Inulin fructotransferase (IFTase), a member of the glycoside hydrolase family 91, catalyzes depolymerization of β-2,1-fructans inulin by successively removing the terminal difructosaccharide units as cyclic anhydrides via intramolecular fructosyl transfer. The crystal structures of IFTase and its substrate-bound complex reveal that IFTase is a trimeric enzyme, and each monomer folds into a right-handed parallel β-helix. Despite variation in the number and conformation of its β-strands, the IFTase β-helix has a structure that is largely reminiscent of other β-helix structures but is unprecedented in that trimerization is a prerequisite for catalytic activity, and the active site is located at the monomer-monomer interface. Results from crystallographic studies and site-directed mutagenesis provide a structural basis for the exolytic activity of IFTase and a functional resemblance to inverting-type glycosyltransferases.

Fructans are polysaccharides composed of linear and branched polymers of fructose linked to sucrose through glycosidic bonds of various linkage types. They have been conceived as one of the principal stored forms of energy in 15% of higher plants, as well as in a wide range of bacteria and fungi (1). Several plant fructosyltransferases, each with a distinct substrate and glycosidic bond linkage-type specificity, have been suggested to be involved in the sequential enzymatic steps that produce fructans such as β-2,6-linked levan and β-2,1-linked inulin. In this process, fructose is first linked to vacuolar sucrose, and then fructosyl units are successively added to the resulting trisaccharide (1, 2). Not only do plant fructans play a major role as storage carbohydrates, but they are also implicated in additional physiological functions in plants, such as drought and cold tolerance (1). By contrast, in bacteria, the multifunctional enzymes levansucrase (3) and inulosucrase (4) catalyze fructan biosynthesis, producing inulin and levan, the predominant bacterial fructans, respectively. Details of the levan biosynthetic mechanism in bacteria were recently revealed by structural studies of levansucrase (3, 5).

Fructan-degrading enzymes that function primarily in the mobilization of stored fructans in plants and microbes have also been characterized. Just recently, the plant fructan hydrolases (EC 3.2.1) were found to catalyze the hydrolysis of levan and inulin via an exclusively exolytic mechanism that releases successive terminal fructose units (6). The presence of these fructan exohydrolases, even in non-fructan-containing plants, suggests an additional, defensive role for these enzymes against pathogenic bacteria (2).

In bacteria, two distinctly different classes of enzymes perform fructan degradation. One of these classes includes two hydrolases, levansucrase (EC 3.2.1.65) and inulinase (EC 3.2.1.7), which exhibit both endo- and exotype hydrolytic activities. Classification based on sequence similarity (7) (CAZY; www.cazy.org/CAZY/index.html) place these plant and microbial hydrolytic enzymes into the GH32 family (O-glycoside hydrolases). Structural analyses of the GH32 enzymes invertase (8, 9), exo-inulinase (10), and plant exohydrolase (11) have revealed substantial structural similarities; these enzymes consist of five-bladed β-propeller domains connected to β-sandwich domains. In addition, active sites for the proposed hydrolytic activity have been identified; they include three acidic amino acid residues.

The second class of bacterial fructan-degrading enzymes, which includes levan fructotransferase (EC 4.2.2.16) (12, 13) and inulin fructotransferase (IFTase; EC 4.2.2.17 or 18) (14, 15), has long been known to catalyze the depolymerization of fructans into difructose dianhydrides (DFAs) via intramolecular fructosyl transfer (Fig. 1A). Because DFAs have physiological effects, including the promotion of human health, and show promise as low calorie sweeteners (reviewed in Ref. 16), IFTase and levan fructotransferase have attracted the attention of the food industry. The linkage types of enzymatically produced DFAs vary with the specific enzymes and substrate fructans employed; IFTase produces DFA-I (α-D-fructofuranose-β-D-fructofuranose 2',1,2,1'-dianhydride) and DFA-III (α-D-fructofuranose-β-D-fructofuranose 2',1,2,3,3'-dianhydride), whereas levan fructotransferase results in DFA-IV (β-D-fructofuranose-
Crystal Structure of Inulin Fructotransferase

β-D-fructofuranose 2,6'-2',6'-dianhydride). Levan fructotransferases are categorized as GH32 enzymes, like other fructohydrolases, and they share considerable sequence identity of 30–44% and several conserved regions with the other members of GH32 (12, 13).

Notably, IFTases do not exhibit any apparent sequence homology with levan fructotransferases, although both enzymes carry out similar reactions. Furthermore, IFTases appear to be unrelated to any other known proteins but are highly similar to members of the family, with 50–98% sequence identity (Fig. 1B), suggesting that the IFTases share a three-dimensional structure common to the family. The products of six bacterial genes have so far been characterized biochemically as IFTases and classified as GH91 enzymes, even in the absence of supporting evidence for hydrolase activity. The DFA-III-producing IFTase from Bacillus sp. snu-7 (BsfIFTase; EC 4.2.2.18) consists of 450 amino acid residues, including a 40-residue, N-terminal signal sequence for secretion. Although biochemical features of BsfIFTase have been characterized (14, 17), the fundamental details of BsfIFTase catalysis, such as the identities of the residues that catalyze intramolecular fructosyl transfer and the mode of enzyme action, remain elusive. To elucidate the structural and functional properties of BsfIFTase, we determined its crystal structure at 1.8-Å resolution and the structure of BsfIFTase.

EXPERIMENTAL PROCEDURES

Purification and Crystallization of BsfIFTase—The gene for BsfIFTase (access number DQ112363) from the Bacillus sp. snu-7 was cloned, and DNA for the mature BsfIFTase (residues 41–450) was subcloned into the expression vector pET15b (Novagen, Madison, WI) using an Ndel and a SalI site (17). The His-tagged, selenomethionine-substituted recombinant BsfIFTase was purified using immobilized metal affinity chromatography with buffer A (50 mM NaH2PO4, pH 7.4, 500 mM NaCl). The His-tagged recombinant BsfIFTase was purified using immobilized metal affinity chromatography with buffer A (50 mM NaH2PO4, pH 7.4, 500 mM NaCl). The His-tagged recombinant BsfIFTase was purified using immobilized metal affinity chromatography with buffer A (50 mM NaH2PO4, pH 7.4, 500 mM NaCl). The His-tagged recombinant BsfIFTase was purified using immobilized metal affinity chromatography with buffer A (50 mM NaH2PO4, pH 7.4, 500 mM NaCl). The His-tagged recombinant BsfIFTase was purified using immobilized metal affinity chromatography with buffer A (50 mM NaH2PO4, pH 7.4, 500 mM NaCl).

Crystalization was accomplished at 22 °C using the hanging drop vapor diffusion method. The crystals of BsfIFTase in space group C2 with three monomers/asymmetric unit were produced using the His-tagged, selenomethionine-substituted protein with a crystallization buffer consisting of 0.1 M NaH2PO4, pH 7.4, 500 mM NaCl. The His-tagged recombinant BsfIFTase was purified using immobilized metal affinity chromatography with buffer B (buffer A + 500 mM imidazole), dialyzed against buffer C (50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM EDTA), and then concentrated to <10 mg/ml.

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nine-substituted BsIFTase were collected at 1.8 Å on Beamline 6B at Pohang Accelerator Laboratory (Pohang, Korea). Diffraction data for the BsIFTase-tetrafructosaccharide complex at 1.8 Å resolution were collected using a single wavelength on Beamline 6B at Pohang Accelerator Laboratory. The data were collected at 100 K, and the crystals were cryoprotected by the addition of 30% glycerol to each crystallization solution. The program HKL2000 was used for data processing (20).

The program SOLVE/RESOLVE (21, 22) was used for phasing and density modification of the native BsIFTase structure. Of 12 possible selenium atoms, a total of 10 sites were identified and used to calculate the initial phases. The presence of three monomers in the asymmetric unit improved density modification in RESOLVE with noncrystallographic symmetry averaging. The initial electron density map was sufficient to trace most of the residues in each monomer, except the highly disordered N-terminal region including the His tag and residues 41–51. Manual model building and refinement were performed using the programs O (23) and CNS (24), respectively.

The substrate-binding site was unambiguously located using a difference map with phases from the substrate-bound and amplitudes between the substrate-bound and native data. The difference map showed that only a disaccharide moiety of the tetrasaccharide was observed, and a conformation of the identified difructosaccharide corresponds to that of substrate, not the product DFA-III. Presumably, a high concentration of substrate (200 mM) led to stabilization of substrate binding in the active site. A model for the bound difructosaccharide was subsequently fit into the density, and the resulting model for the BsIFTase-substrate complex was then subjected to refinement using CNS. The residual density near the binding site could not be explained by any molecular model and was therefore modeled as water molecules (at positions 106, 118, and 159). Two molecules of phosphate were modeled in the putative intersubunit channel. Details of data collection and structure refinement are shown in Table 1. The stereochemistry of refined structures was confirmed using the program PROCHECK (25). With the exception of one residue, Val188, in each monomer, neither the native nor the substrate-bound structures contained residues with disallowed conformations. The figures were prepared using PyMOL (DeLano, W. L. The PyMOL Molecular Graphics System), and the structure was analyzed using the CCP4 suite (25).

Site-directed Mutagenesis and Enzymatic Activity Assay—Genes encoding BsIFTase mutants were generated from plasmid pET15b-BsIFTase using polymerase chain reaction (Table 2), and their sequences were verified by DNA sequencing. The mutant proteins were expressed and purified as His-tagged recombinant proteins in E. coli strain BL21(DE3)pLysS as described above, and each of the purified proteins appeared as a single major band on SDS-PAGE. BsIFTase enzyme activity was assayed as reported previously (14, 17), using 1 μg of wild-type or mutant BsIFTase in a 100-μl reaction mixture containing 0.5% inulin in 50 mM Tris-HCl (pH 7.5). The reaction mixtures were incubated at 37 °C for 5 min, and then their products were analyzed by thin layer chromatography in a solvent system of n-propanol:ethyl acetate:H2O (3:1:1).

Analytical Ultracentrifugation—Equilibrium sedimentation studies were performed using a Beckman ProteomeLab XL-A analytical ultracentrifuge using six-hole, charcoal-filled Epon centerpieces. BsIFTase was dissolved at 1.8 M (0.32 mg/ml) in a buffer containing 50 mM Tris-HCl, pH 7.5, 0.2 m NaCl, and 1 mM EDTA. The concentration of the protein was determined using ε280 nm = 27,765 M−1 cm−1 calculated from its amino acid composition. The radial absorbance distribution was measured at 20 °C and 12,000 rpm at four different wavelengths (230, 235, 240, and 280 nm). After 48 h of centrifugation, five scans were collected at 12,000 rpm at four different wavelengths (230, 235, 240, and 280 nm). After 48 h of centrifugation, five scans were collected at 12,000 rpm at four different wavelengths (230, 235, 240, and 280 nm). After 48 h of centrifugation, five scans were collected at 12,000 rpm at four different wavelengths (230, 235, 240, and 280 nm). After 48 h of centrifugation, five scans were collected at 12,000 rpm at four different wavelengths (230, 235, 240, and 280 nm). After 48 h of centrifugation, five scans were collected at 12,000 rpm at four different wavelengths (230, 235, 240, and 280 nm).
ity of the rotor, and $e$ is a base-line error term. The data were analyzed by mathematical modeling using MLAB (27). All of the measured data fit well to a trimeric model, and a representative result obtained for 1.8 $\mu M$ BsIFTase is presented.

Isothermal Titration Calorimetry—Isothermal titration calorimetric analysis was carried out using MicroCalorimetry System (Microcal) at 25 °C. Two inactive mutants E244Q and D233N, as well as substrate inulin were dialyzed against a buffer containing 50 mM Tris-HCl, pH 7.5, 200 mM NaCl, 1 mM EDTA, and then degassed. The data were analyzed using the Origin software (OriginLab Corp.).

RESULTS

Overall Structure of Monomer—Mature BsIFTase consisting of amino acid residues 41–450 was crystallized in the C2 space group with three monomers/asymmetric unit (A, B, and C) related by noncrystallographic symmetry. The presence of a homotrimeric BsIFTase in a crystalline state is consistent with results obtained from analytical ultracentrifugation experiments (Fig. 2), which indicate that the enzyme exists exclusively as a trimer in solution at concentrations as low as 0.08 mg/ml. In size exclusion chromatography, however, BsIFTase appeared to behave as a dimer, possibly because of its elongated shape (data not shown).

Each monomer forms a right-handed parallel $\beta$-helix (henceforth, “$\beta$-helix”), a fold that was initially identified in the pectate lyase PelC (28). $\beta$-Helical folds have been identified in several classes of enzymes frequently associated with polysaccharides, including pectin lyase-related proteins (29), pectin methyltransferase (30), and phage P22 tailspike protein (31). Recently, they have also been found in other polysaccharide-degrading enzymes in $O$-glycoside hydrolase families 28 (32), 49 (33), and 82 (34), as well as in virulence factors (35). The BsIFTase monomer, which is shown in Fig. 3A, has dimensions of 75 $\times$ 57 $\times$ 30 Å. Each monomer has an overall cylindrical shape consisting of 399 residues that form 13 helical turns of a right-handed coil. The highly disordered N-terminal residues 41–51 are excluded from the final model. The BsIFTase structure exhibits the general structural characteristics of $\beta$-helical proteins (35–37), but it differs in some details. Each turn consists of two, three, or four $\beta$-strands, joined by intervening loops, and the $\beta$-strands

![FIGURE 2. Oligomerization state of BsIFTase. Typical equilibrium sedimentation data obtained at 230 nm and 12,000 rpm with 1.8 $\mu M$ (0.08 mg/ml) BsIFTase were fit to dimer, trimer, and tetramer models. The absorbance distribution is consistent with a thermodynamically ideal trimer (solid line), not with a dimer or tetramer (dashed line). Inset, the distributions of the residuals as a function of radial position indicate that the trimer model (open circles) yielded the smallest root mean square error of 7.54 $\times$ $10^{-3}$, whereas the dimer and tetramer models deviated significantly from the measured data (rectangles and triangles, respectively).](image)

![FIGURE 3. Structure of BsIFTase. A, side view of monomeric BsIFTase is shown in stereo, with PB1 labels for turns 2 and 9. Two active site-forming loops are indicated (see text). Each $\beta$-strand is defined in Fig. 1B, structure-based sequence alignment for equivalent $\beta$-strands, with corresponding turns labeled. The color coding for each $\beta$-strand is identical to that used in Fig. 3A, and stacked side chains are enclosed in a black box. For clarity, residues 126–148 in T2 of turn 2 are indicated only by a dashed line. C, the stacked internal, hydrophobic side chains are presented in a top view from the C terminus, with labeled residues. D, stacked asparagine residues in T1 and T2 are shown with their turn and residue numbers.](image)
Crystal Structure of Inulin Fructotransferase

that are present between adjacent turns associate to form a parallel sheet on the surface of the enzyme. All of the β-strand residues fulfill two strict structural requirements: they are in the β-conformation region of a Ramachandran plot, and the main chain atoms of β-strands in adjacent turns interact by hydrogen bonding. A structure-based sequence alignment of the β-strands along the β-sheet is shown in Fig. 3B. Of particular interest is the fact that the N-terminal five turns (turns 3–7) of the coil involve four β-strands, whereas turns 8 and 9 include three, and the C-terminal four turns involve only two β-strands each (Fig. 3, A and B). Each β-strand consists of two to six residues, except that the β-strands in turns 10 and 11 have ten and nine residues, respectively. As a consequence of variation in the number of β-strands and loop length, the N-terminal turns exhibit wider interiors, with a 19-residue α1-helix capping the N-terminal domain, whereas the last two turns at the C terminus have narrow interiors with only two β-strands.

Structural Characteristics of Turns—The BsIFTase turns are partially homologous to three-β-strand turns of PelC (28, 36, 37). In PelC, the first, second, and third β-strands of the turns are designated PB1, PB2, and PB3, respectively, with loops T1, T2, and T3 following the corresponding strands. In BsIFTase, an additional two-residue β-strand is present within the four-β-strand turn at the region corresponding to T1 of PelC; this strand is designated PB1b. The second strand in the two-β-strand turns is designated PB2 because its location is equivalent to that of PB2 in a three-β-strand turn (see below). The long β-strands in turns 10 and 11 include all three regions (PB2, T2, and PB3; Fig. 3B). In cross-section, the turns resemble an “L” shape, with the β-strands almost perpendicular to each other. The most dramatic changes in β-strand conformation were observed at turns 10 and 11, in which the 10- and 9-residue PB2 strands are so sharply bent that the two PB2 ends are almost perpendicular. As a result, the first half of PB2 in these turns corresponds structurally to PB2, but the second half of PB2 is equivalent to PB3 in other turns.

Although the BsIFTase structure differs from that of most other β-helical proteins in the number and conformation of β-strands in turns, it does resemble these proteins in that the interior of the β-helix is closely packed with hydrophobic or polar side chains along the sheet (35–37). Specifically, the internal hydrophobic residues, which include valine, leucine, and isoleucine predominantly, and occasionally phenylalanine, are stacked between adjacent turns, as indicated in the structure-based sequence alignment of the β-strands shown in Fig. 3B. PB1 has two rows of hydrophobic stacks, and PB2 and PB3 have one stack each, with each stack involving a total of 11–13 residues (Fig. 3C).

With the exception of the 27-residue T2 loop in turn 2, the T2 loops are only two or three residues in length. Sharp kinks in a T2 loop are associated with a stack of seven asparagine residues that is located in the β-helix interior (Fig. 3B); this was first identified in the structure of PelC and is called the “asparagine ladder” (28). In BsIFTase, this asparagine ladder, including Asn372 in turn 10 and Asn406 in turn 11, has a continuous hydrogen-bonded network between the asparagine side chains and forms hydrogen bonds between the main chain amide groups in a T2 loop of adjacent turns and the asparagine side chain (Fig. 3D); consequently, the ladder stabilizes the sharp kinks.

Unlike T2 loops, T3 loops following PB3 strands exhibit a wide range of lengths and conformations. The T3 loops in turns 3, 9, and 10 protrude from the core β-helix, forming a relatively irregular protrusion on one side of the enzyme. In the central portion of the β-helix, the β-helix surface consists primarily of T3 loops. Notably, the protruding 27-residue T2 loop in turn 2 also wraps halfway around the β-helix and is localized in this loop cluster (Fig. 3A).

Trimeric Structure of BsIFTase—The three parallel monomers in the asymmetric unit intertwine to constitute a trimer (Fig. 4A) in which PB2 in one monomer is packed in an antiparallel manner against PB3 in the adjacent monomer. When the three monomers are superimposed, the resulting root mean square deviation for the corresponding Cα atoms is 0.05 Å, indicating that the structures of the independent monomers are essentially identical.

In contrast to the interior side chains, the external side chains of each BsIFTase sheet are relatively heterogeneous in amino acid composition and configuration. The side chains of adjacent subunits interdigitate, which stabilizes the trimer (38, 39). The surface area buried by trimerization is calculated to be 4439 Å2/monomer, or 28% of the monomer surface area. Because of this close packing, no intersubunit channel is present between monomers or at the trimer interface. Specifically, in the channel that otherwise might be present in the center of the trimer, the only electron density observed arose from several water molecules and from two phosphate molecules from the crystallization buffer.

Substrate-binding Site at the Monomer-Monomer Interface—The structure of BsIFTase complexed with a β-2,1-linked tetrafructosaccharide revealed that the substrate is bound in the crevice between two interacting monomers. Therefore, three noncrystallographic symmetry-related, independent, but structurally identical binding sites are present in the trimeric complex (Fig. 4A). Only a disaccharide moiety of the tetrasaccharide was unambiguously observed (Fig. 4B). Although inulin is a nonreducing fructan, the BsIFTase region interacting with the terminal fructosyl residue was assigned as subsite +2 following the nomenclature of Davies et al. (40). The terminal fructose F1 (subsite +2) is linked to another fructose F2 (subsite +1) by a β-2,1 glycosidic bond. Weak residual density corresponding to another glycosidic bond to F3 (subsite −1) was observed, but the density was so disordered that the third fructose unit was not included in the final model.

The structure of the BsIFTase substrate-binding pocket occupied by the observed disaccharide F1–F2 is shown in Fig. 4 (C and D), and the interactions between BsIFTase and the bound substrate are shown schematically in Fig. 4E. In particular, the substrate-binding site is located on the surface between (i) the regions including PB3 and T3 of turns 5–7 and (ii) PB1b of turns 4–6 from the interacting subunit. Two additional structural elements, loop T3 from turn 9 and loop T2 from turn 2, enclose the top and bottom of the substrate-binding site, respectively, generating a pocket between two interacting monomers. The pocket is wide open at the entrance, which is comprised of PB1b and loop T3 from turn 9, and terminated
by a layer of polar residues, including Ser$_{133}$, Arg$_{292}$, and Glu$_{244}$. This 10-Å-deep pocket is lined by hydrophobic residues, including Phe$_{129}$, Ile$_{134}$, Tyr$_{197}$, Phe$_{290}$, Pro$_{291}$, Phe$_{315}$, Tyr$_{343}$, and Phe$_{346}$, as well as polar residues Arg$_{174}^*$ and Asp$_{233}^*$ from the neighboring monomer. All of the active site residues are either invariant or highly conserved within the family (Fig. 1B).

Substrate binding appears to cause insignificant, but systematic, localized structural changes in the loop region including Tyr$_{343}$ (Fig. 4D). Loop T3 residues Glu$_{341}^-$Arg$_{349}$ in the pocket are shifted toward the substrate, with the Cα atoms moving 0.4–1.0 Å for an overall Cα root mean square deviation of 0.16 Å. Except for these variations, no other conformational differences, even in side chain configuration, were observed between the native structure and the substrate complex. An apparent feature of the bound F1–F2 is the relative orientation of the two fructose units; a furanose ring of F2 is not stacked with but rather is oriented perpendicular to F1. This conformation is similar to that previously determined for DFA-III (41).

Only six direct interactions between the bound disaccharide and active site residues are observed in the crystal structure (Fig. 4E). Four of these interactions are localized in F1 bound at the inner site; the hydroxyl group O-1′ (primed numbers refer to F1 atoms) interacts with the side chains of Arg$_{292}$ and Ser$_{133}$ at distances of 3.1 and 2.8 Å, respectively, and the O-3′ hydroxyl group is 2.6 Å from the carboxylate group of Glu$_{244}$. The main chain carbonyl group of Pro$_{291}$ also hydrogen bonds with O-4′. Fructose F2, which is close to the entrance of the active site, hydrogen bonds with Asp$_{233}^*$ from a neighboring monomer; Asp$_{233}^*$ mediates bidentate hydrogen bonds with O-3 and O-4 of F2 at distances of 2.6 and 2.7 Å, respectively. A potential 2.9-Å hydrogen bond with the guanidinium group of Arg$_{174}^*$ may further stabilize the orientation of Asp$_{233}^*$. Solvent-medi-
Of these substrate-interacting residues, Arg174, Asp233, and Glu244 are invariant in the IFTase family (Fig. 1), suggesting that they have important roles in catalysis or substrate binding. The interactions described here were consistently observed at each of the three independent binding sites of the BsIFTase active site.

Site-directed Mutagenesis of BsIFTase—The functional roles of residues Asp233 and Glu244, which interact directly with the bound disaccharide, were examined using site-directed mutagenesis. The enzymatic activities of the resulting mutant proteins were measured using thin layer chromatography (Fig. 5). Wild-type BsIFTase predominantly produced DFA-III, as well as by-products such as GF3 and GF4, whereas mutants D233N and E244Q were essentially inactive.

Binding of Substrate to the Mutant BsIFTase—Because two mutants, D233N and E244Q, were inactive, it was possible to measure the binding affinity of substrate inulin to these mutants by isothermal titration calorimetric analysis (Fig. 6). Substrate inulin readily binds both mutants, with its $K_d$ of 23.1 μM and 68.5 μM for D233N and E244Q, respectively.

**DISCUSSION**

The x-ray crystal structure of BsIFTase reveals that it folds into a β-helix. The program BetaWrap (42) successfully predicted a β-fold structure for BsIFTase, with a raw score of −19.93 and a p value of $3.1 \times 10^{-4}$. The basic structural features of BsIFTase are largely reminiscent of a β-helix. Using DALI server (43), we found a high level of structural similarity between monomeric BsIFTase and 17 other β-helix structures with Z values greater than 10; the root mean square deviation values for the equivalent Ca atoms were 2.2–3.3 Å, suggesting that the arrangements of basic structural units are well conserved within these β-helical structures. Nonetheless, helical turns in the BsIFTase β-helix consist of two to four parallel β-strands. In other β-helix structures, a uniform number of β-strands, typically three β-strands in each turn, is usually observed (35–37).

The most noticeable structural feature of BsIFTase is its oligomerization state, which is functionally and structurally essential for catalysis. The active sites in all known β-helix protein structures are located exclusively in the elongated groove parallel to the helical axis on the surface between T3 and PB1 within a monomer (35), implying that oligomerization is not essential for catalysis in these enzymes. Indeed, most of the β-helix enzymes characterized to date function as monomers. Tailspike protein, which forms a trimer in a manner similar to that of BsIFTase, is the exception, although its proposed catalytic mechanism occurs within a monomeric structural context (31). Therefore, trimerization of the tailspike protein β-helix may not be required for catalysis and might instead be important for thermostability and protease resistance (38, 39). In contrast to that of tailspike protein, the BsIFTase active site is located at the monomer-monomer interface. This structural feature is unprecedented for β-helical proteins and implies that trimerization, which brings the catalytic residues together in the active site, is a prerequisite for catalytic activity. These structural features are consistent with our results from analytical ultracentrifugation and site-directed mutagenesis studies (Figs. 2 and 5). In addition to BsIFTase, other IFTases have also yielded size exclusion chromatography results that suggest a dimeric or trimeric structure (14, 15).

Another structural feature of BsIFTase is the size and shape of the substrate-binding pocket. The current structural evidence clearly reveals that the active site without the exit side is just large enough to accommodate one molecule of difructosaccharide in the pocket. From a structural perspective, therefore, a plausible mechanism for BsIFTase is that it successively removes the terminal difructosaccharide units of inulin as cyclic anhydrides. Hence, our structural data provide a physical basis for the exolytic activity of BsIFTase. These structural findings are analogous to those associated with cellobiohydrolase CbhA of the O-glycoside hydrolase family 9 (44). CbhA is a seven-module enzyme that shares many structural features with other GH9 members associated with cellobiohydrolase CbhA of the O-glycoside hydrolase family 9 (44). CbhA is a seven-module enzyme that shares many structural features with other GH9 members that exhibit endocellulase activity; in these other enzymes, a tunnel-shaped active site catalyzes endolytic cleavage of the substrate. However, the presence of a unique loop at one end of the tunnel in CbhA causes the active site exit to be blocked and thus provides a structural basis for the exclusive exocellulase activity of CbhA. Taken together, our data of the structural features of BsIFTase described here reveal many important features of the enzyme, including its functional trimerization, its exolytic mode of action, and the location of the active site at the monomer-monomer interface.

To date, mechanistic clues about IFTase-mediated catalysis, such as the identity of the active site residues and a possible mechanism for intramolecular fructosyl transfer, remain elusive. Although classified as a hydrolase by CAZy, BsIFTase does not have a pair of acidic residues in the vicinity of a possible glycosidic bond cleavage site between subsite +1 and −1. The absence of
a putative nucleophile and a general acid in this subsite precludes the idea of BsIFTase catalyzing the reaction in a manner similar to that of O-glycoside hydrolases typically employing two acidic residues (45). Instead, our structural and functional analysis suggests that BsIFTase catalyzes an intramolecular fructosyl transfer analogous to that of glycosyltransferases with an inverting mechanism (46, 47).

Glycosyltransferases consist of two homologous $\beta/\alpha/\beta$ domains, and the active site is located in the crevice between the two domains (47). Therefore, neither the overall structure nor the active site arrangement of BsIFTase bears any resemblance to those of glycosyltransferases. The generally accepted explanation for enzymatic glycosyl transfer using the inverting mechanism is that the reaction is carried out using an $S_{N}2$-like single-displacement mechanism, in which the acceptor molecule is activated for nucleophilic attack by a general base. The resulting deprotonated acceptor can then attack at the anomeric center of the donor from the opposite side, with concomitant elimination of the moiety attached to the anomeric carbon of the donor. This attack results in inversion of the anomeric configuration at donor and forms a nascent covalent linkage between the donor and the acceptor. In fact, x-ray crystallographic structure of DFA-III, a product by IFTase, indicated that one of fructose unit forms $\alpha$-configuration on its anomeric center (41), suggesting that there is an inversion of the anomeric center, consistent with the inverting mechanism.

By analogy with the above mechanistic view, we propose that IFTase catalyzes the reaction via the inverting mechanism (Fig. 7). The terminal fructosyl unit $F1$ (subsite +2) bound at the inner site in BsIFTase appears to serve as the acceptor, and the $F2$ close to the entrance (subsite +1) might serve as the donor, given that the remaining fructose units of inulin containing the glucosyl end are expected to be eliminated as a leaving group upon catalysis. Therefore, the $F2$ fructosyl unit should undergo inversion of its anomeric configuration. Notably, the carboxylate group of Glu244 is within 2.6 Å of the O-3' hydroxyl group of $F1$ in our structure (Fig. 4, D and E), which immediately suggests that Glu244 acts as a general base to activate the acceptor. This suggestion is supported by the effect of the E244Q mutation, which essentially inactivates the enzyme (Fig. 5). These structural features are quite similar to those of glycosyltransferase employing an inverting mechanism in that the general base is exclusively either aspartate or glutamate (46, 47), and recently a histidine residue activated by a nearby aspartate (48, 49). The effect of Asp233 mutation is rather complicated. Apparently, Asp233 is involved in recognizing a donor $F2$ via hydrogen bonds (Fig. 4, D and E), and the measured binding affinity of inulin to D233N was almost similar to that of E244Q (Fig. 6). But a mutation of Asp233 eliminated catalytic activity (Fig. 5), suggesting that Asp233 plays a critical role in catalysis. Given that Asp233 is only one of possible residues by Asp233 to orient a donor $F2$ molecule into an optimal configuration for cataly-
The proposed mechanism of BsIFTase is consistent with the results of our structural and functional analysis, which provided mechanistic insights into intramolecular fructosyl transfer. These insights cannot be extended to the homologous fructosyl units in DFA-IV remain in the β-propeller structures. In contrast, the perpendicular orientation of F2 relative to the F1 fructosyl unit appears to alleviate steric hindrance, thus allowing O-3 of F1 and O-1 of F2, resulting in an orientation of O-3 that is unfavorable for an SN2-like reaction (Fig. 4D). The dihedral angles involved in the 2′-1 glycosidic bond would therefore have to be adjusted for this nucleophilic attack to be achieved.

The present study demonstrates that the structurally unrestrained O-3 of F1 can be achieved efficiently. Further experiments will be required to explicitly assign the functional role of Asp233. Interactions between negatively charged residues such as aspartate and glutamate and sugar donors were also observed in other glycosyltransferases, in which mutation of those negatively charged residues abolished catalytic activity (48, 49), as characterized in BsIFTase.

In the binding mode observed for disaccharide F1–F2, the perpendicular orientation of F2 relative to the F1 fructosyl unit appears to alleviate steric hindrance, thus allowing O-3′ of acceptor F1 to more easily gain access to C-2 of donor F2. Although these two atoms are separated by about 5.5 Å, they are constrained by the pre-existing glycosidic bond between C-2′ of F1 and O-1 of F2, resulting in an orientation of O-3′ relative to C-2 that is unfavorable for an SN2-like reaction (Fig. 4D).
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