Crystal Structure and Mutational Analysis of Isomaltodextranase, a Member of Glycoside Hydrolase Family 27*

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Background: Arthrobacter globiformis T6 isomaltodextranase (AgIMD) hydrolyzes a polysaccharide, dextran, but is classified into glycoside hydrolase family (GH) 27, which includes mainly α-galactosidases and α-N-acetylgalactosaminidases.

Results: The crystal structure of AgIMD was determined.

Conclusion: AgIMD has features found in GH13, GH31, and GH66 enzymes.

Significance: The results provide insights into the evolutionary relationships among GH13, -27, -31, -36, and -66.

Arthrobacter globiformis T6 isomaltodextranase (AgIMD) is an enzyme that liberates isomaltose from the non-reducing end of a polymer of glucose, dextran. AgIMD is classified as a member of the glycoside hydrolase family (GH) 27, which comprises mainly α-galactosidases and α-N-acetylgalactosaminidases, whereas AgIMD does not show α-galactosidase or α-N-acetylgalactosaminidase activities. Here, we determined the crystal structure of AgIMD. AgIMD consists of the following three domains: A, C, and D. Domains A and C are identified as a (β/α)8-barrel catalytic domain and an antiparallel β-structure, respectively, both of which are commonly found in GH27 enzymes. However, domain A of AgIMD has subdomain B, loop-1, and loop-2, all of which are not found in GH27 human α-galactosidase. AgIMD in a complex with trisaccharide panose shows that Asp-207, a residue in loop-1, is involved in subsite +1. Kinetic parameters of the wild-type and mutant enzymes for the small synthetic saccharide p-nitrophenyl α-isomaltoside and the polysaccharide dextran were compared, showing that Asp-207 is important for the catalysis of dextran. Domain D is classified as carbohydrate-binding module (CBM) 35, and an isomaltose molecule is seen in this domain in the AgIMD-isomaltose complex. Domain D is highly homologous to CBM35 domains found in GH31 and GH66 enzymes. The results here indicate that some features found in GH13, -31, and -66 enzymes, such as subdomain B, resides at the subsite +1, and the CBM35 domain, are also observed in the GH27 enzyme AgIMD and thus provide insights into the evolutionary relationships among GH13, -27, -31, -36, and -66 enzymes.

Dextran, a polymer of glucose, consists predominantly of α-1,6-glucosidic linkages, and several dextran-hydrolyzing enzymes have been found (1). Isomaltodextranase (α-1,6-d-glucan isomaltodextranase; EC 3.2.1.94) was found in Gram-positive soil bacteria, Arthrobacter globiformis T6 (NRRL B-4425) (2) and Kitasatospora sp. NRRL B-11411 (formerly known as Actinomadura sp.) (3). The A. globiformis T6 enzyme (abbreviated as AgIMD)3 liberates isomaltose from the non-reducing end of dextran and isomalto-oligosaccharides through a retaining mechanism (4, 5). AgIMD also catalyzes transglycosylation, and the enzymatic synthesis of some oligosaccharides using AgIMD has been reported (6).

The most notable feature of AgIMD is that the primary structure of the enzyme is homologous to those of α-galactosidases and α-N-acetylgalactosaminidases, despite the fact that AgIMD hydrolyzes a polymer of glucose. AgIMD is classified as a member of glycoside hydrolase family (GH) 27 in the CAZy database (7). Another enzyme family, GH36, comprises mainly α-galactosidases and α-N-acetylgalactosaminidases. The three-dimensional structures of the two families, GH27 and GH36, share a common structural core (8, 9), a catalytic (β/α)8 barrel domain, and the two families are demonstrated to be evolutionarily related (10). It is interesting to note that GH31 also has a similar catalytic (β/α)8 barrel domain, and the three families, GH27, GH31, and GH36, are categorized into clan GH-D. The main GH31 members function as α-glucosidases and α-xyllosidases (11, 12), but an α-galactosidase that belongs to GH31 has been reported recently (13). Therefore, the structure-function relationships of clan GH-D is complicated, and the study of AgIMD is useful for better understanding of clan GH-D.

In addition to the unique phylogenetic position of the catalytic domain, AgIMD has another intriguing architectural feature belonging to carbohydrate-binding module (CBM) 35. The CBM35 domains have been shown to share a highly similar fold,
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although there is considerable diversity in the biological functions of the enzymes possessing CBM35 (14, 15). We have previously constructed the expression system of AgIMD in *Escherichia coli* (16), and the enzyme has been crystallized (17). Here, we determined the crystal structure of AgIMD and compared it with those of GH27, GH31, and other related enzymes.

**Experimental Procedures**

*Construction of Expression Plasmids for Wild-type AgIMD and the Mutants—* An expression plasmid, pETG2Dsp, which is a derivative of pET3a(+), has been previously obtained (16). To facilitate the purification of AgIMD, an expression plasmid of His-tagged AgIMD was constructed. pETG2Dsp was digested with NdeI and BamHI, and the fragment was ligated into the NdeI-BamHI site of pET28a(+) (16), resulting in plasmid pET28a-AgIMD. Mutant enzymes were generated by site-directed mutagenesis with a QuikChange site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). To construct expression plasmids for mutants, oligonucleotides 5′-C TGG TAC GAG GAC GGA AGG GCC GCC GCG AAT ATT GGG CAG GTC-3′ (for D207A), 5′-TCC TGG TAC GAG GAC GGA AGG GCC GCC GCG GATG GTA CTA GGA GCC CAG G-3′ (for D207A/ N209A), and 5′-GAA GTT TCG GTA GGG ATT AGC CAT ATG TTC-3′ (for M243A) and their complementary strands were used as primers (mutated amino acid residues are underlined). All the constructs used were verified by DNA sequencing.

*Expression and Purification of AgIMD—* *E. coli* BL21(DE3) cells harboring pET28a-AgIMD were grown in 1 liter of Luria-Bertani (LB) medium containing kanamycin (50 μg ml⁻¹) to *A₆₀₀* = 0.6–0.8, and then induced with isopropyl β-D-thiogalactopyranoside at a final concentration of 0.1 mM; the incubation was then continued for 18 h at 20 °C. The cells were harvested by centrifugation at 4000 × g for 5 min, resuspended in 40 ml of 20 mM imidazole in 20 mM Tris-HCl buffer (pH 8.0), and disrupted by sonication. The cells and supernatants of the cultures were separated by centrifugation at 12,000 × g for 20 min. The supernatant obtained was applied onto a nickel-nitritolactriacetic acid (Ni-NTA)-agarose (Qiagen, Hilden, Germany) column equilibrated with 20 mM imidazole in 20 mM Tris-HCl buffer (pH 8.0), and then induced with isopropyl β-D-thiogalactopyranoside at a final concentration of 0.1 mM; the incubation was then continued for 18 h at 20 °C. The cells were harvested by centrifugation at 4000 × g for 5 min, resuspended in 40 ml of 20 mM imidazole in 20 mM Tris-HCl buffer (pH 8.0), and disrupted by sonication. The cells and supernatants of the cultures were separated by centrifugation at 12,000 × g for 20 min. The supernatant obtained was applied onto a nickel-nitritolactriacetic acid (Ni-NTA)-agarose (Qiagen, Hilden, Germany) column equilibrated with 20 mM imidazole in 20 mM Tris-HCl buffer (pH 8.0), and then induced with isopropyl β-D-thiogalactopyranoside at a final concentration of 0.1 mM; the incubation was then continued for 18 h at 20 °C. The cells were harvested by centrifugation at 4000 × g for 5 min, resuspended in 40 ml of 20 mM imidazole in 20 mM Tris-HCl buffer (pH 8.0), and disrupted by sonication. The cells and supernatants of the cultures were separated by centrifugation at 12,000 × g for 20 min. The supernatant obtained was applied onto a nickel-nitritolactriacetic acid (Ni-NTA)-agarose (Qiagen, Hilden, Germany) column equilibrated with 20 mM imidazole in 20 mM Tris-HCl buffer (pH 8.0), and disrupted by sonication. The cells and supernatants of the cultures were separated by centrifugation at 12,000 × g for 20 min. The supernatant obtained was applied onto a nickel-nitritolactriacetic acid (Ni-NTA)-agarose (Qiagen, Hilden, Germany) column equilibrated with 20 mM imidazole in 20 mM Tris-HCl buffer (pH 8.0), and disrupted by sonication. The cells and supernatants of the cultures were separated by centrifugation at 12,000 × g for 20 min. The supernatant obtained was applied onto a nickel-nitritolactriacetic acid (Ni-NTA)-agarose (Qiagen, Hilden, Germany) column equilibrated with 20 mM imidazole in 20 mM Tris-HCl buffer (pH 8.0), and disrupted by sonication. The cells and supernatants of the cultures were separated by centrifugation at 12,000 × g for 20 min.

The recombinant AgIMD was expressed and purified in a manner identical to that used for the wild-type enzyme.

*Crystallization, Data Collection, and Model Building—* The enzyme was crystallized at 20 °C using the hanging drop vapor diffusion method, where 1 μl of AgIMD (30 mg ml⁻¹) was mixed with the same volume of well solution containing 50 mM sodium acetate buffer (pH 3.6), 16% (w/v) polyethylene glycol 8000, and 50 mM potassium dihydrogen phosphate. For phase determination, the crystal was first transferred for 1 min to a solution containing 50 mM sodium acetate buffer (pH 3.6) and 20% polyethylene glycol 8000, and then transferred for 48 h to a solution containing 10 mM lead(II) acetate, 50 mM sodium acetate buffer (pH 3.6), and 20% polyethylene glycol 8000. The obtained crystal was transferred to a cryo-solution of 20% glycerol, 50 mM sodium acetate buffer (pH 3.6), and 20% polyethylene glycol 8000. Crystals of AgIMD-isomaltose and AgIMD-panose were obtained by soaking in well solutions containing 30% (w/v) isomaltose and panose, respectively, for a few seconds. The solution containing the ligand also acted as a cryo-protectant. Diffraction data were collected on the beamlines AR-NW12A and AR-NE3A at the Photon Factory (Tsukuba, Japan). All data were processed and scaled using HKL2000 (18). The initial phases were calculated from the single wavelength anomalous dispersion data set using the AutoSol program in the PHENIX suite (19). The coarse model obtained was applied for the molecular replacement method with MOLREP (20) in the CCP4 program suite (21). The model was refined using REFMAC5 in the CCP4 suite, and manual adjustment and rebuilding of the model were carried out using the program COOT (22). Solvent molecules were introduced using the program ARP/wARP (23). Validation of the structures was performed using the MolProbity server (24). Figures were prepared using PyMOL, Caver (26), and LigPlot (27). The data collection and refinement statistics are summarized in Table 1.

*Measurement of Enzymatic Activity and Protein—* The enzymatic activity of AgIMD was measured as described (16). Briefly, the activity of dextran T2000 (GE Healthcare, Chalfont St. Giles, UK) was measured in 100 mM sodium acetate buffer (pH 5.3) for 30 min at 30 °C. Preparation and measurement of the cleavage of p-nitrophenyl α-D-isomaltoside (pNP-IM) was also performed as described (16). The protein concentration was determined by measuring the absorbance at 280 nm, using the molar extinction coefficient (1 mg/ml = 2.487) calculated by the ExPASy ProtParam server. Kinetic parameters were calculated by nonlinear regression analysis using KaleidaGraph (Synergy Software, Reading, PA).

*Results and Discussion*—

*Overall Structure of AgIMD—* The recombinant AgIMD was expressed in *E. coli* and affinity-purified by Ni-NTA-agarose chromatography. The N-terminal His tag was then removed by thrombin cleavage, and the protein was crystallized. The crystals belonged to the tetragonal space group *I₄₁₂₂*, with one monomer in the asymmetric unit. The structure was determined using the single wavelength anomalous dispersion technique with a crystal soaked in lead acetate solution. A coarse model of AgIMD was initially built with a 2.5-Å resolution dataset, and the model was further refined using a 1.44-Å resolution dataset (Table 1). The 2Fo-Fc electron density contoured at 1 σ showed continuous density for almost all the main chain atoms, but the N-terminal segment, GSHMATAVTARGV, was not visible. The Ramachandran plot calculated with the Molprobity server shows that only one residue, Asp-312, was identified as an outlier. Asp-312 is one of the key amino acid residues in the active site, and the electron density for this residue was well defined.
The structure of AgIMD consists of three domains, designated domain A (residues 21–267), domain C (368–466), and domain D (467–606), as well as an extra loop comprising residues 11–20 in the N terminus (Fig. 1A). Domain A is composed of a (β/α)8-barrel, and domain C is made up of an antiparallel β-structure. This two-domain architecture is commonly found in GH27 enzymes (25). An extra structural component found in GH27 enzymes (25) includes a (β/α)8-barrel, and domain C is made up of an antiparallel β-structure. This two-domain architecture is commonly found in GH27 enzymes (25). An extra structural component found in GH27 enzymes (25) is composed of a (β/α)8-barrel, and domain C is made up of an antiparallel β-structure. This two-domain architecture is commonly found in GH27 enzymes (25).

A structural similarity search of domain A (including subdomain B), domain C, and domain D was performed using the DALI server (28). Domain A shows a significant similarity to the domains of GH27, GH36, GH31, and GH13 enzymes, all of which consist of (β/α)8-barrel folds (Table 2). Among the GH27 enzymes, similarities with β-1-arabinofuranosidases (25, 29) were relatively higher than those with α-N-acetylgalactosaminidases and α-galactosidases (30). Domain C is similar to the antiparallel β-sheet domains of various GH family enzymes.

The results of the similarity search of domain D were markedly different from those of domains A and C, and other GH27 members do not possess CBM35, as described below.

### Interactions with Isomaltose and Panose in the Active Site

The crystal structure of the AgIMD-isomaltose complex is almost isomorphous with that of the unliganded form, and clear electron density maps ($F_o - F_c$) were obtained for two isomaltose molecules (Fig. 1, B and C). One of the molecules is seen in the active site, and the other molecule is bound to domain D (Fig. 1A). The active site structure of AgIMD-isomaltose was compared with that of unliganded AgIMD. GH27 enzymes employ a retaining mechanism, and two Asp residues act as catalytic residues (31, 32). The structural homology with GH27 α-galactosidases indicates that Asp-197 and Asp-265 are identified as a nucleophile and acid/base catalyst, respectively. Based on the position of the catalytic residues, isomaltose is bound to subsites $-2$ and $-1$ in AgIMD-isomaltose, and the two glucose residues are labeled Glc $-2$ and Glc $-1$ (Fig. 2A). The binding of isomaltose is accompanied by conformational changes in Glu-81, Val-242, and Met-243. The side chain of Glu-81 in unliganded AgIMD points away from the active site, whereas atoms OE1 and OE2 of Glu-81 in AgIMD-isomaltose form hydrogen bonds with atoms O6 and O4 of Glc-2, respectively. Also, the main chain atom O of Val-242 is not oriented toward the active site in unliganded AgIMD, whereas in the AgIMD-isomaltose, significant conformational differences of Val-242 and Met-243 are observed, and atom O of Val-242 interacts with atoms O2 of Glc $-1$ via a water molecule.
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A

B

C

D

E

F

G

H

FIGURE 1. Overall structure of AgIMD. A, ribbon model of AgIMD-isomaltose. The N-terminal extra loop (magenta), domain A (yellow), subdomain B (cyan), domain C (blue), domain D (green), and two isomaltose molecules (red) are indicated. B–H, Fc, F2, F3, omit maps contoured at 3σ level for isomaltose in domain A of AgIMD-isomaltose (B), isomaltose in domain D of AgIMD-isomaltose (C), panose in domain A of AgIMD-panose (D), a molecule modeled as isomaltose in domain D of AgIMD-panose (E), Sme243 in unliganded AgIMD (F), Sme243 in AgIMD-isomaltose (G), and Sme243 in AgIMD-panose (H) are shown.

We also determined the crystal structure of AgIMD complexed with a trisaccharide, panose (Glc-α(1→6)-Glc-α(1→4)-Glc). AgIMD hydrolyzes panose, albeit inefficiently, to produce isomaltose and glucose (4). Electron density for two ligand molecules was seen in AgIMD-panose (Fig. 1, D and E), and as with AgIMD-isomaltose, one molecule was found in the active site, and the other molecule was located on domain D. In the active site, three glucose residues were modeled at subsites −2, −1, and +1. The glucose residues at subsites −2 and −1 found in AgIMD-panose and those found in AgIMD-isomaltose are well superimposed (Fig. 3A). The obtained structural models of domain D in the AgIMD-panose complex is nearly identical to that observed in the AgIMD-isomaltose complex, because a glucose residue at the reducing end was disordered, and the molecule bound to domain D was modeled as isomaltose (Fig. 1E).

Tunnel Structures—There are two tunnels, designated tunnel-1 and tunnel-2, near the methionine residue at position 243 in the active site of AgIMD, and several water molecules are found in the tunnels (Fig. 3B). The methionine residue is oxidized based on the interpretation of the electron density map (Fig. 1, F–H), and thus the residue is described as Sme243 in this paper. There is another possibility that residue 243 is present in two conformations simultaneously. However, the distance between an atom in the side chain of the 243th residue and atom N of Ser-200 is 3.0 Å, and also that between the same atom in the side chain of the 243th residue and atom N of Trp-201 is 3.0 Å. The observation suggests that hydrogen bonds are likely to be present between these atoms, and thus the 243th residue is identified as methionine sulfoxide. It is interesting to note that an elaborate conformational change is seen in tunnel-1. The conformational changes of side chains of Ser-200, Sme243, His-245, Phe-247, and the main chain atom O of Pro-244 are induced when the ligand binds to the active site (Fig. 2B). As a result, tunnel-1 is closed in the unliganded form and open in the ligand-bound form.

It is unclear whether Sme243 was oxidized spontaneously or by x-ray radiation. A GH27 α-galactosidase from Trichoderma reesei showed a 12-fold increase in activity when treated with H2O2 (33), and Met-258 is considered to be possibly oxidized based on the structure modeling (34). However, the residue corresponding to Met-258 in T. reesei α-galactosidase is identified as Phe-313 in AgIMD. In a GH26 β-mannanase from the termite Reticulitermes serra-tus, a methionine sulfoxide residue, Sme85, is located in the active site (35), but the role of Sme243 is obviously different from that of Sme85 in the GH26 β-mannanase, because Sme243 does not form any hydrogen bonds with the ligand molecule (isomaltose or panose) in the ligand-bound form, unlike in GH26 β-mannanase.

It is also difficult to explain the role of the tunnels of AgIMD. It is unlikely that the row of water molecules present in the tunnels directly interacts with the acid-base catalyst, Asp-265, as the distance between atom OD1 of Asp-265 and the row of the first water molecule is more than 10 Å. Similar tunnel structures have been reported to be observed in GH13 (36, 37), GH48 (38), and GH68 (39). These water paths have been proposed to function as a water drain and/or a water reservoir, and the tunnels of AgIMD might have similar roles.

Comparison of the Catalytic Domains of AgIMD and GH27 α-Galactosidase—The catalytic domain of GH27 human α-galactosidase (hGAL) is one of the most structurally homologous proteins to that of AgIMD, and its catalytic mechanism has been extensively studied (30, 32). The structures of the catalytic domains of AgIMD and hGAL were superimposed (Fig. 4A). Although the fold of AgIMD is basically identical to that of hGAL, three components comprising residues 123–178, 204–216, and 276–289 were not found in hGAL, and thus these components are designated here as subdomain B, loop-1, and loop-2, respectively. Subdomain B of AgIMD is located between the third β-strand (β3) and the third α-helix (α3) of the (β/α)n-barrel. A short loop comprising residues 136–151 is present instead in the corresponding position of hGAL, and this
Structural similarity search using the DALI server

Summary of structural similarity search using the DALI server

| Enzyme | PDB code | CAZY* | Z-score | r.m.s.d. | Aligned residues | Sequence identity |
|--------|----------|-------|----------|----------|------------------|------------------|
|        |          |       |          | Å        |                  |                  |
| Domain A |          |       |          |          |                  |                  |
| Geobacillus stearothermophilus T6 β-1-arabinopyranosidase | 4NX0 | GH27 | 30.5 | 2.7 | 302 | 21 |
| Thermoanaerobacter tengcongensis α-galactosidase | 17Z9 | GH36 | 26.0 | 3.0 | 281 | 17 |
| Bacillus halodurans putative α-N-acetylgalactosaminidase | 3C61 | GH27 | 25.8 | 2.7 | 279 | 19 |
| Human α-galactosidase (βGal) | 3SSY | GH27 | 25.4 | 2.6 | 262 | 19 |
| L. acidophilus α-galactosidase (LaMel36A) | 2XN0 | GH36 | 20.7 | 3.0 | 268 | 19 |
| E. coli α-xylodase YicI (EcYicI) | 1XSJ | GH31 | 18.8 | 3.1 | 268 | 10 |
| T. vulgaris α-amylose I (TVAI) | 1JI1 | GH13 | 17.6 | 3.9 | 280 | 9 |
| Streptococcus mutans dextranase | 3VMN | GH66 | 9.4 | 4.3 | 262 | 9 |
| Domain C |          |       |          |          |                  |                  |
| Streptomyces avermitilis β-1-arabinopyranosidase | 3A21 | GH27 | 10.5 | 1.6 | 79 | 29 |
| Human α-galactosidase (hGal) | 4NXS | GH27 | 9.2 | 1.9 | 82 | 24 |
| Thermus sp. maltogenic amylase | 1GV1 | GH13 | 8.7 | 2.4 | 79 | 15 |
| Geobacillus stearothermophilus β-xylodase | 1W91 | GH39 | 7.0 | 2.7 | 77 | 12 |
| Paenibacillus polymyxa xylolucanase | 2YKK | GH44 | 6.1 | 2.9 | 79 | 14 |
| G. stearothermophilus α-galactosidase | 4FNQ | GH36 | 4.9 | 2.8 | 70 | 14 |
| Domain D |          |       |          |          |                  |                  |
| L. monocytogenes putative 3-α-isomaltosyltransferase | 2W1W | CE12 | 19.5 | 1.7 | 127 | 24 |
| Ruminoclostridium thermosaccharolyticum β-glucosaminidase | 2VZ8 | GH39 | 18.4 | 1.8 | 126 | 22 |
| Amycolatopsis orientalis exo-β-glucosaminidase | 3VMN | GH66 | 9.4 | 4.3 | 262 | 9 |
| R. thermocellum dockerin type I/putative β-xylodase | 3ZM8 | GH26 | 17.8 | 1.7 | 120 | 25 |

* Domain C and domain D are not catalytic domains; therefore, the CAZY classification of the catalytic domain of each enzyme is shown.

To compare the residues involved in each subsite of AgIMD and those of hGAL, residues interacting with panose (Fig. 3C) and the corresponding residues in hGAL are listed (Table 3). At subsite −1, four residues are conserved between AgIMD and hGAL (Asp-77/92, Tyr-120/134, Arg-261/277, and Asp-312/266), despite the fact that glucose binds at subsite −1 of AgIMD, whereas galactose binds at subsite −1 of hGAL. Residues at subsite −2 of AgIMD are completely different from those of hGAL. At subsite −2 of AgIMD, atoms OE1 and OE2 of Glu-81 directly form hydrogen bonds with O6 and O4 of Glc −2, respectively, and also atom N of Trp-79 directly forms hydrogen bonds with O6 and O5 of Glc −2 (Fig. 3C). The tryptophan residue, Trp-79, appears to be the key residue of subsite −2 and is involved in substrate stacking interactions (Fig. 5A). In contrast, α-galactosidase has been reported to be an enzyme that catalyzes the hydrolysis of galactosyl residues from the non-reducing end of a variety of oligosaccharides and polysaccharides, and thus subsite −2 is unnecessary for hGAL. In fact, the position equivalent to the Glc −2 binding cleft of AgIMD is occupied by residues Cys-142 and Ala-143, which are part of loop-A in hGAL, and therefore no binding cleft for subsite −2 is found in hGAL (Fig. 5B).

Residues involved in subsite +1 of AgIMD are also different from those of hGAL. The report of the hGAL-melibiose structure indicated that few interactions with the glucose portion of melibiose have been found (32). However, five residues appear to be involved in the binding of Glc +1 in AgIMD. Atom O2 and atom O3 of Glc +1 directly form hydrogen bonds with atom OD2 of Asp-207 and atom NE1 of Trp-285, respectively, suggesting that Glc +1 binds tightly to AgIMD (Fig. 3C). The substrate, isomaltoligosaccharide, is expected to form a helix-like structure like panose, and Phe-198 is located at the center of the helical spiral of the substrate (Fig. 5A). Side chains of Asp-207, Asn-209, and Trp-285, which are involved in the binding of Glc +1 of panose, are likely to form hydrogen bonds with Glc +1 of isomaltoligosaccharide either directly or

![FIGURE 2. Stereo views of the active site of AgIMD-isomaltose. A, active sites of unliganded AgIMD (green) and AgIMD-isomaltose (red and magenta). In AgIMD-isomaltose, hydrogen bonds linking atom O2 of Glc -1 to oxygen atom of Val-242 via a water molecule (red ball) are indicated as red dashed lines. B, comparison of the residues comprising tunnel-1 of unliganded AgIMD (green) and AgIMD-isomaltose (red and magenta). Red dashed line, hydrogen bond; red ball, water molecule. Arrows indicate conformational changes from the unliganded form to the ligand-bound form. The subsite numbers of isomaltose are labeled.](image-url)
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FIGURE 3. Active site of AgIMD-panose. A, stereo view of the active sites of unliganded AgIMD (green), AgIMD-isomaltose (magenta), and AgIMD-panose (blue). B, two tunnels (tunnel-1 and tunnel-2) are found near the active site of AgIMD-panose. The routes of the tunnels calculated with the program Caver are shown in blue. Red balls inside the tunnels represent water molecules. The panose molecule (Glc-1, Glc-2, and Glc-3) as well as residues Sme243, His-254, and Phe-247 are indicated. C, schematic drawing of the amino acid residues interacting with panose in domain A of AgIMD-panose. Red circle, carbon atom; black circle, carbon atom; gray circle, nitrogen atom; red circle with S, sulfur atom; dashed line, hydrogen bond. The subsite numbers are labeled. Phe-156 and Phe-198 are involved in hydrophobic interactions with panose.

through water molecules. These residues are located at loops uniquely found in AgIMD, loop-1 (Asp-207 and Asn-209), and loop-2 (Trp-285).

Site-directed Mutagenesis of Residues Unique to AgIMD—To assess the role of Asp-207, Asn-209, and Sme243, which are unique to AgIMD, alanine mutants D207A, D207A/N209A, and M294A were constructed. The kinetic parameters of wild-type and mutant AgIMD were determined (Table 4). For the small synthetic saccharide, pNP-IM, the $k_{cat}$ values decreased significantly (0.02- to 0.06-fold). The ratio of $k_{cat}/K_m$ values of wild type:D207A:D207A/N209A:M294A for pNP-IM decreased but not significantly (0.37- and 0.39-fold, respectively), whereas the M294A mutation drastically affected the $k_{cat}$ value for pNP-IM (8.2 $\times$ 10$^{-3}$-fold). For dextran, the $K_m$ values of all the mutants increased only slightly (less than 2-fold). The $k_{cat}$ values decreased significantly (0.02- to 0.06-fold). The ratio of $k_{cat}/K_m$ values of wild type:D207A:D207A/N209A:M294A for pNP-IM was 100:25:4:0.4, whereas that for dextran was 100:3.6:5.6:2.1. The results suggest that Asp-207 is important for the catalysis of both oligosaccharides and dextran.

Comparison of the Catalytic Domains of AgIMD and the GH13, GH31, and GH36 Enzymes—A search with the DALI server indicated that highly homologous proteins belonging to GH13, GH31, and GH36 are identified as *Thermoactinomyces vulgaris* α-amylase I (PDB code 2D0F; hereafter TVAI) (40), *E. coli* YicI (PDB code 1XS; EcYicI) (11), and *Lactobacillus acidophilus* α-galactosidase (melibiase; PDB code 1ZY9; LaMel36A) (9), respectively (Table 2). The (β/α)$_s$-barrel domains of these proteins were superimposed, indicating that the backbone folds are similar among these enzymes (Fig. 4B). It is noteworthy that subdomain B of AgIMD shares structural homology with those of TVAI and EcYicI (Fig. 4, C–E), despite the low similarities in their primary structures (~10%). In GH13 and GH31 enzymes, subdomain B is found between β3 and α3 of the barrel structure, which is the same position in AgIMD. The superimposition of TVAI-glucopentasaccharide, EcYicI-fluoroxypyrano- 
osyl intermediate, and LaMel36A-galactose shows that the position and orientation of the pyranose ring at subsite −1 of AgIMD is almost identical to those of EcYicI and LaMel36A but is different from that of TVAI, and the structural similarity of the active site between AgIMD and TVAI is low (Fig. 4B).

The active sites of AgIMD, hGAL, EcYicI, and LaMel36A were compared (Fig. 5). In hGAL and LaMel36A, Trp-47(hGAL)–Trp-340(LaMel36A) is located near atom O4 of galactose at subsite −1, and Tyr-40(AgIMD) and Phe-515 (EcYicI) are located near atom O4 of Glc −1 in AgIMD and EcYicI, respectively. The finding suggests that this difference is important for the specificity for the C4-epimeric configuration of pyranose. At subsite −1, the residue equivalent to Phe-198 of AgIMD is found in EcYicI (Phe-417). Also, EcYicI possesses an extra domain in the N terminus, and residues in this N-terminal domain (Asp-185 and Tyr-194) appear to participate in subsite −1, and thus, unlike in α-galactosidase, the N-terminal domain of EcYicI may perform a function similar to Asp-207, Asn-209, and Trp-285 of AgIMD. These observations led us to the conclusion that some key residues found in AgIMD, Phe-198 and Asp-207, are found in only GH31 enzymes and are not conserved in GH27 and GH36 enzymes.
CBM35(AgIMD)-isomaltose was compared with the CBM35 domain of Lmo2446 complexed with glucose (CBM35 (Lmo2446)-glucose; PDB code 4WKU) and also compared with that of BcCIT complexed with isomalto-octaose (CBM35 (BcCIT)-IG8; PDB code 3WNN) (Fig. 6A). The result revealed that CBM35(AgIMD) is highly homologous to CBM35 (Lmo2446) and CBM35(BcCIT), and the positions of their sugar-binding sites are conserved (Fig. 6A). Despite the similarities of the CBM35 structures, CBM35(AgIMD) has some unique features. The most conspicuous feature is that a long loop comprising residues 502–509 is found in CBM35(AgIMD). Atoms OE1 and ND2 of Gln-502 directly form hydrogen bonds with atom O6 of Glc-(a), atom O3 of Glc-(b), and atom O4 of Glc-(b), indicating that Gln-502 is the most critical residue for recognition of the α-1,6-glucosidic linkage (Fig. 6B). In contrast, no equivalent loop and residue were found in CBM35 (Lmo2446) and CBM35(BcCIT).

In CBM35(BcCIT) the second sugar-binding site has been reported, and Tyr-499, Phe-501, Asp-512, and Trp-514 appear to participate in the binding. The corresponding residues in CBM35(AgIMD) are Ser-559, Pro-561, Ala-572, and Gly-574, suggesting that the second sugar-binding site is not conserved in CBM35(AgIMD). Also, most CBM35 structures have one or two calcium-binding sites, whereas no calcium-binding site was found in CBM35(AgIMD). In CBM35(Lmo2446) and CBM35(BcCIT), one conserved calcium-binding site was observed, and two Glu residues (Glu-970 and Glu-972 in Lmo2446(CBM35)); Glu-951 and Glu-968 in CBM35(BcCIT)) form hydrogen bonds with atom O6 of Glc-(a), atom O3 of Glc-(b), and atom O4 of Glc-(b), indicating that Glu-951 and Glu-968 is the most critical residue for recognition of the α-1,6-glucosidic linkage (Fig. 6B). In contrast, no equivalent loop and residue were found in CBM35 (Lmo2446) and CBM35(BcCIT).

What is the role of the CBM35 domain D in AgIMD? CBM35(AgIMD), CBM35(Lmo2446), and CBM35(BcCIT) were superimposed and the CBM35 domains, and the catalytic domains are only illustrated in Fig. 6D to clarify the relations between these two domains. Despite the remarkable similarity of the CBM35 domains, the positions of the catalytic domains...
of AgIMD, Lmo2446, and BcCIT are completely different, suggesting that the CBM35 domains have divergent functions as mentioned previously (14). A surface model of AgIMD shows that there is a cleft, seeming suitable for binding a polysaccharide chain, near the sugar-binding site of CBM35(AgIMD) (Fig. 6E). However, this cleft is disconnected to the catalytic cleft, and thus it is unlikely that a linear polysaccharide chain directly moves from CBM35(AgIMD) to the catalytic cleft. Dextran has been reported to contain \( \beta-1,3 \) and occasionally \( \beta-1,2 \) and \( \beta-1,4 \)-branched linkages (1, 44), and also this polysaccharide is known to function as a component of bacterial biofilm matrix (44). Therefore, the polysaccharide chains could adopt complicated structures and tend to be tangled with each other. Although the overall structure is different, a GH16 \( \beta \)-agarase has an extra substrate-binding site, and an unwinding mechanism for agarose chains has been proposed (45). It is likely that CBM35(AgIMD) could help to unwind the tangled polysaccharide chains, similar as proposed in the \( \beta \)-agarase.

A BLAST homology search was carried out to elucidate the physiological function of AgIMD, resulting in some bacteria possessing genes homologous to the AgIMD gene. The most homologous genes were the Caci_6974 gene from *Catenulispora acidiphila* DSM44928 (48% similarity) (46) and the BN506_02253 gene from *Bacteroides cellulolyticus* CAG:158 (45% similarity), although no CBM35 domain was found in Caci_6974 and BN506_02253. A gene encoding GH66 putative endodextranase (Caci_6973 from *C. acidiphila*) or GH97 putative \( \alpha \)-glucosidase (BN506_02255 from *B. cellulolyticus*) was found near the putative isomalto-dextranase genes, whereas no gene encoding GH70 putative dextranucrase was identified in these bacteria. Some bacteria preferentially utilize uncommon sugars such as cyclodextrins, which has been proposed to be beneficial for surviving in a competitive environment (47). It is likely that Caci_6974 and BN506_02253 are involved in efficient utilization of dextran as a special energy source. Although some dextranases have been reported to influence the formation of bacterial biofilm (48), Caci_6974 and BN506_02253 may not participate in the biofilm formation because the organisms do not possess GH70 enzymes. AgIMD is also likely to play a role similar to that of Caci_6974 and BN506_02253, and perhaps CBM35(AgIMD) is an apparatus for accelerating the hydrolysis of the complicated polysaccharide structure.

CBMs are often found in biomass-degrading enzymes, and engineering of CBMs is expected to be beneficial for the production of biofuels (41, 49). Despite the high homology with other CBM35 domains, CBM35(AgIMD) has unique features, such as no calcium-binding site and the presence of a long loop. Thus, our results provide new information for biotechnological engineering of CBMs.

### Conclusions

The crystal structure of AgIMD was determined. Although the structure most closely resembles GH27 enzymes, domain A of AgIMD has subdomain B, loop-1, and loop-2, all of which are not found in GH27 hGAL. The fold of subdomain B is basically identical to those of GH31 EcYicI and GH13 TVAI. Four residues at subsite \(-1 \) in AgIMD are con-
served in hGAL, whereas the residues involved in subsites /H11002 and /H11001 in AgIMD are completely different from those in hGAL. Site-directed mutagenesis showed that Asp-207 at subsite /H11001 in AgIMD is important for the catalysis of the polysaccharide dextran, and the corresponding aspartic acid residue, Asp-185, is present in GH31 EcYicI. The structural feature of domain D, CBM35(AgIMD), is highly homologous to those of CBM35(Lmo2446) and CBM35(BcCIT), which are domains present in the GH31 enzyme Lmo2446 and the GH66 enzyme BcCIT, respectively. These observations lead us to the conclusion that AgIMD has some features found in GH31, GH13, and GH66 enzymes, despite the fact the overall structure most closely resembles GH27 enzymes. GH27, -31, -36, and -66 enzymes have been proposed to share a common origin with those of the GH13 family, and the architecture of AgIMD appears to provide such evidence.

Author Contributions—T. T., T. M., and A. N. designed and coordinated the study. T. T. wrote the paper. G. Y. prepared the crystal of unliganded AgIMD. Y. O. prepared the crystals of AgIMD-isomaltose and AgIMD-panose and constructed the plasmids encoding D207A, D207A/N209A, and M243A. Y. O. and Y. I. measured the enzymatic activities of wild-type and mutant AgIMD. T. T. and T. M. analyzed the diffraction data and determined the crystal structures. All authors analyzed the results and approved the final version of the manuscript.

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