the ligand solution. The ligand solutions were prepared by dissolving the glucans in dialysis buffer (10 mg/ml for glycogen and 2.5 mM for the cyclic glucans). Data analysis was carried out using the “one binding site” model of the MicroCal version of ORIGIN, leaving all parameters floating.
In Vitro Synthesis of Branched α-Glucan by Tandem Action of Potato Phosphorylase and TtGBE

Synthesis of α-glucan by a tandem reaction of GBE and potato phosphorylase was performed as previously described (15). Briefly, maltotetraose (0.5 mM), Glc-1-P (50 mM), potato phosphorylase (2.2 units/ml), and TtGBE (35 μg/ml) were dissolved in 5 ml of citrate buffer (pH 6.2, 50 mM). This solution, and equilibrated over 500 units/ml of GBE (2.2 units/ml), and TtGBE (35 μg/ml) were dissolved in 5 ml of citrate buffer (pH 6.2, 50 mM). This solution was incubated for 3 days at 38 °C. The reaction was followed by measuring the released amount of phosphate with the modified method of Fiske and Subbarow (16). Upon reaching equilibrium conditions the reaction was stopped by boiling for 5 min. Denatured enzymes were removed by means of centrifugation. Dialysis (Spectra/Por dialysis membrane MWCO 1000) and lyophilization of the remaining solution yielded the branched polysaccharides.

Construction of ΔTTHA1902 Strain

The TTHA1902 gene was disrupted by replacement with the highly thermostable kanamycin nucleotidyltransferase (HTK) gene (17). The plasmid vector containing the TTHA1902 region disrupted by the highly thermostable kanamycin nucleotidyltransferase gene was purchased from RIKEN Biological Resource Center (Tsukuba, Japan) (18). Thermus thermophilus HB8 cells were transformed according to Hashimoto et al. (19) and mutant colonies were selected on a plate containing 500 mg/liter of kanamycin at 75 °C. The replacement of the TTHA1902 gene by the kanamycin-resistance marker was confirmed by genomic PCR.

Crystallization

Crystallization experiments with TtGBE were performed using the hanging drop vapor diffusion method. Drops were prepared by mixing 1.25 μl of protein solution (4.7 mg/ml in 25 mM HEPES-NaOH, pH 7.0) with 1.25 μl of reservoir solution, and equilibrated over 500 μl of reservoir solution at 293 K. From the initial commercial screens, the PEG/Ion2 Screen (Hampton Research, Aliso Viejo, CA) gave many hits. The condition containing 20% (w/v) PEG 3350, 8% (v/v) Tacsimate (pH 7.0) yielded crystals suitable for data collection. These crystals grew to a maximum size of about 400 × 250 × 150 μm; they were cryo-protected using 40% (w/v) PEG 3350, 5% (v/v) Tacsimate (pH 7.0).

Data Collection and Structure Determination

A native data set was collected at 100 K, at beamline ID14-2 of the ESRF (Grenoble, France). Diffraction images were indexed and integrated with the program MOSFLM (20) and scaled with SCALA (CCP4) (21). Statistics are given in supplemental Table S2.

The structure of TtGBE was solved with the MolRep (22) molecular replacement method using diffraction data between 33.2 and 3.0 Å, and the coordinates of TT1467 from Thermococcus litoralis (23) (PDB code 1UFA) as a search model.

Refinement and Model Building

Refinement was performed at a resolution of 1.35 Å, using the program REFMAC5 (24), alternating cycles of restrained refinement and manual building in COOT (25). At a later stage TLS refinement (24) was also included. The final model includes protein residues −1 to 234 (2 extra residues were built at the N terminus that are part of the linker between the His tag and the protein) and 243–517 (see supplemental Table S2) and has R-factors of 0.170 and 0.189 (Rcryst/Rfree). The quality of the model was checked using the program MolProbity (26). Figures were prepared with PyMOL (27). The coordinates and structure factor amplitudes have been deposited with the PDB (accession code 3P0B).

Modeling

A model for bound donor substrate was obtained starting from Thermococcus litoralis GH57 α-glucanotransferase (TIGT)-acarbose complex (28). A maltotriose (G3) was superposed on the acarbose residues in the −1, +1, and +2 subsites and the glycosidic torsion angles were adjusted such that clashes with TtGBE residues were avoided and the hydrogen bond between O2 and O3 of subsites +1 and +2 was maintained. Energy minimization of the TtGBE-G3 complex was performed with REFMAC5 (24); all protein residues except the two TtGBE catalytic residues Glu-184 and Asp-353 were excluded from refinement, and 11 water molecules occupying the 3 subsites were omitted.

RESULTS AND DISCUSSION

The T. thermophilus Genome Encodes a GH57 Glycogen Branching Enzyme (TIGB)—T. thermophilus HB8 TTHA1902 protein (TtGBE) was expressed in E. coli and purified to homogeneity, yielding 7 mg of pure protein/liter of culture. Its molecular mass is 60 kDa as determined by SDS-PAGE, in accordance with the calculated value of 59.2 kDa. TtGBE branching activity was demonstrated by incubating it with amylase as substrate followed by TtGBE inactivation and subsequent isoamylase (which hydrolyzes α1,6-branches) treatment of the reaction products. HPAEC analysis showed the release of maltodisaccharides, thus providing clear evidence that TtGBE is a branching enzyme (Fig. 1C). TtGBE showed maximal activity at pH 6.5 and 65 °C, and the enzyme remained fully active following incubation for 1 h at 80 °C. Mutation of the putative catalytic nucleophile (Glu-184) or the putative catalytic acid/base (Asp-353) into alanine yielded virtually inactive enzymes (Table 1) even though they were properly folded as confirmed by circular dichroism (CD) spectrometry (data not shown).

Specific activity on amylase was measured by the branching assay, which allows discrimination between branching activity and hydrolytic activity (10). The main activity of the enzyme is introduction of new α1,6-glycosidic linkages with an activity of 0.29 units/mg of protein; a secondary activity is the hydrolysis of the substrate, being 4% of the total activity (Fig. 1, A and B). This hydrolysis activity is remarkably high considering that the hydrolytic activity of GH13 GBEs is below the detection limit of this assay (10). Thus, the T. thermophilus HB8 GH57 GBE enzyme is clearly more hydrolytic than GH13 GBEs.
Introduces α1,6-Branch Points via an α-Retaining Mechanism—GH57 family enzymes are believed to utilize the classical Koshland double-displacement mechanism with retention of the anomeric configuration (Fig. 2A) (28, 29). They are thus expected to synthesize products with the α-configuration, but direct experimental evidence for this has been lacking (30). Because GBEs introduce α1,6-branch points that are virtually absent in the amylose substrate it is relatively simple to determine the anomeric configuration of the newly formed glycosidic bonds. 1H NMR analysis of the product mixture obtained after incubation of amylose with TtGBE demonstrated that the enzyme forms α1,6-glycosidic bonds.
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TtGBE-E184A mobility was slowed down in gels supplemented with amylose but not with glycogen. Also ITC showed no binding between glycogen and TtGBE-E184A (data not shown). The fact that TtGBE does not bind highly branched α-glucan is in accordance with the preference of the enzyme for linear substrates (amylose) over branched structures (amylopectin).

ITC was also applied to study the binding affinity of TtGBE for the soluble ligands maltotetraose and (substituted) β-cyclodextrins. TtGBE-E184A bound the cyclic compounds but binding of maltotetraose was not detectable. It is not clear where β-cyclodextrin binds in TtGBE-E184A (active site versus more remote regions) as it is not used as a substrate. None of the mutations introduced in the enzyme (see below) had a major effect on its binding constant, although some of them strongly affected enzyme activity (Table 1). TtGBE is not active on maltotetraose (see above) and the enzyme does not bind it, suggesting that this maltoligosaccharide is too short to generate strong interactions.

The same binding experiments were performed with a catalytically inactive mutant (D322A of the D. geothermalis GH13 GBE (DgGBE)) (10). Interestingly, and opposed to TtGBE-E184A, DgGBE-D322A binds amylose, amylopectin, glycogen, and maltotetraose but not the (substituted) β-cyclodextrins. Thus, these GH13 and GH57 GBEs clearly differ in binding specificity, but whether this is a general phenomena is unclear due to a lack of ligand binding studies in literature.

In Vitro TtGBE Makes a Product with Short Side Chains and a Low Degree of Branching—To characterize the in vitro branching properties of TtGBE, the progress of amylose branching and hydrolysis was followed in time by HPAEC analysis (Fig. 1C). The hydrolysis products had DPs of 3–13 with two local maxima at DP 7 and DP 11. HPAEC analysis after debranching revealed that TtGBE transfers side chains from DP 4 until DP 16 with a clear preference for DP 6 chains. This GH57 enzyme thus abundantly produces short side chains compared with most GH13 GBEs, for which the preferred DP of chains transferred lies between DP10 and DP14 (10). Furthermore, the length of the chains transferred by TtGBE is constant through the reaction.

The TtGBE side chain distribution is in agreement with the average chain length of 7 glucose residues found for the glyco-gen isolated from T. thermophilus (8). In general, short side chains are associated with a high degree of branching. However, its 1H NMR analysis showed a low degree of branching of 3% with amylose as substrate (data not shown). The only other GH57 GBE described, TkGBE (5), displays very similar hydrolytic and branching patterns (regarding chain lengths) but its degree of branching has not been reported. Thus, GH57 TtGBE (and most likely also GH57 TkGBE) converts amylose in an α-glucan product with a low degree of branching and short side chains, that is, with a novel overall structure.

In Vitro Glycogen Synthesis of Branched α-Glucan by Tandem Action of Potato Phosphorylase and TtGBE—The low degree of branching of 3% achieved by TtGBE with amylose raised the question of the in vivo role of the enzyme as it was reported that T. thermophilus makes one of the most highly branched glycogens known with a degree of branching of ~14% (8). In the in vivo glycogen synthesis, GBE branching

TABLE 1
Kinetic properties of TtGBE wild type and mutants

All activities were measured with amylose as substrate; mutant activities are given in percentages compared to TtGBE wild type. All mutants displayed the same melting temperature as TtGBE wild type (95 ± 5°C), implying proper folding.

| Enzyme       | Total activity | Branching activity | Hydrolytic activity | $K_{aa}$ amylose |
|--------------|----------------|--------------------|---------------------|-----------------|
| TtGBE WT     | 100            | 100                | 100                 | 39 ± 18         |
| F23A         | 82             | 81                 | 117                 | 41 ± 8          |
| E184AA      | <0.01          | ND                 | ND                  | 0.01 ND         |
| Y236AA      | 38             | <1                 | 950                 | 50 ± 9          |
| L271A        | 91             | 91                 | 100                 | 49 ± 12         |
| W274A       | 0.4            | ND                 | ND                  | ND              |
| D353A       | <0.01          | ND                 | ND                  | ND              |
| W360A       | 0.6            | ND                 | ND                  | ND              |
| W404A       | 0.4            | ND                 | ND                  | ND              |
| F463A       | 0.5            | ND                 | ND                  | ND              |

$^a$ Catalytic nucleophile.
$^b$ ND, not determined.
$^c$ Catalytic acid/base.
$^d$ Residue conserved in the TtGBE subfamily (5).
$^e$ Residue conserved in GH57 family (7).

The assignment of residues A, B, C, D, and R were confirmed by two-dimensional TOCSY and NOESY (supplemental Fig. S1). The two-dimensional NMR analysis also provided evidence that TtGBE is exclusively branching, i.e. synthesizing only α,1,6-branch points, and is not forming linear α,1,6-bonds. This is the first enzyme from the GH57 family proven to operate via an α-retaining mechanism.

TtGBE Has a Strong Preference for Amylose—As shown above, amylose is a suitable substrate for TtGBE. To explore its substrate spectrum in more detail, the possible utilization of small glucans as donors and/or acceptors was examined by incubating TtGBE with α,1,4-linked oligosaccharides of 2–7 glucose residues, β-CD, 6-O-Glc-β-CD, or 6-O-Mal-β-CD, in both the absence and presence of amylose. Analysis of the reaction products before and after isoamylase treatment revealed that the enzyme uses amylose but none of these small molecules as donor or acceptor substrates (data not shown).

TtGBE also uses amylopectin as substrate, as revealed by iodine staining, although at a 10-fold lower rate than amylose (data not shown). The same preference for amylose has been observed for the TtGBE ortholog in T. kodakaraensis KOD1, TkGBE. The branching assay, surprisingly, revealed that TtGBE did not branch amylopectin further; it only hydrolyzed amylopectin (Fig. 1, A and B). Activity with the even more branched glycogen was also studied; this polymer is not further branched nor is it hydrolyzed by TtGBE. Thus TtGBE prefers long and linear α,1,4-linked substrates.

TtGBE Glucan Binding Pattern—Subsequently, we investigated TtGBE substrate binding. Ligand binding was assessed with a catalytically inactive TtGBE mutant (E184A) using ITC, ultracentrifugation binding assay, and native polyacrylamide gel electrophoresis (Table 2). The ultracentrifugation assay demonstrated that TtGBE-E184A binds amylose but not glycogen (Fig. 3, Table 2). The same observations were made using native polyacrylamide gel electrophoresis, where (Fig. 2), indicated by anomeric signals at δ 3.85 (residue C) and δ 4.970 (residue D), with J coupling constants <4 Hz. The assignment of residues A, B, C, D, and R were confirmed by two-dimensional TOCSY and NOESY (supplemental Fig. S1 and Table S3). The two-dimensional NMR analysis also provided evidence that TtGBE is exclusively branching, i.e. synthesizing only α,1,6-branch points, and is not forming linear α,1,6-bonds. This is the first enzyme from the GH57 family proven to operate via an α-retaining mechanism.

H. Takata and T. Kuriki, personal communication.
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A) Chemical structures showing reaction mechanisms.

B) NMR spectrum with peaks labeled A, B, C, Rα, D, Rβ.

C) Additional NMR spectrum with peak marked with an asterisk.
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revealed a degree of branching of 10% (supplemental Fig. S2), over 3-fold higher than the one obtained by incubating the enzyme with amylose and close to the 14% degree of branching of glycogen reported for T. thermophilus (8). This demonstrates that the properties of the products made by TtGBE are heavily dependent on the substrate characteristics and incubation conditions. The combined observations also provide evidence that TtGBE in vivo is acting as branching enzyme for glycogen synthesis in T. thermophilus. We successfully constructed a gene deletion mutant of T. thermophilus (ΔTTHA1902) revealing that the enzyme is not essential for growth as expected for an enzyme involved in the biosynthesis of a storage compound (data not shown). This provides additional possibilities to further study the in vivo role of GH57 GBEs in future work.

Crystal Structure of TtGBE—TtGBE was crystallized from entirely different conditions than reported for TT1467 in the Protein Data Bank (PDB entry 1UFA by Idaka et al. (23)), but otherwise unpublished, as part of the Structural-Biological Whole Cell Project of T. thermophilus HB8, nevertheless, resulting in the same crystal form as indicated by the space group and cell parameters. However, due to better diffraction properties, the structure could be elucidated at much higher resolution (1.35 Å) allowing a more accurate description of the structure. The final model comprises the complete protein, except residues 235–242 and the three C-terminal residues 518–520, for which no electron density was visible. Nineteen residues were built with alternate side chain conformations.

TtGBE consists of three domains (Fig. 4A; see the supplemental Figs. S3 and S4 for the electron density map of the active site and the topology of TtGBE). The central domain A (residues 1–61 and 122–414) harbors a rare (β/α), barrel formed by two distorted sheets composed of B4/B7/B9 and B10/B1/B2/B3, respectively, with a lack of closure between B9 and B10. The first sheet of the barrel is extended by the parallel addition of B11 interacting with the N-terminal end of B1; the second sheet is extended by the antiparallel addition of B8 and B12. Long loop regions with 3_10-helices and an additional small 2-stranded antiparallel β-sheet (β5/β6) are mainly located at the N-terminal side of the (β/α)_7 barrel. Domain B (residues 62–121) is an insertion into domain A after B2, and contains three α-helices. The C-terminal domain C (residues 415–517) is formed by four α-helices arranged in a tight bundle, and two 3_10-helices.

TtGBE displays the highest structural similarity to the GH57 α-amylase AmyC from Thermotoga maritima (TmAmyC, 31% sequence identity) (32) with a root mean square deviation of 1.06 Å for 326 Ca atoms. Domain A of TtGBE

activity is coupled with chain elongation. This is completely different from incubation of the enzyme with amylose. Therefore, we performed a tandem reaction of TtGBE with potato phosphorylase, using maltoheptaose and glucose 1-phosphate. However, we performed a tandem reaction of TtGBE with potato phosphorylase, using maltoheptaose and glucose 1-phosphate, and found that the enzymes with amylose, had the same degree of branching intermediate. The second deglycosylation step involves the attack by the acceptor molecule assisted by the conjugated base of the general acid residue which protonates the glycosidic oxygen and the catalytic nucleophile attacks the anomeric carbon leading to the formation of a covalent glycosyl-enzyme complex.

Proper folding of the mutants and separate TtGBE domains was confirmed by CD spectroscopy (data not shown). Glycogen binding properties of various T. thermophilus HB8 TtGBE proteins: mutant TtGBE_C1944A, TtGBE wild type with deleted C-domain, and the separately expressed and purified TtGBE C-domain, and the D. geothermalis DgGBE322A protein, determined by different methods. Proper folding of the mutants and separate TtGBE domains was confirmed by CD spectroscopy (data not shown).

TABLE 2

| Protein       | Maltoheptaosea | Amyloseb, c | Glycogene, f | β-CD, g, h | 6-0-Glc-β-CD, i 6-0-Mal-β-CDj |
|---------------|----------------|-------------|-------------|-----------|-------------------------------|
| TtGBE         | –              | +           | –           | +         | –                             |
| TtGBE AC     | –              | –           | NDk         | –         | +                             |
| TtGBE C      | –              | –           | NDk         | –         | –                             |
| TtGBE domain | –              | –           | –           | –         | –                             |
| DgGBE        | +              | +           | +           | –         | –                             |

a Determined by ITC.

b Determined by ultracentrifugation binding assay.
c Determined by native gel.
d ND, not determined.

Protein binding (the relative amount of protein measured in the supernatant after centrifugation from its initial amount of protein).

FIGURE 3. Glucan binding capacity analysis of TtGBE (solid lines, open symbols) and DgGBE (dashed lines, closed symbols) by ultracentrifugation assay. Catalytically inactive mutants of the enzymes were incubated with different concentrations of amylose (circles) and glycogen (diamonds). The amount of protein bound was calculated by subtracting the unbound protein measured in the supernatant after centrifugation from its initial amount of protein.

FIGURE 2. A, schematic representation of the α-retaining mechanism of GH57 TtGBEs. In the first step, the glycosylation step, the general acid catalyst aspartate protonates the glycolic oxygen and the catalytic nucleophile attacks the anomeric carbon leading to the formation of a covalent glycosyl-enzyme intermediate. The second deglycosylation step involves the attack by the acceptor molecule assisted by the conjugated base of the general acid residue leading to the transglycosylation product. The α-anomeric configuration is retained: an α,1,4-bond is cleaved and an α,1,6-linkage is generated. B, one-dimensional 1H NMR spectrum of amylose recorded in D2O at 300 K before the incubation with TtGBE. C, one-dimensional 1H NMR spectrum of the product mixture recorded in D2O at 300 K after the incubation of amylose with TtGBE in phosphate buffer (pH 6.5) at 65 °C for 48 h. The anomeric protons in the one-dimensional spectra are indicated as follows: A corresponds to H-1 of internal -(1-→4)-α-D-Glcp-(1-→4) units; B corresponds to H-1 of terminal -(1-→4)-α-D-Glcp-(1-→4) units; C corresponds to H-1 of branched -(1-→4)-α-D-Glcp-(1-→4) units; D corresponds to H-1 of -(1-→4)-α-D-Glcp-(1-→4) units attached to branching points; Ra corresponds to H-1 of reducing -(1-→4)-α-D-Glcpα units; Rβ corresponds to H-1 of reducing -(1-→4)-α-D-Glcpβ units. Asterisk means non-carbohydrate contaminant.
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A stereo figure of the overall structure of TtGBE in ribbon representation; the catalytic residues are shown in stick representation. Domain A, purple with core β-strands in red and additional β-strands in orange; domain B, blue; domain C, green. The 235–242 loop, not visible in electron density, is indicated as a dotted curve. B, stereo figure of the structure with conserved GH57 regions I-V, colored (I, red; II, orange; III, blue; IV, green; V, purple) and the two lids (1, yellow; 2, magenta). Except region II these motifs are near the active site cleft. The catalytic residues are shown in stick representation; the 235–242 loop is represented as a dotted curve.

has a few extra secondary structure elements compared with TmAmyC. First, β12 extends the second sheet of the (β/α)7 barrel with one more strand. Second, the small β-sheet β5/β6 creates an extra protrusion at the side of the A domain (Fig. 4A). The structural similarity is lower with the TlGT (28) (root mean square deviation of 3.02 Å); TlGT also has a large extra C-terminal domain that is absent in TtGBE.

Active Site Cleft—A long cleft is formed by residues from domains A and C; residues from four of the five conserved GH57 sequence motifs (I, III, IV, and V (7)) line its surface (Fig. 4B). The catalytic nucleophile Glu-184 from motif III and the acid/base Asp-353 from motif IV are located opposite of each other at the base of the cleft, at positions corresponding to the catalytic residues of TlGT and TmAmyC. In addition to the GH57 motifs, the cleft of TtGBE is flanked by residues corresponding to two of the three “lids” described in TlGT (Fig. 4B). Residues 359–361 (HWW) in domain A correspond to lid 1, whereas residues 464–470 (LMETGQ) in domain C correspond to lid 2. The latter stretch shows ambiguous electron density, indicating it may have multiple conformations and possibly form a flexible peptide stretch. The end of the cleft harboring Phe-23 (Fig. 5) is more open than in TmAmyC but less open than in TlGT. TtGBE lacks the tunnel-shaped active site observed in TlGT, because the residues corresponding to “lid 3” in TlGT (provided by its additional C-terminal domain) are absent.

Because soaking and co-crystallization experiments with several oligosaccharides did not show binding of sugars to the enzyme, sugars were modeled in the active site of TtGBE. A modeled G3 bound in subsites −1, +1, and +2 designates residues possibly interacting with a donor substrate (Fig. 5). In this model, the catalytic residues are positioned close to the glycosidic oxygen between the −1 and +1 subsites (Asp-353 O2·O4: 3.0 Å and Glu-184 O·C1: 3.6 Å). At the +2 subsite, an aromatic stacking interaction with Trp-274 is observed. Sufficient space appears to be available for binding of additional α1,4-linked glucose residues. However, docking of an acceptor trisaccharide with its O6 hydroxyl group directed toward the C1 atom of the G3 −1 glucose resulted in serious clashes with the G3 +1 glucose. Therefore, similar to GH13 cyclodextrin glucanotransferase (33) and GH77 amylomaltase (34), we infer that the α1,4-bond cleavage must have taken place and that the aglycon must have left the active site before the acceptor α1,6-branch can bind and react with the covalently bound sugar in subsite −1.

Several of the residues lining the proposed active site cleft (Phe-23, Leu-271, Trp-274, Trp-360, Trp-404, and Phe-463) (Fig. 5) were individually mutated into alanines to investigate their importance for activity of TtGBE. Residues Trp-274 and Trp-404 are located at the side and bottom of the positive subsites; mutating them into an alanine virtually inactivated the enzyme (Table 1). Their aromatic stacking (Trp-274) and hydrogen bonding (Trp-404) interactions with the modeled +2 glucose would be abrogated by the mutation, thus drastically lowering the affinity for an acceptor/donor substrate.
Mutation of the third tryptophan, Trp-360, lowered the activity almost 200-fold (Table 1). This residue is located in conserved region IV, which comprises lid 1 (Fig. 4B). Its aromatic side chain points toward negative subsites and likely interacts with glucose residues in these subsites (Fig. 5). However, there is space for a different rotamer such that it could interact with positive subsites as is observed in the acarbose-bound structure of TtGT where the corresponding tryptophan interacts with subsites +1 to +3 (28); the same interactions have been suggested for the equivalent residue in the GH57 enzyme Pyrococcus furiosus α-α-glucanotransferase (PfαGT) (35). Whether in TtGBE Trp-360 interacts with donor or acceptor substrates (or both) is not clear from our model.

Mutation of Phe-23 and Leu-271 had an insignificant effect on the behavior of TtGBE (Table 1). These residues are located near subsites −2/−3 and +3, respectively, far from the site of catalysis. It is not uncommon that mutations in residues interacting with an α-glucan have almost no effect on the catalytic properties of an enzyme utilizing long polymeric substrates, as reported for several GH13 cyclodextrin glucanotransferase mutants (36).

The 235–242 Flexible Loop Is Essential for the Branching Activity—Residues 235–242 (PYGEAALG) are part of a loop that connects β7 and β8. No density is visible for these residues suggesting that they are flexible. Nevertheless, they are near the end of the substrate-binding cleft that harbors the positive subsites (Fig. 5), and they may interact with substrate. Indeed, a Y236A mutation in this loop abolished all branching activity, with a concomitant 10-fold increase in hydrolysis activity (Table 1). The length of the maltotriosaccharide products by hydrolysis and the \( K_m \) are unaffected by the mutation. Thus, the flexible \( α8/η3 \) loop is essential for branching activity.

Both the N and C Domains Are Essential for Substrate Binding and Catalysis—Domain C contributes several amino acid residues forming the active site cleft, among which are residues from region V, which is conserved in GH57 GBEs (Fig. 4). Deleting the domain yields an inactive enzyme (TtGBE-ΔC), even though the mutant protein is easily expressed and properly folds as confirmed by CD spectroscopy (data not shown). Moreover, a single F463A mutation (part of the conserved region V) located in the C domain virtually inactivates TtGBE, whereas conserving the wild type melting temperature (Table 1), implying no folding defects. The Phe side chain partly shields the catalytic nucleophile Glu-184 from the solvent. An F463A mutation, or complete removal of the C domain, would bring Glu-184 in a more polar environment and thereby could drastically decrease its nucleophilicity. Furthermore, whereas full-length TtGBE binds amylose, neither the separate catalytic domain nor the separate C domain (Tt-GBE-C domain) bound amylose (Table 2). Mutant and three-dimensional structural data thus, convincingly demonstrate that the catalytic domain and domain C together form the substrate binding cleft of TtGBE, and that both domains contribute residues essential for catalysis. These results also refute the hypothesis that domain C might be a new type of carbohydrate binding module (CAZY), because the separate domain C has no affinity for amylose.

Conclusions—Our data provides evidence that the T. thermophilus TtGBE functions in glycogen synthesis. In vitro, its activity rate with amylose is ~10-fold higher than with amylopectin and the degree of branching of its product increases dramatically when the substrate is a growing glucan chain. We have solved the T. thermophilus GBE crystal structure and proven that it synthesizes branches via an α-retain-
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In addition, we have modeled a maltotriose in the active site of the enzyme, which suggests that simultaneous binding of donor and acceptor substrates in the active site is not possible. Although the GH57 TtGBE operates via the same catalytic mechanism as GH13 GBES, TtGBE is different in the sense that it exhibits a relatively high hydrolytic activity and it transfers shorter side chains constantly throughout the reaction progress. Moreover, TtGBE activity on amylose yields a product with a low degree of branching (3%) yet with short side chains, thus with a novel fine structure potentially useful for new applications (37).

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