Levan is β-2,6-linked polymeric fructose and serves as reserve carbohydrate in some plants and microorganisms. Mobilization of fructose is usually mediated by enzymes such as glycoside hydrolase (GH), typically releasing a monosaccharide as a product. The enzyme levan fructotransferase (LFTase) of the GH32 family catalyzes an intramolecular fructosyl transfer reaction and results in production of cyclic difructose dianhydride, thus exhibiting a novel substrate specificity. The mechanism by which LFTase carries out these functions via the structural fold conserved in the GH32 family is unknown.

Here, we report the crystal structure of LFTase from Arthrobacter ureafaciens in apo form, as well as in complexes with sucrose and levanbiose, a difructosaccharide with a β-2,6-glycosidic linkage. Despite the similarity of its two-domain structure to members of the GH32 family, LFTase contains an active site that accommodates a difructosaccharide with a β-2,6-glycosidic linkage. Although the similarity of its two-domain structure to members of the GH32 family is unknown.

Here, we report the crystal structure of LFTase from Arthrobacter ureafaciens in apo form, as well as in complexes with sucrose and levanbiose, a difructosaccharide with a β-2,6-glycosidic linkage. Despite the similarity of its two-domain structure to members of the GH32 family, LFTase contains an active site that accommodates a difructosaccharide using the −1 and −2 subsites. This feature is unique among GH33 proteins and is facilitated by small side chain residues in the loop region of a catalytic β-propeller N-domain, which is conserved in the LFTase family. An additional oligosaccharide-binding site was also characterized in the β-sandwich C-domain, supporting its role in carbohydrate recognition. Together with functional analysis, our data provide a molecular basis for the catalytic mechanism of LFTase and suggest functional variations from other GH32 family proteins, notwithstanding the conserved structural elements.

Fructans are polymeric carbohydrates of fructose linked via O-glycosidic bonds, with a terminal sucrose. The β-2,1-linked inulin and β-2,6-linked levan are the two major types of fructans identified to date, and they represent one of the primary carbohydrate storage molecules in some plants, bacteria, and fungi (1). In addition to their role as an energy reserve, plant fructans also function in cold and drought tolerance (1–3). Their biosynthetic pathways in bacteria are relatively well established; in these pathways, a single multifunctional enzyme of levansucrase (4, 5) and inulosucrase (6) is responsible for the synthesis of levan and inulin, respectively, with sucrose as a substrate. However, those pathways have not yet been conclusive in plants (7). Once the fructosyl moiety is reserved in fructans, its mobilization is achieved by various hydrolytic enzymes (7). For example, plant fructan exohydrolases successively remove one terminal fructose unit from inulin and levan through an exo-type reaction, although in bacteria levanase and inulinase perform both endo- and exo-type reactions toward these fructans.

In contrast, an unusual type of fructan-utilizing enzyme catalyzes an intramolecular fructosyl transfer reaction, in which a terminal fructose and its adjacent moiety form an anhydride linkage, in addition to the existing glycosidic bond (see Fig. 1A). Fructans and their breakdown products are responsible for the synthesis of levan and inulin, respectively, with sucrose as a substrate. However, those pathways have not yet been conclusive in plants (7). Once the fructosyl moiety is reserved in fructans, its mobilization is achieved by various hydrolytic enzymes (7). For example, plant fructan exohydrolases successively remove one terminal fructose unit from inulin and levan through an exo-type reaction, although in bacteria levanase and inulinase perform both endo- and exo-type reactions toward these fructans.

Results:
Levan fructotransferase converts polymeric β-2,6-linked levan into cyclic difructose dianhydrides.

Conclusion:
The shape of the active site pocket dictates substrate specificity and formation of cyclic difructose dianhydrides.

Significance:
Sequence conservation in the loop region among levan fructotransferases is the molecular basis for the exo-type cleavage of difructosaccharide and product formation.
Structural Analysis of Levan Fructotransferase

into DFA-IV (di-β-D-fructofuranose-2,6′:6,2′-dianhydride) (see Fig. 1A) (8, 9, 12). Because DFAs produced by these enzymes have various beneficial effects on human health and as such are used as dietary supplements and low calorie sweeteners, DFA production has gained the attention of food microbiologists and biotechnologists (13, 14).

Although the fructan-utilizing enzymes exhibit diverse substrate specificities and catalytic properties, these enzymes—including LTFase—are either O-glycoside hydrolylase (GH 32 or GH68 family enzymes, based on sequence similarity classifications (CAZy) (15). More specifically, most of these enzymes are members of the GH32 family; however, bacterial levansucrase and inulosucrase are in the GH68 family. Despite their limited sequence homology, these two families exhibit high similarities in their three-dimensional structures and belong to the GH-J clan by a structure-based classification (16). In particular, a five-bladed β-propeller fold that acts as a catalytic domain is common to the GH-J clan, whereas an additional β-sandwich domain has been characterized in the GH32 family C-terminal region. The only exception to these general features is with IFTase, which is a member of the GH91 family and adopts a homotrimeric β-helical fold with its active site located in the intersubunit interface (11). Notably, the proposed catalytic residues and their mechanistic features account for characteristics of IFTase that differ from those of the GH32 and GH68 families.

Unlike IFTase, the DFA-IV-producing LTFase from Arthrobacter ureafaciens K-2032 (AuLTFase) is a member of the GH32 family (12) (supp. Fig. S1). Therefore, how the unusual functional features of AuLTFase can be accommodated within the structural fold conserved in the GH-J clan remains to be resolved. We determined the crystal structure of AuLTFase and conducted a functional analysis to understand its structural and functional features. The structure of AuLTFase was determined in apo form at a resolution of 2.57 Å, its structural and functional features. The structure of AuLTFase and conducted a functional analysis to understand

EXPERIMENTAL PROCEDURES

Cloning, Expression, and Purification—The gene for AuLTFase (12) (GenBankTM accession number AF181254) was amplified by PCR with sequence-specific primers (supp. Table S1). The resulting PCR product contained residues Ala-41 to Ile-521 by excluding the N-terminal 40 residues as a signal peptide, Met-1 to Ala-33 (supp. Fig. S1), and was subcloned into the pET28b expression vector (Merck) with the C-terminal noncleavable His tag.

Seleno-1-methionine-substituted His-tagged AuLTFase (residues Ala-41 to Ile-521) was expressed in Escherichia coli B834 (DE3) methionine auxotroph cells (Merck) for structural studies. In particular, we found that methylation of AuLTFase is essential for crystallization. Therefore, a methylation step was included in the purification, according to a protocol published previously (17, 18). Cells were grown at 37 °C in minimal medium supplemented with seleno-1-methionine to an A600 of 0.7 and then induced for 16 h at 20 °C by the addition of 0.5 mM isopropyl-β-D-thiogalactopyranoside. The cells were harvested and sonicated in buffer A (50 mM HEPES, pH 7.6, 150 mM NaCl). The enzyme was purified using immobilized metal affinity chromatography with buffer A plus 500 mM imidazole. After the purified AuLTFase was dialyzed against buffer A, the methylation reaction using the dimethylamine-borate complex, and formaldehyde was performed overnight at 4 °C with 1–2 mg/ml of AuLTFase. After the soluble fraction from the reaction mixture had been obtained by centrifugation, size exclusion chromatography using a Superdex 200 column (GE Healthcare) was carried out with buffer A to remove any possible aggregated form of the protein. Elution fractions for AuLTFaseMET (hereafter, a superscript of MET denotes the methylated enzyme) were pooled, resulting in a yield of 15 mg/ml, based on its molar extinction coefficient of 151,260 M⁻¹ cm⁻¹ at 280 nm. AuLTFaseMET was catalytically competent (supp. Fig. S3A), and four of the five lysine residues were estimated to be methylated at the ε-amino group of lysine, based on mass spectroscopy-time of flight analysis.

For structural analysis of AuLTFaseMET in complex with either sucrose or levanbiose, we used a mutant AuLTFase(D54N)MET enzyme, in which the catalytic nucleophile Asp-54, common to members of the GH32 family (supp. Fig. S1), was mutated into asparagine to exclude any possible involvement of Asp-54 in catalysis of the ligand used in this experiment. Site-directed mutagenesis was performed using a QuikChange (Agilent Technologies), with mutagenic primers (supp. Table S1), and the resulting plasmid that encodes the AuLTFase(D54N) enzyme was then transformed into the BL21(DE3) codon plus-RIL strain (Agilent Technologies) and expressed in Luria Bertani medium. The purification steps for AuLTFase(D54N)MET were identical to the procedure described above, with the exception of addition of 1 mM dithiothreitol. We also confirmed that mutant AuLTFase(D54N) was catalytically inactive (supp. Fig. S3A). During protein purification, we noticed that both the native and methylated enzyme were monomeric, based on their size exclusion chromatography elution profiles.

Crystallization—The apo form of seleno-1-methionine-substituted AuLTFaseMET was crystallized at 22 °C using the hanging drop vapor diffusion method in a crystallization buffer of 0.1 M Bis-Tris (pH 5.5), 1% (w/v) PEG 3350, 1 M ammonium sulfate, and 3% 2-propanol. Unlike the WT enzyme, crystals for the apo form of the AuLTFase(D54N)MET mutant enzyme were produced at 22 °C in different crystallization buffers using the hanging drop vapor diffusion method; the crystallization buffer consisted of 0.1 M MES (pH 6.5) and 1 mM lithium sulfate. Formation of the AuLTFase(D54N)MET-ligand complex, either with sucrose or levanbiose, was achieved by a soaking procedure. Sucrose is not a substrate of AuLTFase (supp. Fig. S3A), but the enzyme structure in a complex with sucrose reveals crucial structural features of AuLTFase substrate binding.
For the AuLFTase(D54N)MET-sucrose complex, a pregrown crystal of AuLFTase(D54N)MET was soaked for 60 min in a solution of 1 M sucrose in 0.1 M MES (pH 6.5), 1 M lithium sulfate, and 5% (v/v) glycerol. The AuLFTase(D54N)MET-levanbiose complex was obtained by soaking a crystal for 60 min in 30% of the levansucrose reaction product (supplemental “Methods”) in 0.1 M MES (pH 6.5), 1 M lithium sulfate, and 10% (v/v) glycerol. In both experiments, glycerol was included as a cryo-protectant. We also determined the crystal structure of AuLFTaseMET in complex with the DFA-IV product. Specifically, DFA-IV is bound to the C-terminal domain of AuLFTaseMET, as described below, indicated that DFA-IV is bound to the C-terminal domain of AuLFTaseMET, not to the catalytic domain (supplemental Fig. S3B).

**Data Collection and Structure Determination**—Multiwavelength anomalous dispersion data using a crystal of the apo form of seleno-L-methionine-substituted AuLFTaseMET were collected at a resolution of 2.57 Å on Beamline 4A and 6C at the Pohang Accelerator Laboratory (Pohang, Korea). Subsequently, single-wavelength diffraction data were collected on Beamline 17A at the Photon Factory (Tsukuba, Japan), for the AuLFTase(D54N)MET-sucrose complex at a resolution of 2.2 Å and the AuLFTase(D54N)MET-levanbiose complex at a resolution of 2.3 Å. We collected all data at 100 K and processed them using the program HKL2000 (19). All of the crystals belonged to the space group of P212121, except for AuLFTase(D54N)MET, which was crystallized under conditions identical to those for AuLFTase(D54N)MET. Subsequent structural analyses, as described below, indicated that D1A-IV is bound to the C-terminal domains of AuLFTaseMET, not to the catalytic domain (supplemental Fig. S3B).

**Functional Analysis**—Various mutant enzymes were prepared by site-directed mutagenesis using a QuikChange kit (Agilent Technologies), with mutagenic primers (supplemental Table S1). All enzymes used for the functional analysis were purified without methylation; their expressions, as well as the WT enzyme, were carried out in Luria Bertani medium, and the C-terminal His-tagged proteins were purified using immobilized metal affinity chromatography. The purified enzymes were dialyzed against 50 mM HEPES (pH 8.0) and diluted to 0.2 mg/ml for the functional analysis.

Enzyme assays were performed according to a method published previously (26, 27). Briefly, 300 μl of enzyme was incubated for 24 h at 40 °C with 600 μl of 1% (w/v) levan (Sigma), and the reaction was stopped by boiling for 5 min. After centrifugation, the reaction product was analyzed by TLC and HPLC (supplemental “Methods”).

**RESULTS**

**Overall Structure of AuLFTaseMET in Apo Form**—AuLFTase is a monomeric protein in solution as characterized using size exclusion chromatography. Consistent with this observation, we could not identify any possible oligomeric conformations of AuLFTaseMET, either by noncrystallographic or crystallographic symmetry, even with four monomers in an asymmetric unit of the P212121 space group. Those four monomers in the asymmetric unit are structurally identical within a root mean square deviation of 0.19–0.48 Å for 479 Co atoms between Ala-41 and Gln-519. We used Monomer B to describe the overall structure of AuLFTase.

The AuLFTaseMET structure consists of the N-domain (Ala-41 to Val-364) and C-domain (Val-371 to Gln-519) (Fig. 1). The AuLFTaseMET structure has a five-bladed β-propeller fold of 14 β-strands (Blades I–V) (Fig. 1), whereas the β-propeller fold of 14 β-strands (Blades I–V) (Fig. 1), whereas the β-propeller fold of 14 β-strands (Blades I–V) (Fig. 1) is characterized in the C-domain, with the 3_{10}-helix as a linker between the two domains. This overall architecture is common to members of the GH32 family, in which the β-propeller N-domain functions as the catalytic domain (7). The proposed catalytic residues of
Structural Analysis of Levan Fructotransferase

AuLFTase conserved in the GH32 and GH68 family also form a cluster for a "catalytic triad" at the center of the N-domain (Fig. 1B and supplemental Fig. S1), including Asp-54 nucleophile in Blade I, Glu-236 acid/base catalyst in Blade IV, and Asp-186 intermediate-stabilizing residue in Blade III. There are two layers of antiparallel β-sheet structures in the C-domain, with the top layer of the β-sheet composed of β21, β30, β23, β24, β25, and β26 in a spatial order and the other β-sheet consists of β19, β31, β32, β22, β27, β28, and β29. These two β-sheets are packed into a parallel orientation to form the β-sandwich fold of the C-domain, generating a concave surface on the top β-sheet. Notably, the concave surface of the C-domain is on the same face as the entrance to the center of the β-propeller N-domain (Fig. 1B).

Structure of AuLFTase(D54N)MET in Complex with Sucrose—An AuLFTase(D54N)MET mutant enzyme, which is catalytically inactive (supplemental Fig. S3A), was used for the structural analysis of the complex with sucrose. Of the four monomers in the asymmetric unit, two exhibited an ordered electron density for sucrose bound in the β-propeller N-domain pocket (Fig. 2A), as well as on the surface of the β-sandwich C-domain (supplemental Fig. S4A).

The pocket identified in the N-domain begins at the center of the β-propeller fold and extends to the edge of the enzyme enclosed by Blades I and II, resulting in an elongated shape (Fig. 2, B and C). Specifically, the innermost floor of the pocket is laid with residues in β1 (Trp-51, Cys-53, and Asn-54) and β3 (Leu-70 and Ser-72) strands in Blade I, whereas the side walls along the long axis of the pocket consist of two loops; that on the wall includes a loop containing Pro-106 and Trp-108 between Blades I and II, and the other involves a loop with Trp-322 between β16 and β17 in Blade V. One end of the pocket is occluded at the center of the domain by residues from Blades III, IV, and V, including residues Glu-236 and Asp-186, as well as Arg-185 and Tyr-298. The other end of the pocket near the Blade I region is also sealed by a loop between β3 and β4 in Blade I; specifically, residues such as Asn-76, Gly-77, Pro-78, and Gly-79 constitute the end wall of the pocket. However, the arrangement of these residues left the top of the pocket wide open to the surface (Fig. 2B).

In the pocket, sucrose is oriented such that the fructosyl moiety faces the bottom of the pocket as a stacked orientation, and the pyranosyl ring of glucose points upward to the surface of the N-domain, with its conformation almost perpendicular to the fructose furanose ring (Fig. 2, B and C). There are extensive hydrogen bonding and hydrophobic interactions with sucrose. In particular, the catalytic triad clustered at the center of the N-domain remains in the vicinity of the sucrose glycosidic bond; the side chain of Asn-54 in the AuLFTase(D54N)MET mutant enzyme, which replaced the WT nucleophile Asp-54, is located −3.6 Å below the anemic carbon (C2') of fructose, and the carboxyl group of an acid/base catalyst Glu-236 is at 3.1 Å from an oxygen atom of the glycosidic bond. The Asp-186 intermediate-stabilizing residue is located within 2.8 Å from the hydroxyl group of fructose (Fig. 2C). The geometric locations of these three residues fulfill the well established stereo-chemical requirements of catalysis in the GH32 family (7), in which the glycosidic bond near these residues is susceptible to cleavage. Therefore, we tentatively named the binding site for the fructose and glucose units as the −1 and +1 subsites, respectively, according to Davies et al. (28). In addition to these interactions, fructose at the −1 subsite mediates possible hydrogen bonding with Arg-185, Asp-186, and Ser-109, as well as extensive van der Waals interactions within 4.5 Å of the residues in the bottom and wall of the pocket, including Leu-70, Trp-108, Tyr-298, and Trp-322 (Fig. 2C). Extensive interactions were also identified around glucose at the +1 subsite through the residues identified at −1 subsite; residues such as Trp-108, Arg-185, and Trp-322 from the pocket side wall also maintain the interactions. Specifically, the Arg-185 side chain is in a stacking mode within 4.0 Å to the glucose pyranosyl ring, and the side chains of Trp-108 and Trp-322 are almost perpendicular to the pyranosyl ring. Arg-139 and Glu-236 acid/base catalyst mediate several hydrogen bonds at a distance of 2.6–3.5 Å to the hydroxyl groups of glucose (Fig. 2C). In particular, Arg-139 mediates various interactions exclusively with glucose at the +1 subsite. It is noticeable that the binding of sucrose occupied only half of the pocket near the catalytic triad at the center of the N-domain, leaving space for the other half near a region of Blade I (Fig. 2B).

Unlike the catalytic N-domain, the function of the β-sandwich C-domain has been elusive but is proposed to be involved in carbohydrate recognition based on its structural similarity to a lectin domain (7). In this complex, we identified the binding site of one sucrose molecule on the concave surface of the C-do-

![Figure 1](image-url)
main (supplemental Fig. S4A). The fructosyl and glucosyl moieties in this sucrose molecule also adopted a conformation essentially identical to that of sucrose in the \( \beta \)-propeller N-domain and was bound to the concave region that is comprised of \( \beta\)30, \( \beta\)23, \( \beta\)24, and \( \beta\)25. Specifically, the side chains of two tyrosine residues on the edge of this region, Tyr-433 in \( \beta\)25 and Tyr-501 in \( \beta\)30, are in a head to head orientation with a possible hydrogen-bonding distance of 2.8 Å between the two hydroxyl groups. This unique structural feature forms a hydrophobic platform on the surface of the C-domain, on which a fructose unit of sucrose sits in a stacking orientation. In addition to these extensive hydrophobic interactions, the residues around fructose further stabilize its conformation through hydrogen bonding, including Asn-408, Asn-424, Asp-435, and Arg-451. In contrast, a pyranosyl ring of glucose, almost perpendicular to the fructosyl furanosyl ring, has a few interactions; the side chain of Tyr-428 in the fructose furanosyl ring, has a few interactions; the side chain of Asn-76, Gly-77, Pro-78, and Gly-79, is responsible for extensive hydrophobic interactions, mainly with a glucose unit. Further structural analysis indicated that this sucrose-binding site is indeed capable of binding other types of carbohydrates, including DFA-IV, levanbiose, and levantriose (supplemental Figs. S3B and S4, B and C), suggesting a possible role for the C-domain in carbohydrate recognition.

Structure of AuLFTase(D54N)\(^{\text{MET}}\) in Complex with Levanbiose—The crystal structure of the AuLFTase(D54N)\(^{\text{MET}}\)-levanbiose complex revealed a levanbiose-binding site in an N-domain pocket and in the concave surface of the C-domain. Levanbiose, which is derived from a \( \beta\)2,6-linked levan (supplemental Fig. S2), consists of two fructose units, one each of reducing and nonreducing fructoses. The electron density for levanbiose clearly exhibits a typical \( \beta\)2,6-O-glycosidic linkage between the fructose units and an extended conformation with a torsion angle of \( \varphi \) for the C6–O bond in the \( \beta\)2,6-O-glycosidic linkage (Fig. 3A) (29). Because of this stereochemical restraint, the furanosyl ring of the two fructose units is perpendicular, similar to those of glucose and fructose in the AuLFTase(D54N)\(^{\text{MET}}\)-sucrose complex.

However, the binding site of levanbiose differs from that of sucrose in the AuLFTase(D54N)\(^{\text{MET}}\)-sucrose complex. In general, levanbiose remains in the pocket in which sucrose was identified, but its binding site includes the Blade I region (Fig. 3, B and C), which was empty in the sucrose complex. Levanbiose is bound in this elongated pocket with an extended conformation and sits on the floor of the pocket parallel to the \( \beta\)1 and \( \beta\)3 strands. In particular, a reducing fructose with a free hydroxyl group (O\(_1\)') at an anomic carbon is located at the immediate vicinity of the –1 subsite identified in the AuLFTase(D54N)\(^{\text{MET}}\)-sucrose complex, whereas a nonreducing fructose with a free hydroxyl group (O\(_6\)) is bound in the region near Blade I. Therefore, we named this newly characterized site as the –2 subsite for a nonreducing fructose (Fig. 3, B and C; hereafter, the prime and double prime symbol denote atoms belonging to the –1 and –2 subsites, respectively). Many interactions occur along levanbiose; however, interactions around the –1 subsite are similar to those in the sucrose complex. A furanosyl ring with an orientation perpendicular to the bottom of the pocket is surrounded by Trp-108 and Trp-322 at the –1 subsite, and its anomic carbon (C2') is 4.8 Å from the Asn-54 side chain. Except for hydrogen bonding with Cys-53 from the floor of the pocket, fructose at the –1 subsite maintains interactions similar to those at the –1 subsite of the AuLFTase(D54N)\(^{\text{MET}}\)-sucrose complex. In contrast, additional structural features are involved in stabilizing the fructose conformation at the –2 subsite. A furanosyl ring at the –2 subsite is perpendicular to the bottom of the pocket and faces in a distance of 5.5–7.0 Å against the Pro-78 loop residue between \( \beta\)3 and \( \beta\)4 in a stacked orientation. Residues in Blade I make extensive hydrophobic and hydrogen bonding interactions with fructose at the –2 subsite, which are mediated mainly by a loop region between \( \beta\)3 and \( \beta\)4. Specifically, this loop region, including Asn-76, Gly-77, Pro-78, and Gly-79, is responsible for extensive hydrophobic contacts to fructose at the –2 subsite. The elongated pocket in the N-domain is now fully occupied by levanbiose, suggesting that difructosaccharide is a basic unit recognized by this enzyme.

Levanbiose is also bound to the C-domain region identified in the AuLFTase(D54N)\(^{\text{MET}}\)-sucrose complex (supplemental Fig. S4B). However, its conformation is different from that in the N-domain. Specifically, the relative orientation of the two fructosyl rings is completely inverted, with a torsion angle \( \varphi \) of 87°. Our modeling study indicates that this conformation allows the polymeric fructose to take a linear form, rather than a circular form dictated by the torsion angles characterized in the N-domain (Fig. 3A). However, its binding environment is
largely reminiscent of that in the AuLFTase(D54N)MET-levanbiose complex, and more interactions are identified because of the extended conformation of levanbiose.

**Structure Comparison of AuLFTase and Other Members of the GH32 Family**—No significant changes in the conformation of AuLFTase occurred following binding of a ligand; superposition of the three structures described in this study showed a root mean square deviation of less than 0.48 Å for 477 Cα atoms. These structural features extended into the active site residues, including a catalytic triad, the side chains of which differ only by 0.1–0.5 Å. These observations suggest that no noticeable structural rearrangement was induced by mutation of Asp-54 to Asn-54 in AuLFTase(D54N). Therefore, the active site environment of AuLFTase(D54N) is almost identical to the WT enzyme, except for the immediate vicinity of Asn-54. However, some differences were observed in the orientation of the ligand bound to the active site. Specifically, the relative orientation of fructose at the −1 subsite was different in the two complexes (supplemental Fig. S5A); the anomeric carbon (C2′) at the −1 subsite of levanbiose was located ~1.7 Å farther from Asn-54, and its furanosyl ring adopted a different orientation. Our analysis indicated that fructose at the −1 subsite in the complex with sucrose has geometrical features more appropriate for an enzymatic reaction (see “Discussion”).

Consistent with the general features of GH32 family proteins, the AuLFTase structure in this study showed high similarity with the known structures of the GH32 family (see “Discussion”), but noticeable differences in the β-propeller N-domain pocket dimensions were observed. All GH32 family structures determined to date liberate a monomeric unit of carbohydrate, either glucose or fructose, by hydrolysis of the glycosidic bond of various substrates. Accordingly, the substrate-binding pocket, which is located at the center of the N-domain near the catalytic triad, accommodates a monosaccharide (Fig. 4A) (30). Structural comparison of AuLFTase with an exo-inulinase of the GH32 family clearly indicates that the −2 subsite identified in this study is absent in other GH32 family structures (Fig. 4B); there is no space available in other GH32 family proteins for the −2 subsite, because of sequence changes in the loop between blade 3 and blade 4 of blade I. In particular, residues such as Asn-76 and Gly-79 are conserved in the corresponding loop among members of LFTase but are changed in other GH32 proteins with residues for a larger side chain such as tryptophan and isoleucine, respectively (supplemental Fig. S1). These features are likely an essential for the substrate specificity of LFTase, i.e., the production of DFAs that consist of difructosaccharide.

**Functional Analysis**—We carried out a functional analysis of various AuLFTase mutant enzymes by reacting them with levan, the products of which were characterized by TLC and HPLC. Sixteen residues were selected for these analyses, including the proposed catalytic residues for the GH32 family proteins for the −2 subsite, because of sequence changes in the loop between β3 and β4 of blade I. In particular, residues such as Asn-76 and Gly-79 are conserved in the corresponding loop among members of LFTase but are changed in other GH32 proteins with residues for a larger side chain such as tryptophan and isoleucine, respectively (supplemental Fig. S1). These features are likely an essential for the substrate specificity of LFTase, i.e., the production of DFAs that consist of difructosaccharide.

![FIGURE 3. The binding of levanbiose in the AuLFTase(D54N)MET-levanbiose complex. A, the modeled levanbiose is overlaid with an Fo – Fe electron density map contoured at 2.2 σ. Torsion angles of levanbiose (supplemental Fig. S2) are 60° for ω, 176° for ψ, and 171° for φ. B, shown is surface representation of the active site, with levanbiose in a stick model. C, the active site is shown, with neighboring residues forming the active site pocket. This orientation is identical to that in B. Water molecules are not included for clarity. D, represented is a scheme for the interactions around levanbiose. Residues involved in hydrophobic interactions within 4.5 Å and hydrogen bonding are indicated in magenta and black, respectively, together with the interatomic distance values. Water molecules are represented by red circles.](image-url)
(supplemental Figs. S3A and S6A), indicating their involvement in DFA-IV production. In addition to these catalytic residues, many others surrounding the +1 and −1 subsites also play a crucial role in enzyme activity (Figs. 2C and 3C). The catalytically inactive or greatly impaired mutants (supplemental Fig. S6A) are L70A, W108A, S109A, R185A, and W322A, which are involved in hydrogen bonding or van der Waals interactions to ligands, suggesting that these residues are involved in substrate binding. The only exception is Arg-139, which exclusively interacts with the +1 glucose moiety, and the R139A mutant retains enzymatic activity (see “Discussion”). In contrast, many residues surrounding the −2 subsite were to some extent tolerant of mutation (Fig. 5), suggesting their roles in pocket formation, not in catalysis. Only the W51A mutant lost its activity, whereas others, such as C53A and S72A, retained their DFA-IV production activity to a level identical to that of the WT enzyme. In particular, a larger side chain was introduced by mutation at positions 76 and 79, which mimics the structural characteristics of the pocket-forming residues for other GH32 proteins (Fig. 4B). Mutants such as N76A, N76W, G77A, and P78G maintained activities as high as the WT enzyme, but G79I and a double mutant of N76W/G79I produced fructose as a major product, rather than DFA-IV, consistent with the structural features of other monosaccharide-producing GH32 proteins. The HPLC analysis further supports our conclusion (supplemental Fig. S6B), because the major product of both mutant enzymes is indeed fructose. These observations suggest that the presence of a larger side chain at position 79 eliminates the fructosyl moiety binding site at the −2 subsite, and therefore those mutants catalyze hydrolysis of a monomeric fructose, possibly the terminal nonreducing fructose of levan, instead of producing DFA-IV.

**DISCUSSION**

We presented a crystal structure of AuLFTase, a member of the GH32 family, in apo form and in complexes with sucrose or levanbiose. The overall structural features of AuLFTase are essentially identical to those of other GH32 family members, in that the enzyme consists of two domains: a five-bladed β-propeller N-domain and a β-sandwich C-domain (Fig. 1B). A structural similarity search using the DALI program (31) indicated that there are more than a dozen GH32 family proteins homologous with AuLFTase, with Z-scores of 20.0–42.3 and a root mean square deviation of 2.3–4.2 Å. From a functional perspective, the β-propeller N-domain is a catalytic domain as revealed by functional analysis, and our structural data further suggest the possible role of the β-sandwich C-domain in carbohydrate recognition. Given that the carbohydrate-binding site on the concave surface of the C-domain is on the same face as an entrance to the center of the catalytic β-propeller N-domain, it could be considered that the polymeric substrate levan binds the C-domain along the concave surface and that its nonreducing end reaches into the active site for catalysis. Recent structural analysis of β-fructofuranosidase in the GH32 family also indicated the binding of substrate in the β-sandwich C-domain (32).

Despite the marked overall structural similarities, LFTase exhibits novel features in terms of its substrate specificity and the chemical nature of its DFA-IV product compared with other members of the GH32 family that hydrolyze one monosaccharide from the substrate. These functional characteristics of LFTase are imprinted in the active site of the N-domain; it accommodates levanbiose using the −1 and −2 subsites (Fig. 3B). These unique features are due to sequence variations in the Blade I loop region between LFTase and other GH32 proteins (Fig. 4B and supplemental Fig. S1), within which smaller side chains are present at the corresponding region in LFTase, unlike the presence of a bulky side chain in other GH32 proteins, indicating that they are key structural elements for the presence of the −2 subsite in LFTase. Except for those loop residues, most of the other pocket-forming residues are well conserved in the GH32 family (supplemental Fig. S1). In addition to LFTase, other carbohydrate-degrading enzymes, such as cellobiohydrolase (33) and IFTase (11), release a disaccharide or DFAs as products, but these enzymes belong to a different structural fold with unrelated sequences. However, common to these enzymes is the shape and dimension of the active site. It is
Pocket-shaped, with dimensions suitable for a disaccharide, providing a structural basis for the exo-type cleavage of disaccharide from the substrate.

Structural and functional analyses in this study led us to propose a possible mechanism for LFTase. In particular, our structural analysis suggested that fructose at the −1 subsite in the AuLFTase(D54N)MET-sucrose complex, rather than in the levanbiose complex, has an orientation favorable for nucleophilic attack by Asp-54 (supplemental Fig. S5A). Consistent with this suggestion, structures of other GH32 enzymes have a fructose conformation at the −1 subsite identical to that of the AuLFTase(D54N)MET-sucrose complex (supplemental Fig. S5B). Therefore, our proposed mechanism is based on a levanbiose conformation, in which the −1 subsite is superposed on that of the AuLFTase(D54N)MET-sucrose complex (Fig. 6A).

In the proposed mechanism (Fig. 6B), the levan substrate binds to the active site of LFTase, with such an orientation that a nonreducing terminal fructose sits at the −2 subsite, and the preceding fructosyl moiety is located at the −1 subsite near the Asp-54 nucleophile. The β,2′,6′-glycosidic bond between the −1 and +1 subsite is cleaved following a nucleophilic attack by Asp-54 on the anomic carbon (C2′) of the −1 subsite and subsequent reaction by the Glu-236 acid/base catalyst. Consequently, an intermediate between a levanbiosyl moiety and LFTase is formed, in which the anomic carbon of the −1 subsite in levanbiosyl moiety is covalently attached to the −2 subsite fructosyl unit were characterized in the structural analysis indicated the presence of −1 and −2 subites in the β-propeller N-domain pocket, but the +1 subsite, tentatively assigned based on a glucosyl unit, was unlikely to be a cognate site for the levan substrate. In fact, in the structural analysis of WT AuLFTaseMET in complex with levanbiose, we observed a continuous electron density from the −1 subsite to the rim of the β-propeller N-domain in one of the monomers, which was in a different direction from the currently assigned +1 subsite, although its density was not well ordered (supplemental Fig. S5D). A putative +1 subsite is likely located between Trp-322 and Arg-185, suggesting the means by which the R139A mutant retains its activity (supplemental Fig. S6, A and B), even though Arg-139 interacts exclusively with the glucosyl unit at the currently assigned +1 subsite. Notably, mutation of Trp-322 or Arg-185 abolished enzyme activity (supplemental Fig. S6A).

We characterized the structural features of AuLFTase in apo form and in complex with sucrose and levanbiose. The structural analysis indicated the presence of −1 and −2 subites in the active site and revealed the pocket that accommodates a difructosaccharide. This unique pocket dimension feature is conserved among LFTase members through sequence conservation in the loop region between β3 and β4 in Blade I but is not...
applicable to other GH32 enzyme family members. Our analyses suggest a possible reaction mechanism for LFTase in which the catalytic triad conserved in the GH32 family plays an essential role, and the terminal nonreducing fructosyl unit serves as acceptor. Our study thus provides a molecular basis for DFA-IV production by LFTase, which exhibits functional variations compared with GH32 family proteins, even within the conserved structural elements.

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