Xanthan lyase, a member of polysaccharide lyase family 8, is a key enzyme for complete depolymerization of a bacterial heteropolysaccharide, xanthan, in Bacillus sp. GL1. The enzyme acts exolytically on the side chains of the polysaccharide. The x-ray crystallographic structure of xanthan lyase was determined by the multiple isomorphous replacement method. The crystal structures of xanthan lyase and its complex with the product (pyruvylated mannose) were refined at 2.3 and 2.4 Å resolution with final R-factors of 17.5 and 16.9%, respectively. The refined structure of the product-free enzyme comprises 752 amino acid residues, 248 water molecules, and one calcium ion. The enzyme consists of N-terminal α-helical and C-terminal β-sheet domains, which constitute incomplete α5/α5-barrel and anti-parallel β-sheet structures, respectively. A deep cleft is located in the N-terminal α-helical domain facing the interface between the two domains. Although the overall structure of the enzyme is basically the same as that of the family 8 lyases for hyaluronate and chondroitin AC, significant differences were observed in the loop structure over the cleft. The crystal structure of the xanthan lyase complexed with pyruvylated mannose indicates that the sugar-binding site is located in the deep cleft, where aromatic and positively charged amino acid residues are involved in the binding. The Arg612 residue in the loop from the C-terminal domain and the Arg512 residue in the loop from the C-terminal domain directly bind to the pyruvate moiety of the product through the formation of hydrogen bonds, thus determining the substrate specificity of the enzyme.

There is a large number of polysaccharide-degrading enzymes. Generally, they can be classified into two groups, hydrolases and lyases. The former catalyze the hydrolysis reaction responsible for breaking glycosidic bonds in polysaccharides. The properties of glycosyl hydrolases that act on poly- and oligosaccharides have been well documented, and the three-dimensional structures of many polysaccharide hydrolases, such as amylases, chitinases, and cellulases, have already been reviewed (1, 2).

As regards the second group, the lyases, it is known that they recognize uronic acid residues in polysaccharides, catalyze the β-elimination reaction, and produce unsaturated saccharides with C=C double bonds at the nonreducing terminal uronate residues (Fig. 1). These characteristics of lyases indicate that they share common structural features determining their uronate recognition sites and reaction modes (β-elimination reaction). Although structural analyses of lyases for pectate (3–8), alginate (9), hyaluronate (10, 11), and chondroitin (12, 13) have been made, there is little information regarding the structural rules common to polysaccharide lyases.

To determine the structural and functional relationships exhibited by polysaccharide lyases, we have recently been focusing on bacterial heteropolysaccharide lyases (lyases for alginate (14), gellan (15), and xanthan (16)) with either an endotyptic or exotyptic reaction mode and with either a backbone or side chain type of cleavage site. We have already determined the crystal structure of the endotype alginate lyase from Sphingomonas sp. A1 (9).

Xanthan is an exopolysaccharide produced by the plant pathogenic bacterium Xanthomonas campestris (17). This exopolysaccharide consists of a main cellulosic chain with trisaccharide side chains composed of one glucuronyl and two mannoyl residues attached at the C-3 position of alternate glucosyl residues (18) (Fig. 1A). The internal and terminal mannoyl residues of the side chains have an O-acetyl group at the C-6 position and a pyruvate ketal at the C-4 and C-6 positions, respectively, although the extents of acetylation and pyruvration vary with the growth conditions and bacterial strain (19). Because the polymer has the peculiar rheological properties of pseudoplasticity (reversible decrease in viscosity with increase in shear rate), high viscosity at low concentrations, and tolerance to a wide range of pH and temperatures, it is widely utilized as a gelling and stabilizing agent in the food, pharmaceutical, and oil industries (20).

Xanthan lyase produced by Bacillus sp. GL1 acts exolytically on the side chains of xanthan and liberates pyruvylated mannose (PyrMan) through the β-elimination reaction (Fig. 1A) (16). The enzyme is synthesized as a precursor form (99 kDa)

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The atomic coordinates and structure factors of xanthan lyase and its complex with PyrMan (codes 1J0M and 1J0N, respectively) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

† These authors contributed equally to this work.

¶ To whom correspondence should be addressed. Tel.: 81-774-38-3766; Fax: 81-774-38-3767; E-mail: murata@food2.food.kyoto-u.ac.jp.

§ B. Henrissat, P. Coutinho, and E. Deleury, afmb.cnrs-mrs.fr/CAZY/index.html.

** The abbreviations used are: PyrMan, pyruvylated mannoside; Glc, D-glucoside; GlcUA, D-glucuronic acid; Man, D-mannoside; GlcNAc, N-acetyl-D-glucosamine; GalNAc, N-acetyl-D-galactosamine; m.i.r., multiple isomorphous replacement; aa, amino acid; r.m.s., root-mean-square; WAT, water molecule; Ac, acetate; KRP, potassium phosphate buffer; Bicine, N,N-bis(2-hydroxyethyl)glycine; CNS, crystallography NMR software; PDB, protein data bank.
and is then converted into the mature form (~75 kDa) through posttranslational excision of the signal peptide (2 kDa) and C-terminal polypeptide (~22 kDa) (21). On the basis of amino acid (aa) sequence similarity, the enzyme is classified into polysaccharide lyase family 8,1 which contains lyases for hyaluronate and chondroitin AC in addition to xanthan lyase, although xanthan lyase does not act on hyaluronate and chondroitin (21).

Moreover, xanthan lyase is peculiar in that it acts on the side chains of a polysaccharide and releases the nonreducing terminal saccharides of the side chains, because almost all polysaccharide lyases (including those for pectate, alginate, hyaluronate, chondroitin, and heparin) endolytically cleave the glycosidic bonds in the main chains of polysaccharides. Therefore, it is thought that the structural analysis of xanthan lyase will contribute to clarification of the structural features that determine the uronate recognition site, the β-elimination reaction, the reaction mode (endo/exo type), and the cleavage site (main/side chain type).

In this study, the three-dimensional structures of xanthan lyase and its complex with the product were determined by x-ray crystallography at 2.3 and 2.4 Å resolution, respectively. We also identified the active cleft of the enzyme and aa residues responsible for both the recognition of the substrate and the catalytic reaction.

**EXPERIMENTAL PROCEDURES**

**Materials**—Pyruvylated xanthan (average molecular mass, 2 × 10^6; pyruvylated of the terminal mannopyranosyl residue in the side chain, ~50%) was obtained from Kohjin Co., Tokyo, Japan. Polyethylene glycol 4000 was purchased from Nacalai Tesque, Kyoto, Japan. DEAE-Toyopearl 650M and Super Q-Toyopearl 650C were from Tosoh Co., Tokyo, Japan. Bio-Gel P2 was from Bio-Rad. The restriction endonucleases and DNA-modifying enzymes were from Takara Shuzo Co., Kyoto, and Toyobo Co., Tokyo, respectively.

**Assays for Enzyme and Protein**—Xanthan lyase was assayed as described previously (16). Briefly, the enzyme was incubated in 1 ml of a reaction mixture containing 0.05% xanthan and 50 mM sodium acetate buffer, pH 5.5, and then the activity was determined by monitoring the increase in absorbance at 235 nm. One unit of the enzyme activity was defined as the amount of enzyme required to produce an increase of 1.0 in absorbance at 235 nm/min. Protein was determined by the method of Lowry et al. (22) with bovine serum albumin as a standard or by measuring the absorbance at 280 nm, assuming that E280 = 2.06 corresponds to 1 mg/ml, as calculated from the aa sequence using ProtParam (www.expasy.org/tools/protparam.html).

**Purification of Xanthan Lyase**—Unless otherwise specified, all operations were carried out at 0–4 °C. Cells of *Escherichia coli* strain BL21(DE3)pLysS harboring a plasmid (pET17b-XL4) (21) were grown in 6 liters of LB medium (1.5 liters/flask), collected by centrifugation at 6000 × g and 4 °C for 20 min, washed with 20 mM potassium phosphate buffer (KPB), pH 7.0, and then resuspended in the same buffer. The cells were disrupted ultrasonically (Insonator model 201M, Kubota, Tokyo, Japan) at 0 °C and 9 kHz for 20 min, and the clear solution obtained upon centrifugation at 15,000 × g and 4 °C for 20 min was used as the cell extract containing the precursor form (97 kDa) of the enzyme. The cell extract, after supplementation with 1 mM phenylmethylsulfonyl fluoride and 0.1 μM pepstatin A, was fractionated with ammonium sulfate. The precipitate (0–30% saturation) was collected by centrifugation at 15,000 × g and 4 °C for 20 min, dissolved in 20 mM KPB, pH 7.0, and then applied to a DEAE-Toyopearl 650 M column (2.6 × 15 cm) equilibrated with 20 mM KPB, pH 7.0. The enzyme was eluted with a linear gradient of NaCl (0–0.7 M) in 20 mM KPB, pH 7.0 (200 ml), with 2 ml fractions collected every 2 min. The active fractions, which were eluted with 0.4 M NaCl, were combined and dialyzed against 20 mM KPB, pH 7.0. The dialysate was used as the purified precursor form (97 kDa) of the enzyme. To convert the precursor (97 kDa) autocatalytically to the mature form (~75 kDa), the purified precursor was kept at 4 °C for 1 week. After confirmation of the conversion by SDS-PAGE (23), the enzyme solution was applied to a Super Q-Toyopearl 650C column (1.7 × 5 cm) equilibrated with 20 mM KPB, pH 7.0, and eluted with a linear gradient of NaCl (0 to 0.5 M) in 20 mM KPB, pH 7.0 (50 ml). 1-ml fractions were collected every minute. The active fractions, which were eluted with about 0.2 M NaCl, were combined and dialyzed against 20 mM Tris-HCl, pH 7.5, and the dialysate was used as the purified mature form (~75 kDa) of the enzyme.

**Preparation of PyrMan**—Xanthan (0.5%) dissolved in 50 mM sodium acetate (pH 5.5) (200 ml) was treated with the purified xanthan lyase (10 mg). The solution was mixed with ethanol (400 ml) and then centrifuged at 15,000 × g and 4 °C for 20 min. The supernatant was concentrated to 1.5 ml through evaporation and then applied to a Bio-Gel P2 column (0.9 by 122 cm) equilibrated with distilled water. The sugar eluted was determined to be PyrMan by confirming the release of mannose and pyruvate on hydrolysis with trifluoroacetic acid, as described previously (16). The fractions containing PyrMan were collected, freeze-dried, and dissolved in distilled water. The purity and content of PyrMan were determined by TLC analysis as described previously (16).

**Crystallization and X-ray Diffraction**—The mature form (~75 kDa) of xanthan lyase was crystallized by the hanging-drop vapor diffusion...
method. The solution for a crystallization drop was prepared on a siliconized coverslip by mixing 3 μl of protein solution (7.18 mg of protein/ml) with 3 μl of mother liquor comprising 23% polyethylene glycol 4000, 0.2 M ammonium formate, and 0.1 M sodium Bicine buffer, pH 9.0. The crystals were soaked in several heavy atom derivative solutions comprising 2 mM NaAuCl₄, 0.2 mM AgNO₃, 1 mM CuCl₂, 1 mM CdCl₂, 1 mM GdCl₃, and 1 mM HoCl₃ for 1 or 2 h at 20 °C. Crystals were also soaked in a sugar solution containing 75 mM mannitol, 23% polyethylene glycol 4000, 0.2 M ammonium formate, and 0.1 M sodium Bicine buffer, pH 7.4. Diffraction data for the native and derivative crystals were collected with a Bruker HiStar multilayer area detector at 20 °C. The data in the highest resolution shells are given in parentheses. The stereo quality of the model was assessed using the programs PROCHECK (27) and WHAT-CHECK (28). Ribbon plots were prepared using the programs MOLSCRIPT (29), BOBSCRIPT (30), RASTER3D (31), and GRASP (32). The coordinates of lyases for hyaluronate and chondroitin AC were taken from the RCSB Protein Data Bank (33). These molecular models were superimposed by means of fitting the programs RIGID and TOPO included in TURBO-FRODO and CCP4, respectively.

RESULTS AND DISCUSSION

Crystallography and Structure Determination—The mature form (about 75 kDa) of xanthan lyase of Bacillus sp. GL1 was purified from recombinant E. coli cells harboring plasmid pET17b-XL4 (21). A crystal of xanthan lyase (0.3 × 0.2 × 0.05 mm) was obtained by the hanging-drop vapor diffusion method. The space group was determined to be P2₁2₁2₁ (orthorhombic) with unit cell dimensions of a = 54.3 Å, b = 91.4 Å, and c = 160.7 Å; the solvent content was 50.2% assuming one molecule/ asymmetric unit. The results of the x-ray data collection are summarized in Table I. The structure of the enzyme was determined by the m.i.r. method. Table II shows the refinement statistics for the heavy atoms at 3.0 Å resolution. The protein model was built after solvent flattening of the m.i.r. phase with the PHASES program (24), and the model was refined by means of simulated annealing and the restrained least-squares method using CNS (26), as shown in Table I.

Quality of the Refined Model—The refined model of xanthan lyase comprises 752 aa residues, 248 water molecules, and one calcium ion. The N- and C-terminal aa residues of the mature form produced from the preproform through posttranslational processing were confirmed to be Ser³⁸ and Gly⁷⁷⁷, respectively, by electron density mapping. All of the polypeptide chain segments could be well traced, and the electron density of the main and side chains was generally very well defined in the 2Fo − Fc map. The final overall R-factor for the refined model was 0.175, with 30,582 unique reflections within the 50.0–2.3 Å resolution range. The final free R-factor calculated with the randomly selected 10% data was 0.240. The final root-mean-square (r.m.s.) deviations from the standard geometry were 0.0060 Å for bond lengths and 1.29° for bond angles. Based on the theoretical curves in the plot calculated according to Luz-
zati (34), the absolute positional error was estimated to be close to 0.25 Å of 5.0–2.3 Å resolution. Judging from the results of Ramachandran plot analysis, in which the stereochemical correctness of the backbone structure is indicated by the (φ, ψ) torsion angles (35), most of the non-glycine residues (86.1%) lay within the most favored regions, and the other residues (13.6%) fell in the additional and generously allowed regions, except for the Thr247 and Asp495 residues. The Thr247 and Asp495 residues are in β-turns. In particular, the latter turn, containing the Asp495 residue, is similar to a β-hairpin consisting of four amino acid residues with the conformation of β-ε-γ-β in a Ramachandran plot (36), because the Ala694, Asp695, Leu696, and Ile696 residues fall in or near the β, ε, γ, and β regions of the plot, respectively. Furthermore, there is one cis-peptide between the Ala776 and Pro774 residues.

**Overall Structure of Xanthan Lyase**—Figs. 2 and 3 depict a ribbon model of the overall structure and topology of the secondary structure elements of xanthan lyase, respectively. The enzyme has approximate dimensions of 100 × 70 × 50 Å and is composed of two globular domains (N- and C-terminal domains) that form α- and β-structures, respectively. The N-terminal domain comprises the 352 aa residues from Ser26 to Asp338 and is composed predominantly of 13 α-helices, 10 of which form an α/α-barrel structure. The C-terminal domain comprises the 389 aa residues from Leu389 to Gly777 and one calcium ion and consists of 30 β-strands arranged in five anti-parallel β-sheets. A peptide linker composed of the 11 aa residues from Asp377 to Asp383 connects the N- and C-terminal domains. In the structure of xanthan lyase, 25.7% of all aa residues are in α-helices, 26.2% in β-strands, and the remaining 48.1% in turns and coils.

**N-terminal α-Helical Domain**—The N-terminal domain is composed primarily of two short β-strands and 13 α-helices. The latter contribute to the formation of an α/α-barrel structure with a deep cleft, which is considered to be an active site (Figs. 2 and 4A). The 13 major α-helices, numbered sequentially from HA1 to HA13 (HA1, aa residues 28–41; HA2, 51–69; HA3, 90–106; HA4, 118–131; HA5, 153–165; HA6, 173–184; HA7, 192–208; HA8, 212–221; HA9, 255–270; HA10, 284–291; HA11, 318–333; HA12, 338–354; and HA13, 366–376), vary in length between 8 and 19 aa residues and consist of 187 turns; and one (HA2) with five turns; and one (HA10) with two turns.

There are 12 loops (from LA1 to LA12) connecting an α-helix to the following α-helix in the N-terminal domain (Fig. 3). The loop (LA8) between HA8 and HA9 includes two short β-strands (S1, aa residues 235–238, and S2, 243–245). Therefore, in the N-terminal domain, 52.3% of all aa residues are in α-helices, 20.3% in β-strands, and the remaining 45.7% in turns and coils.

The N-terminal α-helical domain includes an incomplete α/α-barrel formed by five inner and five outer α-helices, and the 10 α-helices (from HA3 to HA12) constitute the α2/α2-barrel located within the core of the domain (Fig. 4A). These 10 helices are connected by short and long loops in a nearest neighbor, up-and-down pattern. This arrangement is described as a “twisted α/α-barrel” with five inner α-helices (HA3, HA5, HA7, HA9, and HA11), which are oriented in roughly the same direction, and five outer α-helices (HA4, HA6, HA8, HA10, and HA12) running in the opposite direction.

**C-terminal β-Sheet Domain**—The C-terminal domain consists predominantly of 30 β-strands (SA1, aa residues 389–396; SA2, 398–402; SA3, 407–411; SA4, 437–441; SB1, 456–467; SC1, 482–487; SC2, 491–499; SC3, 506–513; SC4, 518–526; SB2, 533–541; SC5, 550–552; SC9, 554–557; SB5, 563–567; SC7, 571–575; SC6, 585–589; SB4, 593–604; SB3, 620–632; SC5, 638–646; SD1, 662–676; SD2, 671–676; SD3, 681–686; SE1, 692–694; SE2, 697–699; SD4, 703–710; SD5, 714–720; SE3, 729–734; SD6, 740–743; SE5, 747–751; SE4, 756–761; and SD7, 769–775) (Figs. 2, 3, and 4B). The β-strands vary in length between 3 and 13 aa residues and consist of 190 aa residues.

There are 29 loops (from LB1 to LB29) connecting a β-strand to the following β-strand in the C-terminal domain (Fig. 3). The loop (LB18) between SC5 and SD1 includes one α-helix (HB1, aa residues 650–658) with two turns. Therefore, in the C-terminal domain, 48.8% of all aa residues are in β-strands, 2.3% in α-helices, and the remaining 49.8% in turns and coils.

The C-terminal domain contains a calcium ion (Fig. 2). The site is located within a loop (LB8) and a β-strand (SD2) (Figs. 3 and 4B). The six oxygen atoms, OD1 of Asp516, OD2 of Asp516, OE1 and OE2 of Glu517, and O of Wat957, are coordinated to the calcium ion, and the coordination geometry comprises a distorted octahedron (Fig. 5). The distance between the calcium ion and the oxygen atoms ranges from 1.92 to 2.73 Å (average, 2.31 Å).

In the C-terminal domain, five anti-parallel β-sheets (sheets A–E) are formed by the 30 β-strands, all of which are anti-parallel in the β-sheets (Fig. 3). The β-sheets are composed of four to nine β-strands (sheet A, SA1–4; sheet B, SB1–5; sheet C, SC1–9; sheet D, SD1–7; and sheet E, SE1–5). Sheet A is parallel to the small β-sheet consisting of S1 and S2 in the N-terminal α-helical domain. As a result, the C-terminal

### Table II

| Derivative   | Conditions | Res. (A) | R_{cal} | R_{crst} | Phasing power | Heavy | Site | Occupancy | B |
|--------------|------------|----------|---------|----------|---------------|-------|------|-----------|---|
| CuCl₂        | 2, 2       | 4.0      | 0.753   | 0.086    | 0.52          | Cu-1  | 0.826| 0.173     | 0.180 | 0.550 | 49.2 |
| AgNO₃        | 0.2, 2     | 4.0      | 0.789   | 0.060    | 0.38          | Ag-1  | 0.248| 0.521     | 0.042 | 0.537 | 67.0 |
| UO₂Ac₂       | 1, 1       | 3.0      | 0.610   | 0.145    | 1.17          | U-1   | 0.963| 0.959     | 0.684 | 1.155 | 2.0  |
| SmCl₃        | 2, 1       | 3.0      | 0.677   | 0.095    | 1.43          | Sm-1  | 0.119| 0.060     | 0.581 | 0.800 | 71.8 |
| GdCl₃        | 1, 1       | 3.0      | 0.671   | 0.085    | 1.47          | Gd-1  | 0.539| 0.041     | 0.184 | 1.716 | 12.1 |
| HoCl₃        | 1, 1       | 3.0      | 0.674   | 0.083    | 1.53          | Ho-1  | 0.538| 0.041     | 0.184 | 1.323 | 2.0  |
| CdCl₂        | 1, 1       | 3.0      | 0.731   | 0.084    | 1.31          | Cd-1  | 0.536| 0.042     | 0.184 | 1.674 | 2.0  |
|              |            |          |         |          |               | Cd-2  | 0.963| 0.041     | 0.200 | 0.253 | 12.4 |

* The soaking solution comprised 23% polyethylene glycol 4000, 0.2 M ammonium formate, and 0.1 M Tris-HCl, pH 7.4.
**Structure of Xanthan Lyase**

![Diagram of Xanthan Lyase](image)

**Fig. 2.** Overall structure of xanthan lyase (ribbon stereodiagram). The colors denote elements with a secondary structure (blue, α-helices; red, β-strands; cyan, turns and coils). A calcium ion in the C-terminal domain is shown as a yellow ball. This figure was prepared using the programs MOLSCRIPT (29) and RASTER3D (31).

**Beta-sheet domain shows a five-layered β-sheet sandwich structure** (Fig. 4B).

**Structural Comparison of Polysaccharide Lyases**—On the basis of their sequence similarity, polysaccharide lyases are classified into 12 families. The three-dimensional structures of lyases belonging to families 1, 3, 5, 6, and 8 have been determined and divided into three groups (parallel β-helix, α/α-barrel, and α/α-barrel + anti-parallel β-sheets). The xanthan lyase belongs to polysaccharide lyase family 8, together with lyases for hyaluronate and chondroitin AC, although the sequence identity of xanthan lyase with the other lyases is less than 30% (Fig. 6).

The overall structure of xanthan lyase is similar to that of the family 8 lyases for hyaluronate (10, 11) and chondroitin AC (13), which consist of N-terminal α-helical and C-terminal β-sheet domains. The crystal structures of xanthan lyase and the other enzymes were superimposed by means of a fitting program, RIGID, included in TURBO-FRODO (Fig. 7). The aa sequences of the lyases for xanthan, hyaluronate, and chondroitin AC were aligned through analyses of the aa similarity and three-dimensional structures (Fig. 6). The r.m.s. deviations of Cα atoms between xanthan lyase and the other enzymes were determined by means of a fitting program, TOP, included in CCP4 (37) (Table III). The overall structure of xanthan lyase is more similar to that of hyaluronate lyase than of chondroitin AC lyase, and among the family 8 lyases, the geometries of the β-domains are more well conserved than those of the α-domains.

However, the following structural differences were observed among the lyases for xanthan, hyaluronate, and chondroitin AC. Hyaluronate lyase of *Streptococcus agalactiae* has a small N-terminal β-domain composed of seven β-strands preceding the α-helical domain (11). It should be noted that in the case of hyaluronate lyase of *Streptococcus pneumoniae*, the presence of an N-terminal β-domain has not been reported due to the crystal structure of the truncated enzyme (10). The C-terminal β-sheet domain of xanthan lyase is slightly larger than those of the lyases for hyaluronate and chondroitin AC; i.e., xanthan lyase has 30 β-strands in the C-terminal domain, whereas the C-terminal domains of the lyases for hyaluronate and chondroitin AC contain 24 and 28 β-strands, respectively (10, 13). The α-helical domain of xanthan lyase is similar to that of hyaluronate lyases but differs from that of chondroitin AC lyase, as the latter has 12 α-helices in its N-terminal domain (Fig. 6).

Although the C-terminal domain of chondroitin AC lyase contains a calcium ion (13), the localization and coordination of the calcium ion in xanthan lyase are different from those in the AC lyase in that the calcium ion of the AC lyase coordinates with seven oxygen atoms in two water molecules and in the side chains of four aa residues (Glu605, Asp607, Asp116, and Tyr117) in strand β5 corresponding to SB1 of xanthan lyase. Furthermore, structural differences between xanthan lyase and the other enzymes were found in the loops over the deep cleft formed in the N-terminal domain. In particular, loop LB16 of xanthan lyase connecting SB4 and SB3 in the C-terminal domain protrudes from the C-terminal domain and covers the cleft in the N-terminal domain (Fig. 2). The extreme protrusion of the loop is caused by the Arg612–Thr615 residues; there are several gaps in the corresponding sites of the lyases for hyaluronate and chondroitin AC (Fig. 6). The significance of the loop is described below.

The topology of the secondary structure elements of the N-terminal α-helical domain of xanthan lyase resembles that of...
alginate lyase, A1-III (polysaccharide lyase family 5), with an \(\alpha/\beta\)-barrel structure, which we have determined to be an example of an endotype polysaccharide lyase (9). The \(\alpha/\beta\)-barrel structure is found in sugar-related enzymes such as glucoamylases (38, 39) endoglucanase (40, 41), endo/exocellulase (42), N-acyl-D-glucosamine 2-epimerase (43), \(\beta\)-1,2-mannosidase (44), and maltose phosphorylase (45), as well as polysaccharide lyases. These enzymes form the \(\alpha/\beta\)-toroid family in the SCOP data base (scop.berkeley.edu/data/scop.b.b.bbc.html). Although these enzymes catalyze different reactions (hydrolysis, epimerization, and \(\beta\)-elimination), their \(\alpha/\beta\)-barrel structures possibly are responsible for the binding of polysaccharides in the hydrolytic and eliminative depolymerization reactions and for the production of common intermediates in the epimerization and \(\beta\)-elimination reactions. In fact, the occurrence of a common step in the catalytic reactions of two types of alginate-modifying enzymes, lyases and epimerases, has been reported (46).

Structure of Xanthan Lyase Complexed with PyrMan—Although almost all polysaccharide lyases analyzed thus far, including those for hyaluronate and chondroitin AC, attack the main chains of polysaccharides in an endolytic manner and release the oligosaccharides from the polysaccharides, xanthan lyase is characteristic in that it attacks the side chains of a polysaccharide exolytically. To identify the substrate-binding site, and to clarify the structural features causing the different substrate specificities of xanthan lyase and the other lyases, the crystal structure of the enzyme complexed with PyrMan (product) was determined at 2.4 Å resolution.

PyrMan was soaked in a native crystal of xanthan lyase. Diffraction data for the crystal of xanthan lyase complexed with the product up to 2.40 Å resolution were collected and refined to 2.4 Å resolution with CNS (26) using the refined
Structure of the native enzyme as a primary model. The results of the x-ray data collection and refinement are summarized in Table I.

The refined model of the enzyme-product complex consists of 752 aa residues (Ser 26–Gly 777), 261 water molecules, one calcium ion, and one PyrMan. A stereodiagram of a ribbon presentation of the complex is shown in Fig. 8A. The final overall R-factor for the refined model was calculated as 0.169 (free R-factor, 0.242) using the data from 50 to 2.4 Å resolution (26,294 reflections).

The difference Fourier map contoured at the 3σ level calculated with the 10–2.4 Å resolution data exhibited the highest densities in the product region (Fig. 8B). The average B-factor of PyrMan was 20.6 Å². The nomenclature for the sugar-binding site in the enzyme proposed by Davies et al. (47) was used here, and the sugars are numbered starting from the cleavage site, with positive numbers increasing toward the reducing terminus. Because xanthan lyase cleaves the glycosidic bond between PyrMan and GlcUA residues (Fig. 1), the position of PyrMan is designated as “−1.”

Xanthan lyase and its complex with PyrMan were superimposed by means of a fitting program included in TURBOFRODO. The r.m.s. deviation was 0.305 Å for the 752 common Cα atoms. There was no significant conformational change between the protein structures with and without PyrMan. PyrMan in the complex structure is bound in the deep cleft formed on the N-terminal domain of the enzyme facing the interface between the N- and C-terminal domains (Fig. 8A), indicating that the active center (substrate-binding site) is located in the cleft. Some aromatic and positively charged aa residues are arranged in the active cleft, suggesting that these aa residues are responsible for the binding and depolymerization of acidic polysaccharide xanthan (Fig. 9). This feature is common to family 8 polysaccharide lyases, which depolymerize acidic polysaccharides (10, 13).

Structure of the Active Cleft—Several aa residues and water molecules have been shown to be crucial for the binding of PyrMan (Fig. 10A). The data listed in Table IV represent the interaction of the enzyme with the bound PyrMan molecule in the complex. There are six direct hydrogen bonds between the protein and PyrMan atoms (Table IV). In particular, the carboxyl group of the pyruvate moiety in PyrMan is directly bound
to the Arg<sub>313</sub>, Tyr<sup>315</sup>, and Arg<sup>612</sup> residues through the formation of four hydrogen bonds. This conformation accounts for why the xanthan lyase of Bacillus sp. GL1 specifically liberates the nonreducing terminal saccharide, PyrMan, from the side chains of xanthan and is inactive on nonpyruvylated xanthan (16). The Arg<sub>313</sub> and Tyr<sup>315</sup> residues are located in the LA10 loop of the N-terminal domain, whereas the Arg<sub>612</sub> residue is in the LB16 loop of the C-terminal domain. As we described above, there are differences between xanthan lyase and family 8 lyases, because the Trp<sub>127</sub> and Trp<sub>292</sub> residues of chondroitin AC and hyaluronate lyases, respectively, correspond to the Arg<sub>313</sub>, Tyr<sup>315</sup>, and Arg<sup>612</sup> residues.

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**Fig. 7.** Superpositioning of the overall structures of xanthan lyase (blue, C<sub>a</sub> backbone) and hyaluronate lyase (PDB code, 1EGU: purple, C<sub>a</sub> backbone) (stereodiagram). This figure was prepared using the programs MOLSCRIPT (29) and RASTER3D (31).

**Table III**

| r.m.s. deviation/Å | C<sub>a</sub> atoms between xanthan lyase and other lyases |
|-------------------|----------------------------------------------------------|
| Whole<sup>a</sup> | 1.32/616, 1.45/330, 1.02/284 |
| α-Domain<sup>b</sup> | 1.43/502, 1.76/219, 1.19/305 |
| β-Domain<sup>c</sup> | |

<sup>a</sup> Whole, overall structures of the lyases were superimposed.

<sup>b</sup> Only the α-domains of the lyases were superimposed.

<sup>c</sup> Only the β-domains of the lyases were superimposed.

Hyaluronate lyase of S. pneumoniae (Lys<sup>171</sup>–Lys<sup>269</sup>) (PDB code, 1EGU).

Chondroitin AC lyase of F. heparinum (Gln<sup>23</sup>–Leu<sup>299</sup>) (PDB code, 1CB8).

Carbon-carbon (C-C) contacts were also observed between the protein (Trp<sup>148</sup>, Trp<sup>197</sup>, Tyr<sup>255</sup>, and Arg<sup>313</sup> residues) and PyrMan atoms (Table IV). The Trp<sup>148</sup> residue is parallel to the pyranose ring of PyrMan through the formation of hydrogen bonds. The side chain (OH) of the Tyr<sup>255</sup> residue forms a hydrogen bond with an oxygen atom (O-1) of PyrMan, which forms a glycosidic bond with the GlcNA residue before the enzyme reaction, suggesting that the Tyr<sup>255</sup> residue plays an important role in the catalytic reaction. In addition to direct hydrogen bonds, there are six hydrogen bonds between water molecules and PyrMan atoms (Table IV). Furthermore, there are six water-mediated hydrogen bonds between the protein and PyrMan atoms: O-1 = WAT<sub>110</sub> = Tyr<sup>255</sup>OH (2.9 Å), O-2 = WAT<sub>118</sub> = Ser<sup>256</sup> OG (3.0 Å), O-2 = WAT<sub>102</sub> = Glu<sup>312</sup> OE2 (2.8 Å), O-3 = WAT<sub>118</sub> = Glu<sup>312</sup> OE2 (2.8 Å), O-6 = WAT<sub>1073</sub> = Arg<sup>612</sup> NH2 (3.1 Å), and O-8 = WAT<sub>1073</sub> = Tyr<sup>315</sup> OH (3.2 Å).

**Fig. 8.** A, overall structure of xanthan lyase complexed with PyrMan (ribbon stereodiagram). The colors denote the secondary structure elements (blue, α-helices; red, β-strands; cyan, turns and coils). PyrMan is shown as a gray ball-and-stick model (red, oxygen atom). B, stereodiagram of the electron density map of PyrMan and the surrounding aa residues. The omit and 2Fo–Fc maps in PyrMan are shown as thin cyan and red lines, respectively. PyrMan is shown as a gray ball-and-stick model (red, oxygen atom). Aromatic and positively charged aa residues are shown in yellow and purple, respectively. This figure was prepared using the programs MOLSCRIPT (29), BOBSCRIPT (30), and RASTER3D (31).

**Fig. 9.** Molecular surface of the active cleft. Aromatic, positively, and negatively charged aa residues are colored yellow, cyan, and purple, respectively. PyrMan is shown as a stick model. A, the mannose moiety of PyrMan is on the front side, and the pyruvate moiety of PyrMan on the back side. The catalytic site is thought to be located in front of the tunnel. B, this view is from the opposite direction of A. This figure was prepared using the program GRASP (32).
Fig. 10. A, PyrMan bound in the active site of xanthan lyase (ribbon stereodiagram). The figure shows the bound sugar and the surrounding aa residues (positive, purple; aromatic, yellow) and water molecules (as, black ball) interacting with the sugar. The sugar is represented by a gray ball-and-stick model (red ball, oxygen atom). Direct hydrogen bonds (≤3.2 Å), shown as dotted lines, are formed between the sugar atoms (red) and the aa residues. B, superpositioning of the active-site structures of xanthan lyase and hyaluronate lyase (PDB code, 1C82; stereodiagram). The aa residues of xanthan lyase (purple) and hyaluronate lyase (green) are responsible for the direct interaction with sugars and the catalytic reaction. PyrMan at position −1 of xanthan lyase is shown as a gray ball-and-stick model (red ball, oxygen atom). GlcNAc at position −1 of hyaluronate lyase is shown as a black ball-and-stick model (red ball, oxygen atom; blue ball, nitrogen atom). C, superpositioning of the active-site structures of xanthan lyase and chondroitin AC lyase (PDB code, 1HM2; stereodiagram). The aa residues of xanthan lyase (purple) and chondroitin AC lyase (yellow) are responsible for the direct interaction with sugars and the catalytic reaction. PyrMan at position −1 of xanthan lyase is shown as a gray ball-and-stick model (red ball, oxygen atom). The GalNAc and GlcUA at positions −1 and +1, respectively, of chondroitin AC lyase are shown as a black ball-and-stick model (red ball, oxygen atom; blue ball, nitrogen atom; yellow ball, sulfur atom). This figure was prepared using the programs MOLSCRIPT (29) and RASTER3D (31).

Trp^{148} residue of xanthan lyase, show a stacked interaction with the sugar positioned at −1 (48, 49) (Fig. 10, B and C).

The arrangements of aa residues at the −1 position of the family 8 lyases were compared (Fig. 10, B and C). The active-site architecture has been reported to be well conserved in lyases for hyaluronate and chondroitin AC (48). The geometry of the Arg^{313} residue of xanthan lyase is similar to that of the Arg^{306} and Arg^{392} residues of lyases for hyaluronate and chondroitin AC, respectively, which are essential for the direct binding of sugars and which correspond to the Arg^{313} residue of xanthan lyase (Figs. 6 and 10, B and C). Recently, the Arg^{392} residue of chondroitin AC lyase responsible for the binding of GlcNAc positioned at −1 was found to be involved in the subsequent, processive, stepwise, and exolytic cleavage reaction of the enzyme (50). On the other hand, the following differences among the lyases for xanthan, hyaluronate, and chondroitin AC were observed in the arrangement of aa residues. In the case of xanthan lyase, the Arg^{313} and Tyr^{315} residues in the N-terminal domain and the Arg^{612} residue in the C-terminal domain directly bind to the carboxyl group of PyrMan. However, no direct interaction of lyases for hyaluronate and chondroitin AC with the sugar positioned at −1 involves residues in the C-terminal domain (11, 48, 49), although the Asn^{374}, Glu^{376}, Ser^{392}, and His^{393} residues of the C-terminal domain of chondroitin AC lyase associate with the sugar via water molecules (49). No aa residues corresponding to the Tyr^{315} and Arg^{612} residues of xanthan lyase are conserved in lyases for hyaluronate and chondroitin AC (Fig. 6). The Arg^{462} residue of hyaluronate lyase directly binds to O-4 of GlcNAc positioned at −1, whereas the Arg^{306} residue of xanthan lyase, corresponding to the Arg^{462} residue of hyaluronate lyase, undergoes no interactions with PyrMan. Therefore, these differences in the arrangement of aa residues at the −1 position determine the substrate specificities of the family 8 lyases, because the substrates of lyases for xanthan, hyaluronate, and chondroitin AC have PyrMan, GlcNAc, and GalNAc residues, respectively, at-
tached to the common GlcUA residues (Fig. 1).

The Tyr 255 residue interacts directly in the active cleft with the O-1 oxygen atom of PyrMan involved in the formation of the glycosidic bond between the -1 and +1 sugars. This finding indicates that the residue is responsible for the catalytic reaction. Several structural studies on catalytic residues in the clefts of lyases for hyaluronate and chondroitin AC have been reported (11, 48, 49). More recently, the Asn 349, His 399, and Tyr 408 residues of hyaluronate lyase from S. pneumoniae were shown to participate in the catalytic reaction through x-ray crystallographic analysis of a mutant enzyme (Y408F) complexed with hyaluronate tetra- and hexasaccharides (51). Asn 349 interacts with the carboxyl group of the GlcUA residue, His 399 functions as a base and withdraws a proton from the C-5 carbon of the GlcUA residue, and Tyr 408, acting as an acid, donates a proton to the glycosidic oxygen to be cleaved. These three residues are conserved in xanthan lyase of Bacillus sp., GL1, and the Asn 194, His 246, and Tyr 255 residues of the xanthan lyase correspond to the respective residues of hyaluronate lyase (Fig. 6). The His 225 and Tyr 234 residues of chondroitin AC lyase corresponding to the His 399 and Tyr 408 residues of hyaluronate lyase have also been reported as crucial for the catalytic reaction (49). The arrangement of these residues is highly conserved (Fig. 10, B and C) in the family 8 lyases; xanthan lyases (N194A, H246A, and Y255F) in which the Asn 194, His 246, and Tyr 255 residues were substituted with Ala, Ala, and Phe, respectively, exhibited little enzymatic activity. These results suggest that the Asn 194, His 246, and Tyr 255 residues play a crucial role in the β-elimination reaction of xanthan lyase, as seen for the lyases for hyaluronate and chondroitin AC. To clarify the reaction mechanism of the exotype xanthan lyase in more detail and to establish common structural rules for polysaccharide lyases, we have attempted to determine the structures of the wild type and mutant enzymes complexed with xanthan-branched pentasaccharide as a substrate (52).

Conclusions and Implications—To the best of our knowledge, this is the first report on the determination of the crystal structure of an exotype polysaccharide lyase that can act on the side chains of a polysaccharide. The enzyme consists of N-terminal α-helical and C-terminal β-sheet domains and has a deep cleft in the N-terminal domain facing the interface between the N- and C-terminal domains. Because the basic frames of lyases for xanthan, hyaluronate, and chondroitin AC are similar to each other, all of the polysaccharide lyases belonging to family 8 are considered to share a common structure consisting of N-terminal α-helical and C-terminal β-sheet domains. Furthermore, their active sites are all located in a deep cleft.

Based on the crystal structure of the enzyme complexed with PyrMan, the deep cleft in the enzyme was revealed to be responsible for the recognition of the substrate and the catalytic reaction. The enzyme specifically binds to the nonreducing terminal PyrMan of xanthan side chains in the cleft, as the aromatic and positively charged aa residues in the active cleft directly interact with the carboxyl group of PyrMan through the formation of hydrogen bonds. The arrangement of aa residues of xanthan lyase in the recognition site for PyrMan attached to the GlcUA residue differs from that in lyases for hyaluronate and chondroitin AC in the corresponding sites for GlcNAc and GalNAc, respectively, attached to the GlcUA residue. These differences in the aa arrangement are thought to determine the substrate specificity.

As seen for lyases for hyaluronate and chondroitin AC, the Asn 194, His 246, and Tyr 255 residues in the active cleft of xanthan lyase are thought to be involved in the catalytic reaction. As regards family 5 alginate lyase A1-III, similar to the case of the N-terminal α-helical domain of family 8 lyases, we have clarified that the activated Tyr 246 residue homologous to the Tyr 255 residue of xanthan lyase is bifunctional as a base and an acid (53). Therefore, the Tyr residue is considered important for the catalytic reactions of these polysaccharide lyases including an α/β-barrel structure.

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\[^{a}\] Distance ≤ 4.2 Å.

\[^{b}\] Distance ≤ 3.2 Å.
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