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Crystall structure of a 117 kDa glucansucrase fragment provides insight into evolution and product specificity of GH70 enzymes

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Glucansucrases are large enzymes belonging to glycoside hydrolase family 70, which catalyze the cleavage of sucrose into fructose and glucose, with the concomitant transfer of the glucose residue to a growing α-glucan polymer. Among others, plaque-forming oral bacteria secrete these enzymes to produce α-glucans, which facilitate the adhesion of the bacteria to the tooth enamel. We determined the crystal structure of a fully active, 1,031-residue fragment encompassing the catalytic and C-terminal domains of GTF180 from Lactobacillus reuteri 180, both in the native state, and in complexes with sucrose and maltose. These structures show that the enzyme has an α-amylase-like β/α-barrel catalytic domain that is circularly permuted compared to the catalytic domains of members of glycoside hydrolase families 13 and 77, which belong to the same GH-H superfamily. In contrast to previous suggestions, the enzyme has only one active site and one nucleophilic residue. Surprisingly, in GTF180 the peptide chain follows a "U"-path, such that four of the five domains are made up from discontinuous N- and C-terminal stretches of the peptide chain. Finally, the structures give insight into the factors that determine the different linkage types in the polymeric product.

Crystal structure complexes | exopolysaccharide | Lactobacillus reuteri | dental caries

Dental infections such as tooth decay and periodontal diseases are amongst the most common bacterial infections in humans. The causal connection between dental caries and the intake of dietary sugar is well established (1). Fermentation of carbohydrates by plaque-forming oral bacteria produces acids, which cause the dissolution of calcium phosphate in the tooth enamel (2). In addition, dietary sucrose serves as a substrate for fermentative bacteria secrete these enzymes to produce α-glucans, which facilitate the adhesion of the bacteria to the tooth enamel. We determined the crystal structure of a fully active GH70 glucansucrase of Lactobacillus reuteri 180, encompassing the catalytic and C-terminal domains, but not its ~740 residues N-terminal domain (17). This 117 kDa GTF180-NΔ (residues 742–1,772) produces a high molecular mass (~36 MDa) glucose polymer, with both α(1 → 6) and α(1 → 3) glucosidic linkages (18). Crystal structures with bound sucrose (substrate) and maltose (acceptor) combined with site-directed mutagenesis experiments showed that GTF180 glucansucrases possess only one active site and have one nucleophilic residue. These results provide a solid basis for structure-based inhibitor design, and could facilitate the search for unique specific anticaries drugs. Surprisingly, the GTF180 peptide chain follows a "U"-path, such that four of its five domains are made up from discontinuous N- and C-terminal stretches of the peptide chain, respectively.


The authors declare no conflict of interest.

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Data deposition: The atomic coordinates and structure factors have been deposited in the Protein Data Bank, www.pdb.org (PDB ID codes 3KLK, 3HZ3, and 3KLL).

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Results
Overall Structure. Lactobacillus reuteri 180 GTF180-ΔN, elucidated at 1.7 Å resolution, has an elongated shape of dimensions 150 × 60 × 60 Å² and folds into five distinct, linearly arranged domains (Fig. 1A; for crystallographic details see Tables S1 and S2). Three of these domains structurally align with the A, B, and C domains of family GH13 α-amylases and therefore were named accordingly. The other two domains do not show structural similarity to domains occurring in the GH13 family and were thus named IV and V. The five domains are not arranged consecutively along the polypeptide chain. From the N to the C terminus, the polypeptide chain contributes first to domain V, then to domain IV, next to B, then to A and finally to domain C, and then returns back to domain V via domains A, B, and IV, respectively. The polypeptide chain thus follows a “U”-path (Fig. 1B).

The A, B, and C Domains. Domain A is the catalytic domain; it contains a (β/α)₇-barrel, which is indeed “circularly permuted” (10); the domain starts with the helix that is equivalent to the α₃ helix of the GH13 (β/α)₇-barrel, while the last strand is equivalent to strand β₃ of the GH13 enzymes (α₃, β₄α₄, ..., β₆α₃, β1α1, β2α2, β3 arrangement, Fig. S2). Domain B is inserted between helix α₁ and strand β₈ of the (β/α)₇-barrel. The domain contributes several amino acid residues to the substrate donor and acceptor binding sites (see below). Because of the equivalence of the GH70 β₈/α₁ and GH13 β₈/α₃ secondary structure elements domain B occupies the same relative position in both enzyme families (Fig. S2). A single heptacoordinated Ca²⁺ ion is bound in the interface between domains A and B (Fig. 2 and Video S1). Treatment of the enzyme with the Ca²⁺-chelating agent EDTA (ethylenediaminetetraacetic acid) resulted in ~40% lower activity (see Table S3), which might be due, among others, to a compromised binding of the substrate/acceptor by N1029, and Q1140 (Fig. 2A). D1025N mutant protein with bound sucrose substrate showed that sucrose binds in the active site, with its glucosyl moiety in subsite −1 and its fructosyl part in subsite +1 (Fig. 2A and Video S1; for a definition of the subsites, see Davies et al. (21)). Among these strictly conserved residues are the putative catalytic acid/base (E1063 in GTF180) and nucleophile (D1025; mutated to N in the sucrose-bound structure), which, in agreement with their proposed role, are oriented towards the glycosidic oxygen and anomeric carbon of the −1 glucosyl moiety, respectively. Mutating these residues virtually inactivates the enzyme (Table S4). The putative transition state stabilizer D1136 is at 2.8 Å from O2, and may reduce the electronegativity of O2 (14, 24). The crucial role in catalysis of D1136 is affirmed by the drastic decrease in activity of the GTF180-ΔN D1136N mutant (Table S4). The conserved H1135 contacts the glucosyl O2 and O3 hydroxyl groups, and R1023 hydrogen bonds the O2 hydroxyl group, similar to the equivalent residues in GH13 enzymes. The sixth conserved residue, D1504, is not located in the catalytic center, but it makes a H-bond with the hydroxyl group of the conserved Y1465, which has hydrophobic stacking interactions with the sucrose glucosyl moiety in subsite −1.

The seventh conserved residue in GH13 enzymes is a histidine that hydrogen bonds to the O6 hydroxyl group of the −1 glucose (22). In GTF180 this residue is Q1509, which fulfills, however, the same function of hydrogen bonding to O6. In subsite +1 the β-D-fructosyl moiety makes hydrogen bonds with E1063, W1065, N1029, and Q1140 (Fig. 2A). Importantly, no evidence was obtained for a second sucrose-binding site near the active site, as required for the two catalytic-site/double-nucleophile mechanism (16). A second sucrose molecule is bound near W1531, approximately 25 Å from the catalytic site (Fig. S3A), but lack of conservation of the binding residues suggests that sucrose binding at this distant site is not of general significance for the GH70 family. Moreover, a W1531S mutation had no effect on the product size (Fig. S4).

Maltose Binding. The disaccharide maltose is a good acceptor substrate (25–27). A structure of native GTF180-ΔN crystals with bound maltose (2.0 Å resolution) revealed four maltose-binding sites, M1, M2, M3, and M4 (see Tables S1 and S2 for crystallographic statistics). The M2–M4 binding site residues are not conserved in other GH70 family enzymes and therefore seem

Fig. 1. Overall structure of L. reuteri 180 GTF180-ΔN. (A) Crystal structure of GTF180-ΔN, the N- and the C-terminal ends of the polypeptide chain are indicated, the Ca²⁺ ion is shown as a magenta sphere; (B) Schematic presentation of the “U-shaped” course of the polypeptide chain. Domains A, B, C, IV, and V are coloured in blue, green, magenta, yellow, and red, respectively, with dark and light colors for the N- and C-terminal stretches of the polypeptide chain.
of no importance to the GH70 family in general. A description of the M3 and M4 binding sites can be found in the SI Text.

Maltose M1 is bound at subsites +1 and +2. The +1 glucosyl moiety of M1 is sandwiched between W1065 on one side and L938 and L981 on the other side (Fig. 2B and Video S2). The +1 C6 hydroxyl group is at hydrogen bonding distance from the catalytic acid/base, E1063, in a productive position to attack a covalently bound enzyme-glucosyl-intermediate. The +1 C4 hydroxyl group makes a direct, short hydrogen bond (2.6 Å) with O6 hydroxide residue of maltose (Fig. 2B). The disaccharide shows no deviations from the low energy maltose torsion angles and also the hydrogen bond normally present between the C2 and C3 hydroxyl groups of adjacent α(1→4)-linked glucose molecules is formed. Interestingly, maltose M2 binds near the active site (Fig. 2B). However, the M2 binding mode is rather aspecific (via H-bonding interactions with the +1 glucose, and water-mediated hydrogen bonds with the protein) and therefore unlikely to mimic a bona fide acceptor substrate.

Analysis of Products Synthesized by GTF180-ΔN. Product analysis of GTF180-ΔN incubated with both sucrose and maltose present in a 1:1 ratio revealed that ~42% of maltose was converted into the trisaccharide panose (Glc-α(1→6)-Glc-α(1→4)-Glc; Glc is glucose) and ~11% into the tetrasaccharide α(1→6)-panose (Glc-α(1→6)-panose) (Fig. S5 and Table S5, for details on product analysis see SI Text). These results show that the glucosyl moiety of sucrose is transferred to the nonreducing end of maltose and panose. Transfer to the nonreducing end is also in agreement with other studies (13, 15) as well as with the structure of GTF180-ΔN with a bound maltose, which shows that maltose binds adjacent to subsite −1 with its nonreducing end C6 hydroxyl group pointing towards the catalytic center. Thus, the O6 atom can act as the nucleophile in the second reaction step, leading to the extension of an α-glucan at its nonreducing end by one glucose residue.

Docking studies in a model of the covalent glucosyl-GTF180-ΔN intermediate (in subsite −1), which was made based on the structure of the glucosyl-intermediate of Neisseria polysaccharea amylase (PDB code 1S46; (23)). Like maltose, isomaltose (Glc-α(1→6)-Glc) binds with its nonreducing glucose residue in subsite +1; its O6 hydroxyl group points towards the position of the C1 atom of the covalently bound sugar in subsite −1 (Fig. 3A). The reducing end glucose binds in subsite +2, and has stacking interactions with W1065. The isomaltose binding mode shows how an α(1→6)-linked glucan can be extended by another glucose residue via an α(1→6)-linkage.

A different mode of binding was shown by isomaltotriose (Glc-α(1→6)-Glc-α(1→6)-Glc), which binds in subsites +1II, +1 and +II” (Fig. 3B). The O3 atom of the central, +1 glucose residue is directed towards the C1 atom of the glucosyl-enzyme intermediate. This binding mode illustrates how an α(1→3) branch point can be introduced in the glucan chain. In principle, isomaltose could also bind in subsites +1 and +II” with O3 pointing towards the C1 atom at subsite −1. This binding mode would allow elongation of a linear α(1→6)-glucan chain with an α(1→3)-linked glucose.

Docking of the disaccharide nigerose (Glc-α(1→3)-Glc) resulted in the +1 glucose predominantly being bound with its O6 hydroxyl group pointing towards the C1 atom of the glucosyl-enzyme intermediate (Fig. 3C), while the reducing end glucose occupies subsite +2 (Fig. 3C). This binding mode shows how a glucosyl moiety can be added to the O6 hydroxyl group of an acceptor saccharide, which the terminal nonreducing sugar is linked α via an α(1→3) linkage. The docking of nigerose did not result in a binding mode allowing elongation at the O3 hydroxyl group.

Discussion

Discontiguous Domain Arrangement. Our results have revealed the structural details of a GH70 glucansucrase. In contrast to the previously proposed linear arrangement of domains along the polypeptide chain (7, 8), the structure of L. reuteri 180 GTF180-ΔN shows that four of the five domains are built up from discontiguous parts of the polypeptide chain. Only the C-domain is
formed from one continuous stretch of amino acids. Neverthe-
less, heterologous expression of the enzyme in
Escherichia coli
is not problematic, showing that folding intermediates are stable
and that the N-terminal variable domain, which was absent in
our construct, is not needed for correct folding of the enzyme.
The structure confirms that, compared to GH13
α-amylases,
the catalytic
(β/α)₈-barrel domain is circularly permuted as was
previously proposed (10).

The “permutation per duplication model” proposes a se-
quence of gene arrangements that could have led to a circularly
permuted gene via gene duplication, in-frame fusion, and partial
 truncation (28, 29). In this way a GH70 glucansucrase precursor
consisting of a circularly permuted domain A, a discontiguous do-
main B, and a contiguous domain C could have arisen from the
GH13 α-amylase fold (Fig. 4). Insertion of this precursor into the
gene of domain IV, followed by insertion into the gene encoding
domains V and N, could then have led to the present day protein.
Alternatively, domain IV may have inserted into the ancestor
α-amylase before the circular permutation occurred. In both
cases various intermediate forms must have existed during evolu-
tion. The requirement that such intermediate forms are stable
and should offer some form of advantage to the organism to pre-
vent removal from the gene pool, can explain why the number of
circular permutations observed in proteins is rather small (30).

Active Site and Formation of the Covalent Glucosyl-Enzyme Intermedi-
ate. The active site of GTF180-ΔN is a pocket-shaped cavity, in
which the glucose moiety of sucrose binds (subsite −1). The cavity
is closed off by residues Q1140, N1411, and D1458, which prevent
the binding of longer oligosaccharides (see Fig. 24 and
Video S1). These residues are strictly conserved in GH70 family
enzymes, indicating that the binding of a single glucose residue in
subsite −1 is a general property of GH70 glucansucrases. In the
GH13 amylosucrase from N. polysaccharea, which produces an
α(1 → 4)-linked glucan polymer from sucrose, the glucose-bind-
ing pocket is also closed off, but by a salt-bridge between R509
and D144 (31).

Six residues that interact with the −1 glucose in GTF180-ΔN
are strictly conserved in GH70 and GH13 enzymes, and exhibit
comparable interactions with the −1 glucose residue (see Results).
In addition, Q1509 has a similar hydrogen bonding interaction
with the O6 hydroxyl group of the −1 glucose as the equivalent
His in the GH13 family. Thus, the active site of GTF180-ΔN is
very similar to those of GH13 family members. No space is avail-
able for a glucose covalently bound to D1136, as required for the
two-nucleophile mechanism proposed by Robyt et al. (16).

The similarity of the active sites of GH70 and GH13 enzymes
suggests a very similar reaction mechanism for the cleavage of the
α-glycosidic bond of sucrose and the formation of the β-glucosyl-
enzyme intermediate. Cleavage of the glycosidic bond of sucrose
is likely started by protonation of the glycosidic oxygen by E1063,
resulting in bond cleavage, release of fructose, and formation of a
partly planar oxocarbenium ion-like intermediate (12, 14, 24, 32).
Conceivably, D1136 pushes the −1 O2 atom towards the inter-
mediate position, while Q1509 holds the −1 glucose O6 atom.
Similar to GH13 enzymes, the C2-O2 bond of the −1 glucose resi-
due is polarized by interactions of the O2 atom with H1135,
D1136, and R1023, which lowers the energy of the transition state

Fig. 3. Docking of isomaltose (A), isomaltotriose (B), and nigerose (C) in the active site of a modeled glucosyl-GTF180 intermediate. For modelling and docking
procedure see SI text. The covalently bound glucosyl moiety is shown in white stick representation, isomaltose, nigerose, and isomaltotriose are shown in
yellow stick representation. Dashed lines show hydrogen bonding distances of less than 3.5 Å, solid yellow lines show where the chain may extend; subsites
are numbered with Arabic and Roman white numerals. Domains A and B of GTF180 are shown in surface presentation and are coloured blue and green,
respectively.
are α end glucose binds in subsite II although our docking studies with isomaltose did not provide evi-
sible (Fig. 3). At subsite ing studies showed that these two binding modes are indeed pos-
sible stacking interactions with W1065; therefore the en-
zyme must thus be able to transfer the glucosyl residue to either the O6 or the O3 hydroxyl group. The enzyme must thus be able to transfer the glucosyl moiety attached to D1025 via a β-glycosidic linkage. Because the +1 fructosyl moiety of sucrose has fewer interactions with the enzyme than the −1 glucosyl moiety, it can easily be released from the active site in order to allow the reaction to proceed by binding the acceptor molecule at subsite +1.

Transfer of Glucose to the Acceptor Saccharide and Product Specificity. The next step in the reaction mechanism is the transfer of the covalently bound glucosyl residue to the acceptor saccharide. GTF180-ΔN produces a branched dextran built up from different lengths of α-(1 → 6)-linked glucose residues, interconnected by single α-(1 → 3)-bridges. No consecutive α-(1 → 3) glucosidic bonds are present (35). The enzyme must thus be able to transfer the glucosyl residue to either the O6 or the O3 hydroxyl group. This requirement implies that the accepting glucose must be able to bind in two different orientations, either with its O6 or with its O3 hydroxyl group pointing towards the catalytic site. Our docking studies showed that these two binding modes are indeed possible (Fig. 3). At subsite +2 the acceptor sugars are mostly bound via aspecific stacking interactions with W1065; therefore the en-
zyme is not very particular about the specific glycosidic linkage between the sugars in subsites +1 and +2, and can accommodate α(1 → 3), α(1 → 4), or α(1 → 6)-linked glucose. In all cases the accepting O6 hydroxyl group will be near the C1 atom of the covalent intermediate.

In case the O3 hydroxyl group is the acceptor, a different bind-
ing mode of the acceptor is needed, as exemplified by the docking results with isomaltotriose (Fig. 3B). In this case, the reducing end glucose binds in subsite II′ rather than subsite +2, and the nonreducing end extends towards subsite −II′. In this binding mode an α(1 → 3) branch point is introduced in the glucan chain. Although our docking studies with isomaltose did not provide evidence, it can be envisaged that occasionally also a terminal α(1 → 6)-linked glucan may bind in subsites +1 and +II′, with O3 pointing into the active site. Such a binding mode would allow linear glucan chain elongation with an α(1 → 3)-linked glucose.

The docking results for both isomaltose and nigerose suggest that the most favorable glucan conformer is the one allowing formation of the α(1 → 6) linkage. Indeed, the majority (69%) of the glycosidic bonds in the α-D-glucan synthesized by GTF180 from sucrose are α(1 → 6)-bonds and only about 31% are α(1 → 3)-bonds (18, 35).

Conclusions
The crystal structures of GTF180-ΔN have revealed the molecular details of a GH70 enzyme and its interaction with sucrose and the acceptor maltose. The data show that, in contrast to previous proposals, only a single active site is present, and no space for a second covalently bound glucose residue or glucan polymer is available. This finding invalidates the proposal of a double ac-
tive-site/double-nucleophile insertion mechanism (16). Also the conservation of key amino acids involved in substrate binding and catalysis at subsite −1 in both GH13 and GH70 enzymes confirms that the mechanism of glycosidic bond cleavage by GH70 glucansucrases is similar to that of the α-amylase super-family enzymes (14, 23).

The crystal structure of GTF180-ΔN with bound maltose revealed how an acceptor substrate binds to receive a glucosyl group at its O6 atom. Modelling showed that transfer of the glucosyl group to the O3 atom requires an entirely different binding mode (Fig. 3B).

Finally, in view of the conservation of the residues involved in subsite −1 in enzymes from families GH13, GH70, and GH77, the design of specific antacaries glucansucrase-inhibitors is predicted to require the exploitation of features of the enzymes more remote from subsite −1.

Experimental Procedures
Cloning, Mutagenesis, Purification, Crystallization, and Soaking Experiments. For cloning and mutagenesis of gtf180 see SI text. Purification and crystallization of native L. reuteri GTF180-ΔN were performed as described previously (17). Selenomethionine (SeMet) labeled GTF180-ΔN was produced as formerly reported (36). Both the D1025N mutant and SeMet labeled GTF180-ΔN crystallized in the same conditions as the native protein. Crystals of the D1025N mutant were soaked for 30 min in mother liquor containing 25% (w/v) PEG 3530, 50 mM NaCl, 50 mM 1,3-bis[tris(hydroxymethyl)methylamino]propane HCl, pH 6.0, 2 mM CaCl2) containing 25 mM sucrose, and cryoprotected in mother liquor containing 35% (w/v) PEG 3350 and 25 mM sucrose. For maltose soaking studies a crystal of SeMet-GTF180-ΔN was trans-
ferred first to mother liquor supplemented with 25 mM maltose (15 min), and then soaked for 2 h in mother liquor containing 250 mM maltose. Crystals were cryoprotected in 35% (w/v) PEG 3350 and 250 mM maltose.
Data Collection. All datasets were collected at 100 K. Multiple wavelength anomalous dispersion (MAD) and “native” datasets were collected at beam line ID29 at the European Synchrotron Radiation Facility (ESRF), Grenoble, France and processed using MOSFLM and SCALA (37). The “sucrose” and “maltose” datasets were collected at beam lines BM16, ESRF, Grenoble, France, and BW7A, European Molecular Biology Laboratory (EMBL) outstation at the Deutsches Elektronen-Synchrotron, Hamburg, Germany, respectively. The latter two datasets were processed using the XDS package and SCALA (37, 38). All crystals belonged to space group P1, with one molecule per unit cell and 52% solvent content. The presence of one molecule per unit cell is in agreement with gel filtration (Fig. S6) and dynamic light scattering experiments (estimated molecular mass is 110 KDa with 13.1% polydispersity), which indicate that the protein is monomeric in solution. For data collection and processing statistics see Table S1.

Structure Determination. The GTF180-ΔN structure was solved with the program SOLVE/RESOLVE (39) using MAD data collected around the Se edge. The experimental phase information for further automated model building and refinement using the programs ARP/WARP (40) and REFMAC5 (41), respectively.

Model building and corrections were done using the program COOT (42). The final native model consists of 1,006 amino acid residues (residues 746–1,751). The first 4 (residues 742–745) and the last 21 residues (residues 1,752–1,772) are invisible in the electron density maps. Both sucrose and maltose were included in the model in the final stages of the refinement. The resulting structures were validated with MolProbity (43). For refinement statistics see Table S2. Coordinates and structure factors have been deposited with the Protein Data Bank (entries 3KLL, 3Z3K, 3KLL). Figures were created using PyMol (44).

Product Analysis. Products synthesized by GTF180-ΔN were analyzed as described in SI text.

Docking Studies. The automatic docking calculations were done with the program AutoDock 4 (45). More details can be found in SI text.

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