

Metabolic Fate of Unsaturated Glucuronic/Iduronic Acids from Glycosaminoglycans

MOLECULAR IDENTIFICATION AND STRUCTURE DETERMINATION OF STREPTOCOCCAL ISOMERASE AND DEHYDROGENASE*

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Background: Some streptococci target host extracellular matrix glycosaminoglycans for infection.

Results: The streptococcal metabolic pathway of glycosaminoglycan-derived unsaturated uronates was identified. The crystal structures of its isomerase and dehydrogenase were determined.

Conclusion: Streptococci include an assembled genetic cluster for depolymerization, import, degradation, and metabolism of glycosaminoglycans.

Significance: This study contributes to understanding of the streptococcal degradation/metabolism mechanism of glycosaminoglycans.

Glycosaminoglycans in mammalian extracellular matrices are degraded to their constituents, unsaturated uronic (glucuronic/iduronic) acids and amino sugars, through successive reactions of bacterial polysaccharide lyase and unsaturated glucuronyl hydrolase. Genes coding for glycosaminoglycan-acting lyase, unsaturated glucuronyl hydrolase, and the phosphotransferase system are assembled into a cluster in the genome of pathogenic bacteria, such as streptococci and clostridia. Here, we studied the streptococcal metabolic pathway of unsaturated uronic acids and the structure/function relationship of its relevant isomerase and dehydrogenase. Two proteins (gbs1892 and gbs1891) of *Streptococcus agalactiae* strain NEM316 were overexpressed in *Escherichia coli*, purified, and characterized. 4-Deoxy-L-threo-5-hexosulose-uronate (Dhu) nonenzymatically generated from unsaturated uronic acids was converted to 2-keto-3-deoxy-D-gluconate via 3-deoxy-D-glycero-2,5-hexodiulosonate through successive reactions of gbs1892 isomerase (DhuI) and gbs1891 NADH-dependent reductase/dehydrogenase (DhuD). DhuI and DhuD enzymatically corresponded to 4-deoxy-L-threo-5-hexosulose-uronate ketol-isomerase (KduI) and 2-keto-3-deoxy-D-gluconate dehydrogenase (KduD), respectively, involved in pectin metabolism, although no or low sequence identity was observed between DhuI and KduI or between DhuD and KduD, respectively. Genes for DhuI and DhuD were found to be included in the streptococcal genetic cluster, whereas KduI and KduD are encoded in clostridia. Tertiary and

quaternary structures of DhuI and DhuD were determined by x-ray crystallography. Distinct from KduI β -barrels, DhuI adopts an $\alpha/\beta/\alpha$ -barrel structure as a basic scaffold similar to that of ribose 5-phosphate isomerase. The structure of DhuD is unable to accommodate the substrate/cofactor, suggesting that conformational changes are essential to trigger enzyme catalysis. This is the first report on the bacterial metabolism of glycosaminoglycan-derived unsaturated uronic acids by isomerase and dehydrogenase.

Mammalian extracellular matrices are important for the connection of neighboring cells and protection of cells against physicochemical stresses, such as osmotic pressure or invasion by pathogenic microbes (1). Glycosaminoglycans, one of the major components of extracellular matrices, are highly negatively charged polysaccharides with a repeating disaccharide unit comprising a uronic acid residue (glucuronic (GlcUA)³ or iduronic acid (IdoUA)) and an amino sugar residue (glucosamine or galactosamine), frequently N-acetylated (2). Based on the sugar composition, mode of glycosidic bond, and sulfation level, glycosaminoglycans are classified as hyaluronan, chon-

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³ The abbreviations used are: GlcUA, D-glucuronic acid; IdoUA, L-iduronic acid; UGL, unsaturated glucuronyl hydrolase; Δ GlcUA, unsaturated GlcUA; Δ IdoUA, unsaturated IdoUA; KDG, 2-keto-3-deoxy-D-gluconate; Dhu, 4-deoxy-L-threo-5-hexosulose uronic acid; DhuI, 4-deoxy-L-threo-5-hexosulose-uronate ketol-isomerase; DhuD, 2-keto-3-deoxy-D-gluconate dehydrogenase; SagDhuI, *S. agalactiae* gbs1892; SpnDhuI, *S. pneumoniae* spr0289; SpyDhuI, *S. pyogenes* SPY0637; SagDhuD, *S. agalactiae* gbs1891; SpnDhuD, *S. pneumoniae* spr0290; SpyDhuD, *S. pyogenes* SPY0636; KduI, 4-deoxy-L-threo-5-hexosulose-uronate ketol-isomerase; EcoKduI, *E. coli* KduI; KduD, 2-keto-3-deoxy-D-gluconate dehydrogenase; PcaKduD, *P. carotovorum* KduD; SagUGL, *S. agalactiae* UGL; BacillusUGL, *Bacillus* sp. GL1 UGL; Ga5DH, *S. suis* gluconate 5-dehydrogenase; PDB, Protein Data Bank; PTS, phosphotransferase system; IdnO, 5-keto-D-gluconate dehydrogenase/reductase; KdgK, 2-keto-3-deoxygluconate kinase; KdgA, 2-keto-3-deoxy-6-phosphogluconate aldolase; SDR, short-chain dehydrogenase/reductase; RpiB, ribose 5-phosphate isomerase B; FabG, *E. coli* β -ketoacyl carrier protein reductase.

Streptococcal Glycosaminoglycan-metabolizing Enzymes

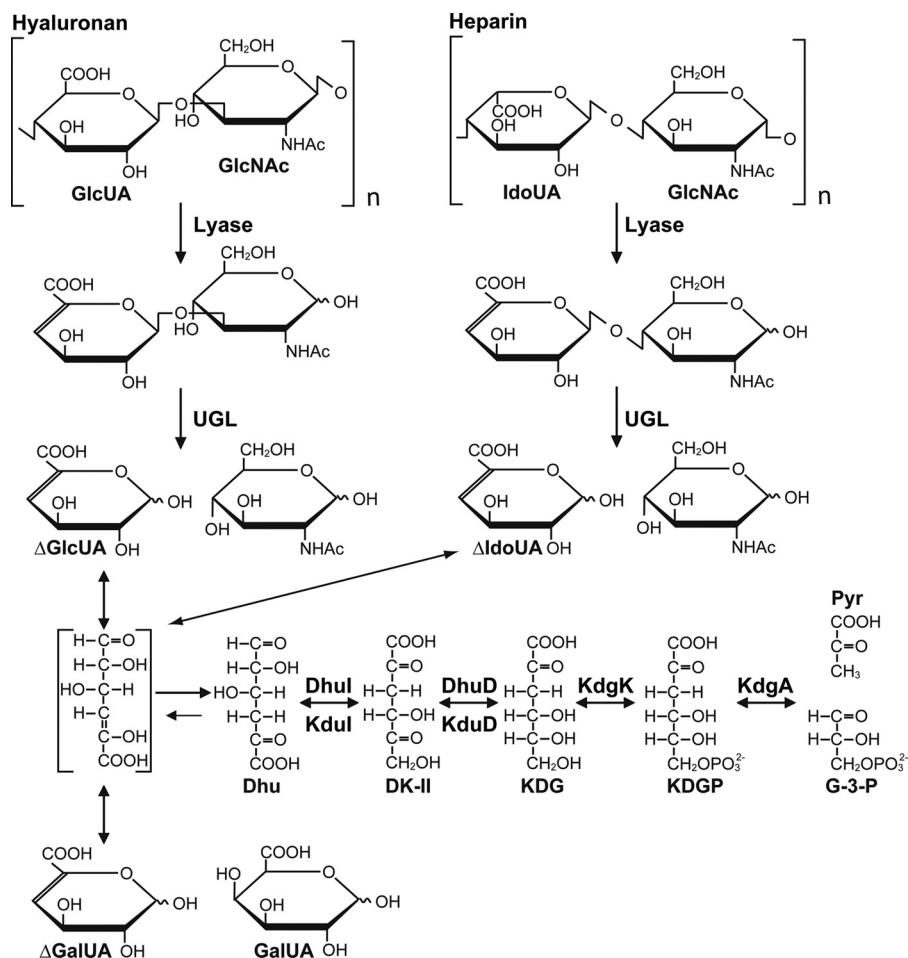


FIGURE 1. **Bacterial degradation and metabolism of glycosaminoglycans.** Glycosaminoglycans (e.g. hyaluronan and heparin) are depolymerized by lyase, and the resultant unsaturated disaccharides are subsequently degraded to unsaturated uronic acids and amino sugars by UGL. Unsaturated glucuronic/ iduronic acids are nonenzymatically converted into Dhu. Dhu is metabolized to pyruvate (Pyr) and glyceraldehyde-3-phosphate (G-3-P) through the subsequent reactions of four enzymes (i.e. isomerase (DhuI or Kdul), dehydrogenase (DhuD or KduD), kinase (KdgK), and aldolase (KdgA)). *GlcNAc*, *N*-acetylglucosamine; *DK-II*, 3-deoxy-D-glycero-2,5-hexodiulosonate; *KDGP*, 2-keto-3-deoxy-6-phosphogluconate. Dhu is also generated from pectin via unsaturated galacturonic acid (Δ GalUA).

droitin sulfate, dermatan sulfate, keratan sulfate, heparin, and heparan sulfate, which are widely present in human tissues, such as the arterial wall, cartilage, heart valve, intestinal mucosa, liver, lung, and skin (3, 4). Except for hyaluronan, most glycosaminoglycans are bound to core proteins as a proteoglycan (5).

Some pathogenic bacteria are known to recognize host extracellular matrices (e.g. glycosaminoglycans) as targets for adhesion and/or degradation (6). A large number of streptococci depolymerize hyaluronan to unsaturated disaccharides by hyaluronate lyase (7) (Fig. 1). We have identified unsaturated glucuronyl hydrolase (UGL), which is essential for degrading unsaturated disaccharides to constituent monosaccharides (i.e. unsaturated uronic acids and amino sugars) in bacteria, including streptococci (8, 9), and found that a genome segment termed the UGL genetic cluster is responsible for the depolymerization, import, and degradation of glycosaminoglycans in streptococci, such as *Streptococcus agalactiae*, *Streptococcus pneumoniae*, and *Streptococcus pyogenes* (10) (see Fig. 2A). The UGL genetic cluster of *S. agalactiae* is inducibly transcribed in the presence of hyaluronan (10). Recently, the disruption of the UGL genetic cluster in

S. pneumoniae has been demonstrated to reduce the bacterial attachment to host cells and the ability to cause infectious diseases (11). These reports indicate the significance of the UGL genetic cluster in bacterial infections.

Two streptococci, *S. pneumoniae* and *S. pyogenes*, have been shown to grow on hyaluronan as the sole carbon source (11–13), but little knowledge about the bacterial assimilation mechanism of glycosaminoglycans has been accumulated. More recently, streptococcal UGL has been demonstrated to act on various unsaturated disaccharides, such as hyaluronan, chondroitin sulfate, heparin, and heparan sulfate, and to release unsaturated uronic acids and amino sugars (9, 14). Due to the lack of the hydroxyl group at C4, unsaturated glucuronic (Δ GlcUA) and iduronic acids (Δ IdoUA) are chemically identical (Fig. 1). The utilization pathway of amino sugars has already been identified in bacteria, such as *Escherichia coli* (15) and *Streptococcus mutans* (16). In contrast, the metabolism of unsaturated uronic acids from glycosaminoglycans remains to be clarified, although these unsaturated uronic acids are known to be nonenzymatically converted to 4-deoxy-L-threo-5-hexosulose uronic acid (Dhu) (Fig. 1). Our previous study (10) suggested that these unsaturated uronic acids are converted to

2-keto-3-deoxy-D-gluconate (KDG) because the streptococcal UGL genetic cluster includes two homologous genes coding for KDG kinase and aldolase.

The elucidation of the metabolic pathway of unsaturated uronic acids will facilitate the development of novel drugs against streptococcus-induced infectious diseases. The present study deals with the molecular identification of the streptococcal isomerase and dehydrogenase involved in the metabolism of unsaturated uronic acids from glycosaminoglycans, and the structures of these enzymes are also determined by x-ray crystallography.

EXPERIMENTAL PROCEDURES

Materials—Unsaturated chondroitin disaccharide was purchased from Seikagaku Biobusiness. Oligonucleotides used in this study were synthesized by Hokkaido System Science or GeneDesign, and their nucleotide sequences are listed in Table 1. Restriction endonucleases and DNA-modifying enzymes were purchased from Toyobo. All other analytical grade chemicals were obtained from commercial sources.

Microorganisms and Culture Conditions—*S. agalactiae* strain NEM316 (CIP 82.45) was purchased from the Institut Pasteur. *S. pneumoniae* strain R6 (ATCC BAA-255) and *S. pyogenes* strain M1 GAS SF370 (ATCC 700294) were from the American Type Culture Collection. For isolating the genome DNAs, the three streptococci were statically grown at 30 °C under 5% (v/v) CO₂ in tryptic soy broth (Difco) containing 5% (v/v) defibrinated sheep blood (Nippon Bio-Test Laboratories). As the host for plasmid amplification, *E. coli* strain DH5 α (Toyobo) cells were routinely cultured at 37 °C in Luria-Bertani (LB) medium (1% (w/v) tryptone, 0.5% (w/v) yeast extract, and 1% (w/v) NaCl) containing sodium ampicillin (0.1 mg/ml). *E. coli* strain BL21 (DE3) pLysS (Novagen) was used as the host for the expression of streptococcal proteins. For expression in *E. coli*, cells were aerobically precultured at 30 °C in LB medium supplemented with sodium ampicillin (0.1 mg/ml). When the turbidity reached about 0.5 at 600 nm, isopropyl- β -D-thiogalactopyranoside was added to the culture at a final concentration of 0.1 mM, and the cells were further cultured at 16 °C for 44 h.

DNA Manipulations—Genomic DNA isolation, subcloning, transformation, and gel electrophoresis were performed as described previously (17). The nucleotide sequences of the streptococcal genes amplified by PCR or constructed through site-directed mutagenesis were determined by the dideoxy-chain termination method using the automated DNA sequencer model 3730xl (Applied Biosystems) (18).

Construction of the Overexpression System—Overexpression systems for streptococcal isomerases (DhuIs) (*S. agalactiae* gbs1892 (SagDhuI), *S. pneumoniae* spr0289 (SpnDhuI), *S. pyogenes* SPy0637 (SpyDhuI)) and dehydrogenases (DhuDs) (*S. agalactiae* gbs1891 (SagDhuD), *S. pneumoniae* spr0290 (SpnDhuD), *S. pyogenes* SPy0636 (SpyDhuD)) were constructed in *E. coli* as follows. To clone the streptococcal genes, PCR was conducted in a reaction mixture (10 μ l) consisting of 0.2 units of KOD Plus polymerase (Toyobo), 40 ng of genomic DNA, 0.3 pmol of forward and reverse primers, 2 nmol of dNTPs, 10 nmol of MgCl₂, 1 μ l of dimethyl sulfoxide, and the commercial reaction buffer supplied with KOD Plus polymerase. A restriction site (NdeI or XhoI) was added to each of the 5' regions of

the forward and reverse primers. The PCR conditions were as follows: 94 °C for 2 min followed by 30 cycles at 94 °C for 15 s, 50 °C for 30 s, and 68 °C for 1 min. The PCR products were ligated with HincII-digested pUC119 (Takara Bio), and the resultant plasmids were digested with NdeI and XhoI to isolate the streptococcal genes. Further, DNA fragments of the streptococcal genes were ligated with NdeI- and XhoI-digested pET21b (Novagen). The pET21b vector was designed to express proteins with a hexahistidine-tagged sequence at the C terminus. An overexpression system for *E. coli* 4-deoxy-L-threo-5-hexosulose-uronate ketol-isomerase (KduI) (EcoKduI) was also constructed as described above.

Purification—Unless otherwise specified, all procedures were performed between 0 and 4 °C. *E. coli* cells harboring each plasmid were grown in 3.0 liters of LB medium (1.5 liters/flask), collected by centrifugation at 6,000 \times g and 4 °C for 5 min, washed with 20 mM Tris-HCl (pH 7.5), and then resuspended in the same buffer. The cells were ultrasonically disrupted (Insonator Model 201M, Kubota) at 0 °C and 9 kHz for 20 min, and the clear solution obtained by centrifugation at 20,000 \times g and 4 °C for 20 min was used as the cell extract. Recombinant streptococcal proteins and EcoKduI were purified to homogeneity from *E. coli* cells harboring each plasmid using column chromatography with three different separation media: affinity (TALON (Clontech, 1.0 \times 10 cm)), anion exchange (Toyopearl SuperQ-650 M (Tosoh, 1.0 \times 9.5 cm)), and gel filtration (Sephacryl S-200HR (GE Healthcare, 2.6 \times 65 cm)). Protein purity was confirmed using SDS-PAGE (19). The fractions containing each protein were combined and dialyzed against 20 mM Tris-HCl (pH 7.5). The dialysate was then concentrated to about 10–30 mg/ml by ultrafiltration using a Centriprep (molecular weight cut-off, 10,000) (Millipore). The concentrate was used as the purified enzyme source. *Pectobacterium carotovorum* 2-keto-3-deoxy-D-gluconate dehydrogenase KduD (PcaKduD) was obtained from recombinant *E. coli* cells as described previously (53). The UGLs of *S. agalactiae* (SagUGL) and *Bacillus* sp. GL1 (BacillusUGL) were purified from recombinant *E. coli* cells as described previously (10).

Assays for Enzymes and Proteins—Because both Dhu and 3-deoxy-D-glycero-2,5-hexodiulosonate are commercially unavailable, Dhu was nonenzymatically obtained from Δ GlcUA prepared from 10 mM unsaturated chondroitin disaccharide sulfated at the C6 position of N-acetylgalactosamine residue through the reaction of SagUGL or from 10 mM unsaturated gellan tetracarbohydrate (unsaturated glucuronyl-glucosyl-rhamnosyl-glucose) (20) after treatment with BacillusUGL. The standard dehydrogenase activity was assayed at 30 °C in a reaction mixture (0.5 ml) consisting of 0.2 mM Dhu, 0.2 mM cofactor (NADH or NADPH), 50 mM Tris-HCl (pH 7.5), and an appropriate amount of enzymes. The activity was measured by continuously monitoring the decrease in the absorbance at 340 nm, which corresponds to the oxidation of NADH or NADPH. One unit of enzymatic activity was defined as the amount of enzyme required to oxidize 1 μ mol of cofactor per min at 30 °C. The kinetic parameters (K_m and k_{cat}) were determined using the Michaelis-Menten equation with the KaleidaGraph program (Synergy Software). In the case of the purified enzyme, the protein concentration was estimated by measuring the absorbance at 280 nm. The absorbance coefficient for 1 mg/ml of each

Streptococcal Glycosaminoglycan-metabolizing Enzymes

TABLE 1

Oligonucleotides used in this study

Single and double underlines show restriction and mutation sites, respectively.

Oligonucleotide	Sequence
gbs1892NdeI	5'-GGC <u>CATATG</u> AAAAATGCATTAATCAACGAAAAATAGC-3'
gbs1892XhoI	5'-GGCTCGAGGTCTAAGACAGATTTCAAGTAGTCTGC-3'
spr0289NdeI	5'-GGC <u>CATATG</u> AAAAATCGCATTAATCAATGAAAAATAGT-3'
spr0289XhoI	5'-GGCTCGAGCTTGGCTAATACTTCTTTCAAATAAGC-3'
SPy0637NdeI	5'-GGC <u>CATATG</u> AAAAATGCATTAATTAATGAAAAATAGC-3'
SPy0637XhoI	5'-GGCTCGAGCTGTTCAAGTACTGATTTCAAATAAGT-3'
gbs1891NdeI	5'-GGC <u>CATATG</u> ACTGAAACAATTCCTTAAAAGACAACCTTT-3'
gbs1891XhoI	5'-GGCTCGAGTTGAGGTGTTTACCAGATGTAAGCTAA-3'
spr0290NdeI	5'-GGC <u>CATATG</u> CAAAATACATCATTTCTCAATTGAGCAG-3'
spr0290XhoI	5'-GGCTCGAGCTCAGGTGTTTCCGATGTAGGCTAA-3'
SPy0636NdeI	5'-GGC <u>CATATG</u> CAAAATATGTTTTCGTTACAAGGTAAG-3'
SPy0636XhoI	5'-GGCTCGAGAGGTGTTTTCCAATATAAGCTAAAAT-3'
EcoKduINdeI	5'-GGC <u>CATATG</u> CAGCTAAGACAGAGCATCCACAGTCGG-3'
EcoKduIXhoI	5'-CCCTCGAGGCGCAAACTTTTAAACGGCCACATGGTC-3'
gbs1892 C72A sense	5'-GCAGATTTTGTATTACAGGCGCTGGGACAGGAATTGGAGCGATG-3'
gbs1892 C72A antisense	5'-CATCGCTCCAATTCCTGTCCCAGCGCCTGTAATAACAAAATCTGC-3'
gbs1892 T74A sense	5'-GTTATTACAGGCTGTGGGCGAGGAAATTGGAGCGATGCTTGC-3'
gbs1892 T74A antisense	5'-GCAAGCATCGCTCCAATTCTGCCCCACAGCCTGTAATAAC-3'
gbs1892 N106A sense	5'-GCTTACTTATTCCTCAAGTAGCCGGAGGAATGCTCTTTCCC-3'
gbs1892 N106A antisense	5'-GGAAAAGAGCATTTCCTCCGGCTACTTGGAGAGAATAAGTAAGC-3'
gbs1891 S150A sense	5'-GGTAAATCATCAATATTTGGCCCATGATGAGTGAGCTTGGACGC-3'
gbs1891 S150A antisense	5'-GGCTCCAAGCTCACATCATATGGCGCAAAATATGATGATTTACC-3'
gbs1891 Y163F sense	5'-GGACGCGAACAGTTGCTGCCTTGGCTGCTGCCAAAGGGGACTT-3'
gbs1891 Y163F antisense	5'-AAGTCCCCCTTTGGCAGCAGCAAGGCGCAACTGTTCCGCTCC-3'
gbs1891 K167A sense	5'-GTTGCTGCCTATGCTGCTGCCAGGGGGACTTAAAATGTTAAC-3'
gbs1891 K167A antisense	5'-GGTTAACATTTTAAAGTCCCCCTCGGCGCAGCAGCATAGGCGCAAC-3'

protein with His tag is as follows: SagDhuI, 0.840; SpnDhuI, 0.888; SpyDhuI, 0.845; SagDhuD, 0.586; SpnDhuD, 0.636; SpyDhuD, 0.600.

Determination of Molecular Mass—In order to determine the molecular weight of streptococcal proteins, SDS-PAGE, blue native PAGE (Invitrogen), and gel filtration chromatography (Sephacryl S-200HR) were performed.

Optimal pH and Temperature and Thermal Stability—The substrate (3-deoxy-D-glycero-2,5-hexodiulosonate) for SagDhuD was prepared as follows. Dhu was reacted with the purified SagDhuI, and the resultant product was heated at 95 °C for 3 min to inactivate SagDhuI. Experiments were performed using the purified SagDhuD. For optimal pH, reactions were performed at 30 °C in the following 46 mM buffers: sodium acetate, potassium phosphate, Tris-HCl, and glycine-NaOH. For optimal temperature, reactions were performed at different temperatures in 50 mM Tris-HCl (pH 7.5). For thermal stability, after preincubating the enzyme at different temperatures for 5 min, residual activity was measured at 30 °C in 50 mM Tris-HCl (pH 7.5).

Site-directed Mutagenesis—Cys⁷², Thr⁷⁴, and Asn¹⁰⁶ of SagDhuI were substituted with Ala (C72A), Ala (T74A), and Ala (N106A), respectively, and Ser¹⁵⁰, Tyr¹⁶³, and Lys¹⁶⁷ of SagDhuD were substituted with Ala (S150A), Phe (Y163F), and Ala (K167A), respectively, using the QuikChange site-directed mutagenesis kit (Stratagene) except that KOD Plus polymerase was used as the PCR enzyme. Site-directed mutagenesis was conducted using each expression plasmid as the template and synthetic oligonucleotides as sense and antisense primers (Table 1). The mutations in the resultant plasmids were confirmed by DNA sequencing. *E. coli* (i.e. BL21 (DE3) pLysS) was transformed with the plasmids. Moreover, the mutant enzymes were purified from the *E. coli* transformants approximately as described under "Purification."

Crystallization and Structure Determination—To determine the three-dimensional structures of SagDhuI and SagDhuD,

each purified enzyme was crystallized using the sitting drop vapor diffusion method. The SagDhuI T74A mutant (1 μl; 26.4 mg/ml protein in 20 mM Tris-HCl (pH 7.5) and 10 mM dithiothreitol) was mixed with an equal volume of reservoir solution (15% (w/v) polyethylene glycol 10,000, 2% (v/v) dioxane, and 0.1 M sodium citrate (pH 6.5)). The SagDhuI T74A mutant (1 μl) was also mixed with an equal volume of reservoir solution (2.0 M ammonium sulfate, 0.2 M potassium sodium tartrate, and 0.1 M sodium citrate (pH 5.6)). Furthermore, the SagDhuD S150A mutant (1 μl; 9.7 mg/ml protein in 20 mM Tris-HCl (pH 7.5)) was mixed with an equal volume of reservoir solution (20% (w/v) polyethylene glycol monomethyl ether 550, 0.1 M sodium chloride, and 0.1 M sodium N,N-bis(2-hydroxyethyl)glycine (pH 9.0)). Protein solutions were then incubated at 20 °C, and single crystals were allowed to grow for about 1 month. The crystals were flash-frozen under the cold nitrogen gas stream at -173 °C after soaking in the mother liquor containing 20% glycerol as the cryoprotectant.

X-ray diffraction data were collected at λ = 1.00 Å using a Quantum 210 or Quantum 315 CCD detector (Area Detector Systems Corp.) at BL38B1 station of SPring-8 (Hyogo, Japan). Data were processed and scaled up to 1.55 or 2.00 Å for SagDhuI and 2.90 Å for SagDhuD using the HKL2000 program (21). The structures were determined by molecular replacement using the Molrep program (22) supplied in the CCP4 program package (23) using the coordinates of the *S. pneumoniae* uncharacterized ribose 5-phosphate isomerase (Protein Data Bank (PDB) code 2PPW) as the initial model for SagDhuI and *Streptococcus suis* gluconate 5-dehydrogenase (Ga5DH) (PDB code 3CXR) as the initial model for SagDhuD. Structure refinement was conducted using the refmac5 program supplied in the CCP4 program package (24). Randomly selected 5% reflections were excluded from the refinement and used to calculate the R_{free}. After each cycle of refinement, the model was manually adjusted using the winCoot program (25). Water molecules

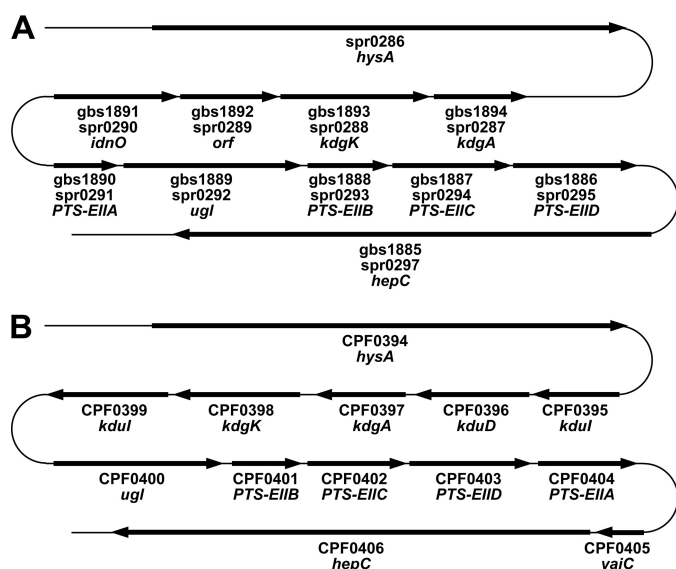


FIGURE 2. Genetic cluster for depolymerization, import, degradation, and metabolism of glycosaminoglycans. *A*, streptococcal genetic cluster. *gbs1885–1894* and *spr0286–0297*, gene annotations of *S. agalactiae* strain NEM 316 and *S. pneumoniae* strain R6, respectively. *PTS-EIIA–D*, subunit domains of the phosphotransferase system for amino sugar import. *B*, clostridial genetic cluster. *CPF0394–0404*, gene annotations of *C. perfringens* ATCC 13124.

were incorporated into isolated electron densities exceeding 3σ in the $F_o - F_c$ map and/or 1.2σ in the $2F_o - F_c$ map. Final model quality was checked with the PROCHECK program (26). Figures of protein structures were prepared using PyMOL (27). Coordinates used in this work were taken from the Protein Data Bank (28).

RESULTS AND DISCUSSION

Metabolism of Unsaturated Uronic Acids from Glycosaminoglycans

Streptococci such as *S. agalactiae*, *S. pneumoniae*, and *S. pyogenes* include the UGL genetic cluster responsible for the depolymerization (by lyases (HysA and/or HepC) for hyaluronan and/or heparan sulfate), import (by the phosphotransferase system; PTS), and degradation (by UGL) of glycosaminoglycans in the bacterial genomes (10, 11) (Fig. 2A). In addition to these glycosaminoglycan-related genes, the putative genes coding for 5-keto-D-gluconate dehydrogenase/reductase (IdnO), 2-keto-3-deoxygluconate kinase (KdgK), and 2-keto-3-deoxy-6-phosphogluconate aldolase (KdgA) involved in the metabolism of KDG are situated upstream of the UGL gene (29–31) (Fig. 2A). A gene for a hypothetical protein is inserted between *idnO* and *kdgK* in the streptococcal genomes. KDG has been demonstrated to convert to pyruvate and glyceraldehyde-3-phosphate through succeeding reactions by KdgK and KdgA (32) (Fig. 1).

The metabolic pathway of alginate-derived unsaturated mannuronic/guluronic acids and pectin-derived unsaturated galacturonic acid has been identified in bacteria (33, 34). α -Keto acid, 4-deoxy-L-erythro-5-hexoseulose uronic acid, from unsaturated mannuronic/guluronic acids is converted to KDG by NADPH-dependent reductase. We have recently identified the enzyme (A1-R) gene in an alginate-assimilating bacterium (35). In pectin-assimilating bacteria, such as *Dickeya*

and *Pectobacterium*, formerly known as *Erwinia*, α -keto acid, Dhu, from unsaturated galacturonic acid is converted to KDG via 3-deoxy-D-glycero-2,5-hexodiulosonate through subsequent reactions of KduI and 2-keto-3-deoxy-D-gluconate dehydrogenase (KduD) (36) (Fig. 1). In *E. coli*, KduI and KduD are also demonstrated to be involved in the metabolism of galacturonate and GlcUA (37). To the best of our knowledge, no reports have been published on the metabolism of Δ GlcUA/ Δ IdoUA derived from glycosaminoglycans, although unsaturated galacturonic acid as well as Δ GlcUA/ Δ IdoUA are nonenzymatically converted to Dhu (Fig. 1).

The gene arrangement in the UGL genetic cluster and the metabolism of unsaturated uronic acids suggest that α -keto acid (Dhu) generated from Δ GlcUA/ Δ IdoUA through the UGL reaction is also converted to KDG by certain enzymes. Thus, hypothetical proteins (*i.e.* *gbs1892*, *spr0289*, and *SPy0637*) and *IdnO* homologues (*i.e.* *gbs1891*, *spr0290*, and *SPy0636*) from *S. agalactiae*, *S. pneumoniae*, and *S. pyogenes* were expressed in recombinant *E. coli* cells and purified to homogeneity (Fig. 3, A–C). Purified proteins were subjected to enzyme assay using Dhu as the substrate. Three *gbs1892* mutants (C72A, T74A, and N106A) with a mutation at a possible catalytic residue were also constructed based on the crystal structure of *gbs1892* in complex with the substrate analog and its structural comparison with sugar isomerase, as described below. In the case of *gbs1891*, three mutants (S150A, Y163F, and K167A) with a mutation at the catalytic triad well conserved in the short-chain dehydrogenase/reductase (SDR) family were subjected to the enzyme assay and crystallization.

No oxidation of NADH or NADPH occurred in the presence of Dhu and *IdnO* homologues, whereas these proteins slightly reduced 5-keto-D-gluconate, depending on the presence of NADH. On the other hand, NADH was readily oxidized in the presence of Dhu and *IdnO* homologues as well as hypothetical proteins, indicating that Dhu was metabolized through subsequent reactions of the hypothetical protein and *IdnO* homologue. These reactions seem to correspond to those by KduI and KduD involved in the metabolism of unsaturated galacturonic acid (Fig. 1). The purified *EcoKduI* and *PcaKduD* with molecular masses of 31 and 27 kDa, respectively, were therefore used instead of the streptococcal enzymes (Fig. 3D). NADH was readily oxidized in the reaction mixtures of the combinations Dhu, *EcoKduI*, and *PcaKduD*; Dhu, *EcoKduI*, and *gbs1891*; and Dhu, *gbs1892*, and *PcaKduD*, demonstrating that the enzyme reactions by *gbs1892* and *gbs1891* are identical to those by *EcoKduI* and *PcaKduD*, respectively. These results indicated that Dhu was first converted to 3-deoxy-D-glycero-2,5-hexodiulosonate by *gbs1892* (EC 5.3.1.17; 4-deoxy-L-threo-5-hexosulose-uronate ketol-isomerase) and subsequently to KDG by *gbs1891* (EC 1.1.1.127; 2-keto-3-deoxy-D-gluconate dehydrogenase). The resultant KDG is considered metabolized to pyruvate and glyceraldehyde 3-phosphate through the subsequent reactions of KdgK and KdgA (Fig. 1). Because *S. agalactiae* genes encoding four enzymes are inducibly expressed in the presence of hyaluronan (10), these enzymes are suggested to be crucial for the metabolism of Δ GlcUA from the polysaccharide. In addition to *gbs1892* and *gbs1891*, *spr0289* and *SPy0637* were found to enzymatically correspond to *EcoKduI*, and *spr0290*

Streptococcal Glycosaminoglycan-metabolizing Enzymes

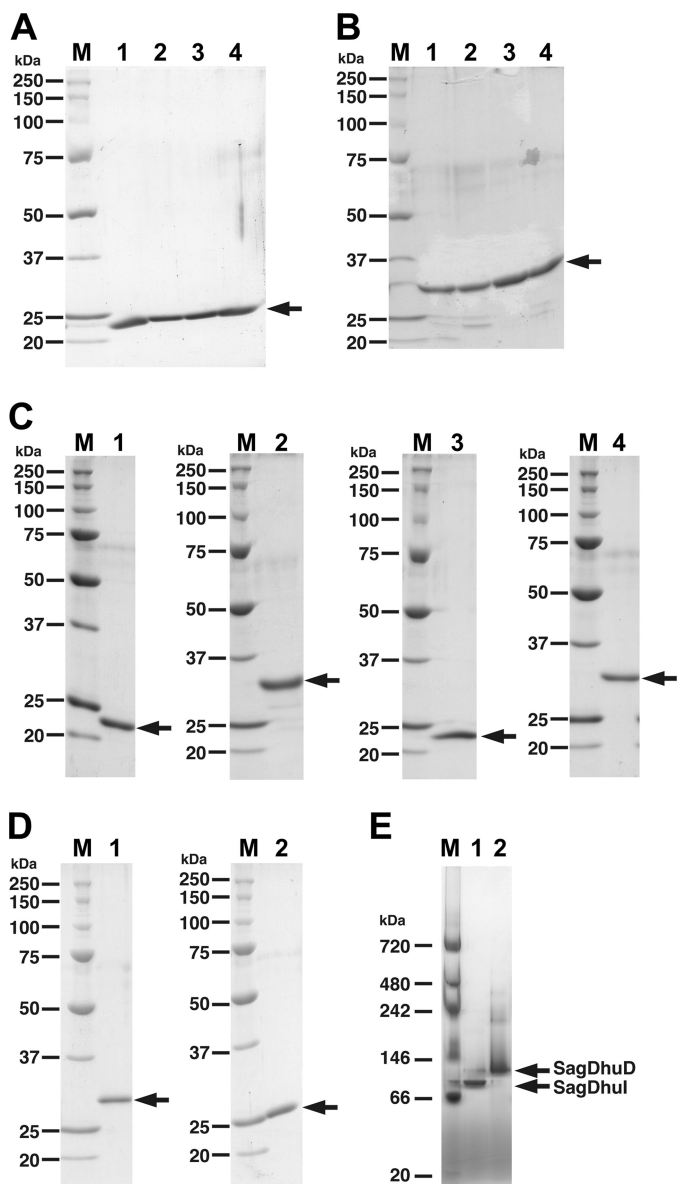


FIGURE 3. Electrophoretic profile of purified enzymes. A, SDS-PAGE profile of gbs1892 (SagDhuI). Lane 1, wild-type SagDhuI; lane 2, SagDhuI C72A; lane 3, SagDhuI T74A; lane 4, SagDhuI N106A. B, SDS-PAGE profile of gbs1891 (SagDhuD). Lane 1, wild-type SagDhuD; lane 2, SagDhuD S150A; lane 3, SagDhuD Y163F; lane 4, SagDhuD K167A. C, SDS-PAGE profile of spr0289 (SagDhuI, lane 1), spr0290 (SagDhuD, lane 2), SPY0637 (SpyDhuI, lane 3), and SPY0636 (SpyDhuD, lane 4). D, SDS-PAGE profile of EcoKduI (lane 1) and PcaKduD (lane 2). Lane M in A–D, molecular weight standards (from top): recombinant polypeptides with molecular weights of 250,000, 150,000, 100,000, 75,000, 50,000, 37,000, 25,000, and 20,000. E, blue native PAGE. Lane 1, gbs1892 (SagDhuI); lane 2, gbs1891 (SagDhuD). Lane M, molecular weight standards (from top): polypeptides with molecular weights of 720,000, 480,000, 242,000, 146,000, 66,000, and 20,000.

and SPY0636 were found to correspond to PcaKduD. Therefore, streptococcal hypothetical proteins (*i.e.* gbs1892, spr0289, and SPY0637) and IdnO homologues (*i.e.* gbs1891, spr0290, and SPY0636) encoded in the UGL genetic cluster should be redefined as 4-deoxy-L-threo-5-hexosulose-uronate ketol-isomerase and 2-keto-3-deoxy-D-gluconate dehydrogenase, respectively.

Classification of the UGL Genetic Cluster

The primary structure of gbs1892 is significantly different from that of EcoKduI, and the sequence identity between

gbs1891 and PcaKduD is also low (35.6%). The low sequence identity suggests that streptococcal genes coding for isomerase and dehydrogenase involved in the metabolism of glycosaminoglycans have been separately evolved from *kduID*, which is the operon for KduI and KduD, thus postulating that the streptococcal isomerase and dehydrogenase are designated as DhuI and DhuD, respectively, distinct from KduI and KduD. The operon *kduID* was first identified in *Dickeya dadantii* (36) and distributed in *Escherichia*, *Klebsiella*, *Salmonella*, *Vibrio*, and *Yersinia* (38). We also found *kduID* and *kdgAK* in the UGL genetic cluster in pathogenic *Clostridium* (39) and *Enterococcus* (40) (Fig. 2B), suggesting that these bacteria convert $\Delta\text{GlcUA}/\Delta\text{IdoUA}$ to KDG through the successive reactions of KduI and KduD. Similar to clostridia and enterococci, most bacteria having the UGL genetic cluster include *kduID*, but not *dhuDI*. Based on the type of isomerase and dehydrogenase, bacterial UGL genetic clusters are classified into two groups (*i.e.* *dhuDI* and *kduID*), although *dhuDI* is rather specific to streptococci.

Enzyme Characteristics

Because, compared with the well characterized KduI (41) and KduD (42), the enzyme properties of DhuI and DhuD remain unclear, streptococcal enzymes, especially DhuD, were further characterized.

Molecular Weight—The molecular masses of DhuI (*i.e.* gbs1892, spr0289, and SPY0637) and DhuD (*i.e.* gbs1891, spr0290, and SPY0636) were determined to be 24 and 29 kDa, respectively, by SDS-PAGE (Fig. 3, A–C). These values were almost comparable with the theoretical ones deduced from the predicted amino acid sequences of the enzymes. Blue native PAGE indicated that SagDhuI (gbs1892) and SagDhuD (gbs1891) have molecular masses of 94 and 115 kDa, respectively (Fig. 3E), indicating that both SagDhuI and SagDhuD are tetrameric. This result was also supported by gel filtration chromatography on Sephacryl S-200HR (data not shown).

pH and Temperature—SagDhuD was most active at around pH 8.0 in potassium phosphate (Fig. 4A) at 45 °C (Fig. 4B). Over 50% of the enzyme activity was lost after preincubation at 40 °C for 5 min in 20 mM Tris-HCl (pH 7.5) (Fig. 4C).

Substrate Specificity—The substrate specificity of SagDhuD was investigated using 3-deoxy-D-glycero-2,5-hexodiulosonate, 5-keto-D-gluconate, 2-keto-D-gluconate, and gluconate. A low concentration (16.8 μM) of SagDhuD readily oxidized NADH when using about 0.2 mM 3-deoxy-D-glycero-2,5-hexodiulosonate as a substrate, whereas a high concentration (10 mM) of 5-keto-D-gluconate was reduced in the presence of NADH by a high concentration (1.68 mM) of SagDhuD. The specific activity (4.28 units/mg) of SagDhuD for 5-keto-D-gluconate was extremely low compared with that (175 units/mg) for 3-deoxy-D-glycero-2,5-hexodiulosonate. The intrinsic substrate (3-deoxy-D-glycero-2,5-hexodiulosonate) at more than 4 mM interfered with the measurement of absorbance at 340 nm, and determination of kinetic parameters for the substrate was difficult. On the other hand, K_m and k_{cat} for 5-keto-D-gluconate were determined to be 260 ± 51 mM and 0.521 ± 0.038 s⁻¹, respectively. 2-Keto-D-gluconate and gluconate were inert substrates for SagDhuD. The reaction product (KDG) or its

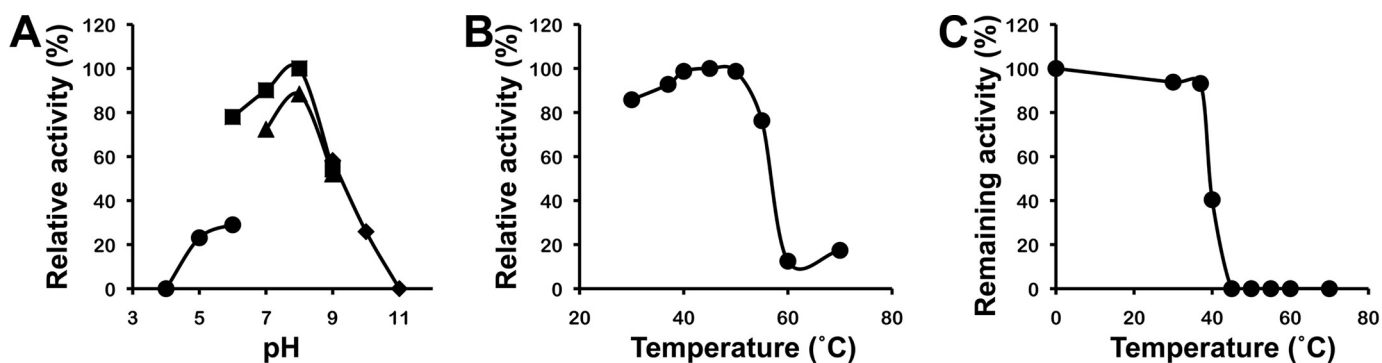


FIGURE 4. **Optimal pH and temperature and thermal stability of SagDhuD.** A, optimal pH. Experiments were performed at 30 °C using the following buffers: sodium acetate (circles), potassium phosphate (squares), Tris-HCl (triangles), and glycine-NaOH (diamonds). The maximum activity at pH 8.0 (potassium phosphate) was relatively taken as 100%. B, optimal temperature. Reactions were carried out at various temperatures in 50 mM Tris-HCl (pH 7.5). The maximum activity at 45 °C was relatively taken as 100%. C, thermal stability. After preincubation for 5 min at various temperatures, the residual activity was measured at 30 °C in 50 mM Tris-HCl (pH 7.5). The activity of the enzyme preincubated at 0 °C was taken as 100%.

TABLE 2
Statistics for data collection and structure refinement

	SagDhuI T74A	SagDhuI T74A/tartrate	SagDhuD S150A
Data collection			
Wavelength (Å)	1.00	1.00	1.00
Resolution range (Å)	50.00–2.00 (2.07–2.00) ^a	50.00–1.55 (1.61–1.55)	50.00–2.90 (2.95–2.90)
Space group	<i>I</i> 222	<i>P</i> 4 ₁ 2 ₁ 2	<i>P</i> 6 ₂ 22
Unit cell parameters (Å)	<i>a</i> = 53.23, <i>b</i> = 87.97, <i>c</i> = 90.30	<i>a</i> = <i>b</i> = 92.96, <i>c</i> = 129.35	<i>a</i> = <i>b</i> = 83.94, <i>c</i> = 181.10
Total observations	88,502	615,489	201,293
Unique reflections	14,717	82,052	8964
Completeness (%)	100 (100)	99.4 (95.7)	99.8 (100)
<i>I</i> /σ(<i>I</i>)	28.6 (5.3)	47.3 (4.8)	78.5 (13.1)
<i>R</i> _{merge}	0.148 (0.443)	0.084 (0.385)	0.069 (0.414)
Refinement			
<i>R</i> _{cryst}	0.183 (0.282)	0.198 (0.256)	0.211 (0.323)
<i>R</i> _{free}	0.227 (0.255)	0.211 (0.268)	0.288 (0.449)
No. of subunits per ASU ^b	1	2	1
No. of non-hydrogen atoms			
Proteins	1670	3381	1994
Water molecules	121	525	5
Ligand atoms		20	
Average <i>B</i> -factor (Å ²)	27.31	17.40	76.08
RMSD ^c from ideal			
Bond length (Å)	0.0057	0.0045	0.0073
Bond angle (degrees)	0.971	0.932	1.195
Ramachandran plot (%)			
Most favored	96.2	95.1	89.9
Additionally allowed	3.8	4.9	10.1

^a Data for the highest shells is given in parenthesis.

^b Asymmetric unit.

^c Root mean square deviation.

derivative (2-keto-3-deoxy-6-phosphogluconate) exhibited no inhibitory effect on the SagDhuD activity.

Cofactor Dependence—SagDhuD showed a preference for NADH (295 units/mg, 100%) rather than for NADPH (3.75 units/mg, 1.27%). Thus, the kinetic parameters (*K_m* and *k_{cat}*) of the enzyme for cofactor were measured using 3-deoxy-D-glycero-2,5-hexodiulosonate as the substrate. *K_m* and *k_{cat}* of the enzyme for NADH were determined to be $9.62 \pm 1.05 \mu\text{M}$ and $2.67 \pm 0.07 \text{ s}^{-1}$, respectively. When 5-keto-D-gluconate was used as the substrate, *K_m* and *k_{cat}* for NADH were determined to be $26.3 \pm 16.2 \mu\text{M}$ and $1.57 \pm 0.30 \text{ s}^{-1}$, respectively, whereas the kinetic parameters for NADPH could not be determined due to its low activity. This result indicated that SagDhuD is an NADH-dependent enzyme. There is a significant difference in the cofactor preference between DhuD and KduD, because KduD is known to use both NADH and NADPH (42).

Crystal Structure of SagDhuI

To clarify the structure/function relationship, SagDhuI proteins (wild type and mutant (C72A, T74A, and N106A) enzymes) were subjected to x-ray crystallography. Although all of the wild type and mutants of SagDhuI were crystallized and subsequently employed for structure determination, we focused on the SagDhuI T74A mutants because their crystals diffracted to higher resolution.

Two types of SagDhuI T74A crystals formed under different crystallization conditions. The crystal in droplet A containing polyethylene glycol as the major precipitant showed a space group of *I*222, whereas the space group of the other crystal in droplet B with ammonium sulfate as the major precipitant was *P*4₁2₁2. The structures of the SagDhuI crystals in droplets A and B were determined at 2.00 and 1.55 Å resolutions, respectively. Data collection and model refinement statistics are sum-

Streptococcal Glycosaminoglycan-metabolizing Enzymes

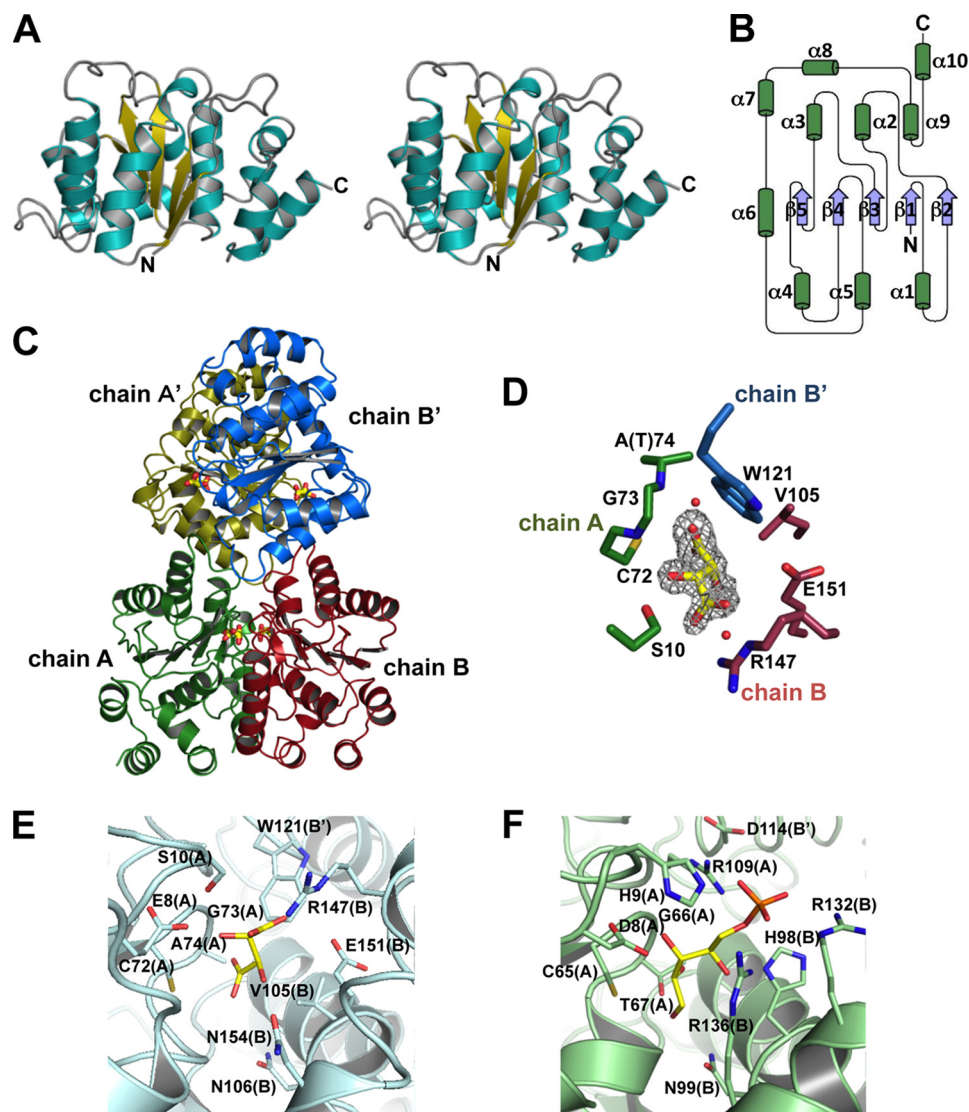


FIGURE 5. Structure of sugar isomerases. *A*, overall structure of monomeric SagDhul (stereo diagram). *B*, topology of monomeric SagDhul. *C*, quaternary structure of SagDhul. The four monomeric subunits are colored in green (chain A), red (chain B), yellow (chain A'), and blue (chain B'). Stick models indicate tartrate molecules. *D*, tartrate-binding residues in SagDhul T74A. Shown is an omit map ($F_o - F_c$) of tartrate (gray mesh with a contour of 3σ). *E*, active site of SagDhul T74A. *F*, ribose 5-phosphate-bound active site of clostridial RpiB (PDB code 3HEE). Subunit molecules (chains) are given in parenthesis. Atoms of carbon, nitrogen, oxygen, and phosphorus are colored in yellow, blue, red, and orange, respectively.

marized in Table 2. Both crystal structures were identical except that the electron density map for tartrate included in the droplet B was also observed in the enzyme. SagDhul adopts a Rossmann fold-like $\alpha/\beta/\alpha$ -barrel structure as the basic scaffold (Fig. 5A), and a parallel β -sheet composed of five strands is sandwiched by α -helices (Fig. 5B). Four crystallographically symmetrical molecules of SagDhul are assembled into a tetramer (Fig. 5C). There is no structural homology between SagDhul and EcoKdul, although both catalyze the same reaction. EcoKdul is a homohexameric enzyme, each subunit of which consists of two β -barrels and shows structural similarity to the cupin superfamily (43).

The structural homologues of SagDhul were sought in the PDB using the Structure navigator program. The top seven proteins having the Rossmann fold displayed structural homology with SagDhul (Table 3). In the Pfam database, these proteins belong to the RpiB/LacAB family (44), although the structural

homologues of SagDhul are divided into groups I and II based on sequence identity and amino acid length. The RpiB/LacAB family includes sugar isomerases, and its typical members are ribose 5-phosphate isomerase B (RpiB) and galactose isomerase subunits A and B (LacAB). Due to the high sequence identity, the overall structure of SagDhul was highly similar to three group I protein structures from *S. pneumoniae* (PDB code 2PPW), *Novosphingobium aromaticivorans* (PDB code 3C5Y), and *Vibrio parahaemolyticus* (PDB code 3ONO). These proteins, composed of ~ 200 residues, are assigned to RpiB based on the Pfam motif, although their enzyme characteristics and crystal structures have not yet been published. In contrast, group II proteins (PDB codes 1NN4, 3K8C, 3HEE, and 3SDW), which consist of ~ 150 residues, moderately resemble the tertiary structure of SagDhul, although little sequence identity is observed. Distinct from group I proteins, group II proteins are experimentally identified as RpiB, which are involved in the

TABLE 3
Structural homologs of SagDhuI

PDB entry	Organism	No. of residues	RMSD ^a to SagDhuI (no. of matching C ^α atoms)	Sequence identity with SagDhuI	References
			Å	%	
2PPW	<i>S. pneumoniae</i>	213	0.823 (192)	74	Unpublished
3C5Y	<i>N. aromaticivorans</i>	212	0.887 (192)	57	Unpublished
3ONO	<i>V. parahaemolyticus</i>	211	1.031 (198)	52	Unpublished
1NN4	<i>E. coli</i>	149	2.045 (115)	21	Ref. 44
3K8C	<i>T. cruzi</i>	159	1.823 (124)	17	Ref. 45
3HEE	<i>C. thermocellum</i>	149	2.315 (112)	21	Ref. 46
3SDW	<i>C. immitis</i>	163	2.191 (117)	14	Ref. 47

^a Root mean square deviation.

TABLE 4
Interactions between SagDhuI T74A and tartrate

Tartrate atom	Protein/Water	Chain	Atom	Distance ^a
				Å
Hydrogen bonds				
O1A	Wat ⁴⁴			2.9
	Arg ¹⁴⁷	B	NH2	2.8
O1B	Arg ¹⁴⁷	B	NE	2.8
	Arg ¹⁴⁷	B	NH2	3.0
O2	Ser ¹⁰	A	OG	3.0
	Gly ⁷³	A	N	2.9
O3	Wat ⁴⁴			2.6
O4A	Wat ⁶⁶			2.8
	Cys ⁷²	A	SG	3.3
O4B	Wat ⁶⁶			3.1
	Ala ⁷⁴ (Thr)	A	N	2.9
C-C contacts				
C1	Ser ¹⁰	A	CB	3.7
	Arg ¹⁴⁷	B	CZ	3.9
C2	Ser ¹⁰	A	CB	4.4
	Gly ⁷³	A	CA	4.2
	Trp ¹²¹	B'	CH2	4.3
C3	Val ¹⁰⁵	B	OG1	3.8
	Glu ¹⁵¹	B	CG	4.2
	Trp ¹²¹	B'	CH2	4.2
C4	Val ¹⁰⁵	B	CG1	3.7
	Cys ⁷²	A	CB	4.1
	Gly ⁷³	A	CA	4.4

^a For hydrogen bonds, the distance was <3.3 Å, and for C-C contacts, the distance was <4.4 Å.

pentose phosphate pathway (45–48). Group I proteins, including SagDhuI, have four additional α-helices at the C terminus. Genes coding for group I proteins from *S. pneumoniae* and *V. parahaemolyticus* are located at the genetic clusters for the metabolism of glycosaminoglycan and pectin, respectively, in the bacterial genomes. These structural and genetic features suggest that group I proteins should be reclassified as DhuI.

Active Site of SagDhuI

Four molecules of tartrate are bound to the homotetrameric SagDhuI (Fig. 5C), and each tartrate is accommodated at the interface formed by three subunits of SagDhuI (Fig. 5, D and E). The binding mode of tartrate to SagDhuI through hydrogen bond formation and van der Waals contacts is listed in Table 4. This binding site corresponds to the active site of RpiB (Fig. 5F), suggesting that tartrate was bound to SagDhuI as a substrate analog. Thus, three mutants (*i.e.* SagDhuI C72A, T74A, and N106A) carrying a mutation at the binding site were constructed based on the binding mode of tartrate and structural comparison between SagDhuI and RpiB (Fig. 3A). In the conjugation assay of SagDhuI and SagDhuD, three mutants caused a significant reduction of SagDhuD activity due to their low substrate production. When the enzyme activity using wild-type SagDhuI was taken as 100%, the relative activities using SagD-

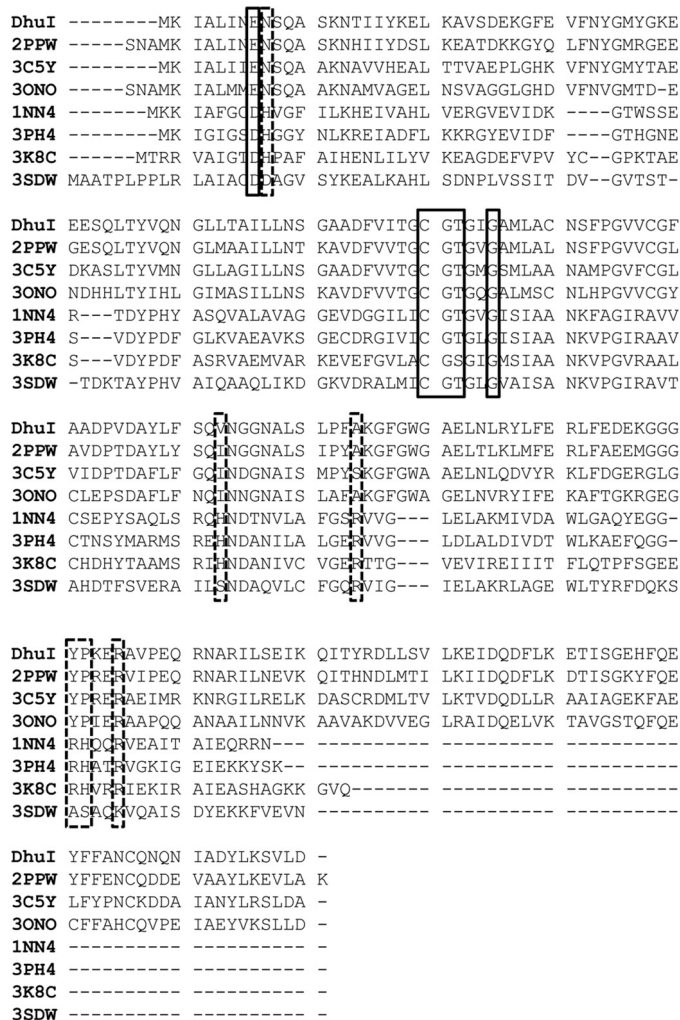


FIGURE 6. Sequence alignment of SagDhuI and its structural homologs. DhuI, SagDhuI; 2PPW, *S. pneumoniae* uncharacterized RpiB; 3C5Y, *N. aromaticivorans* putative RpiB; 3ONO, *V. parahaemolyticus* RpiB; 1NN4, *E. coli* RpiB; 3PH4, *Clostridium thermocellum* RpiB; 3K8C, *Trypanosoma cruzi* RpiB; 3SDW, *Coccidioides immitis* RpiB. Solid lined box, catalytically important residues; broken lined box, phosphate group-recognizing residues in RpiB.

huI C72A, T74A, and N106A were determined to be 4.15, 0.474, and 6.29%, respectively. This site-directed mutagenesis indicated that tartrate was bound to the active site of SagDhuI. In fact, the catalytically important residues in group II RpiBs are also conserved in group I proteins, whereas phosphate-recognizing residues are specific to group II proteins (Fig. 6), thus suggesting that group I proteins are defined as DhuI but not as RpiB.

Streptococcal Glycosaminoglycan-metabolizing Enzymes

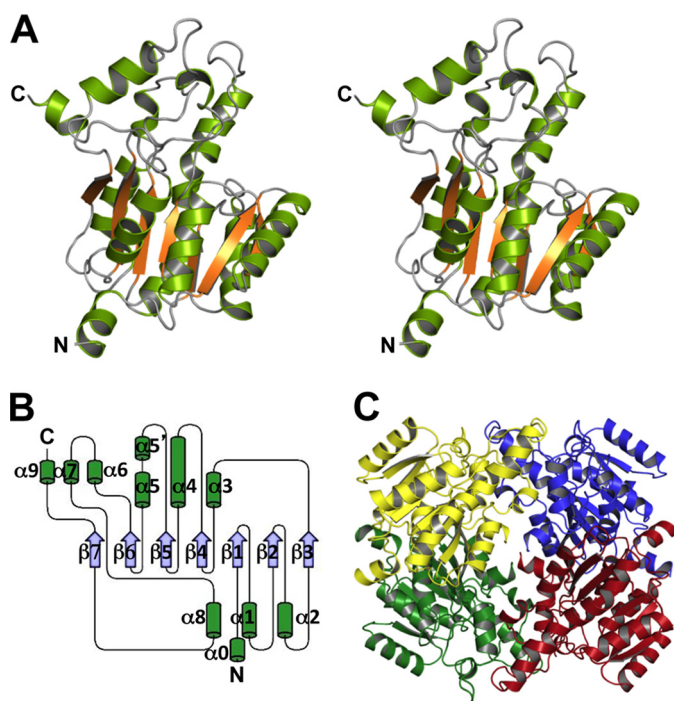


FIGURE 7. **Structure of SagDhuD.** *A*, overall structure of monomeric SagDhuD (stereo diagram). *B*, topology of monomeric SagDhuD. *C*, quaternary structure of SagDhuD. The four monomeric subunits are colored in green, red, yellow, and blue.

Crystal Structure of SagDhuD

The crystal structure of SagDhuD S150A was determined at 2.90 Å resolution. The data collection and model refinement statistics are summarized in Table 2. Similar to SagDhuI, SagDhuD adopts a Rossmann fold-like $\alpha/\beta/\alpha$ -barrel structure as the basic scaffold (Fig. 7, *A* and *B*), and a parallel β -sheet composed of seven strands is surrounded by α -helices (Fig. 7*B*). Four crystallographically symmetrical molecules of SagDhuD are assembled into a tetramer (Fig. 7*C*).

Based on the primary structure, SagDhuD belongs to the SDR family (49). A large number of SDR enzymes have been structurally analyzed, and their structure/function relationships have been well documented (50). Catalytic triads (Ser, Tyr, and Lys) are identified to be crucial for the enzyme reaction. Ser¹⁵⁰, Tyr¹⁶³, and Lys¹⁶⁷ are also conserved in wild-type SagDhuD, and the SagDhuD mutants (S150A, Y163F, and K167A) constructed in this study (Fig. 3*B*) exhibited extremely lower activity (wild type, 100%; S150A, 0.240%; Y163F, 0.0581%; K167A, 0.0682%). Although three mutants were subjected to the kinetics analysis, their kinetic parameters for 5-keto-D-glucuronate could not be determined due to their extremely low activity. The crystal structure of *S. suis* Ga5DH, one of the SDR enzymes, has been determined (51), although no description regarding its activity as dehydrogenase is included in the literature. Based on its gene location in the bacterial genome (the UGL genetic cluster) and high sequence identity (83%) with SagDhuD, the enzyme is considered to function as DhuD.

Structural Comparison in the SDR Family

Interesting structural features specific to SagDhuD were found. Compared with Ga5DH (PDB code 3CXR), SagDhuD

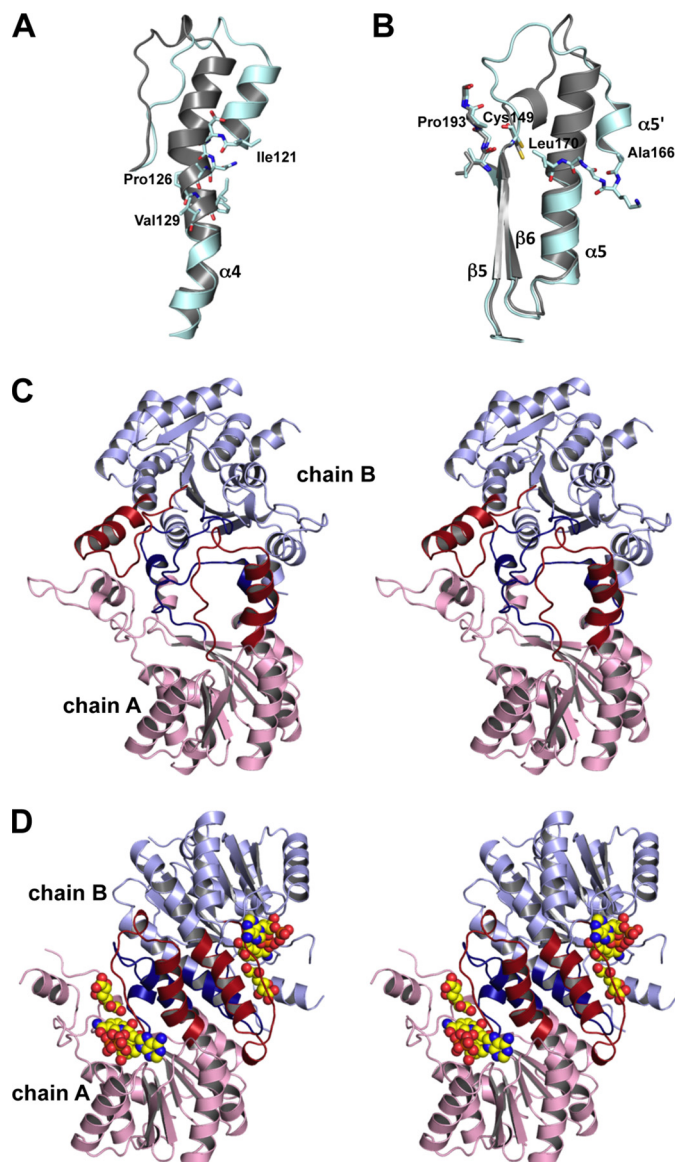


FIGURE 8. **Structural comparison.** *A*, the bent helix 4 in SagDhuD. Cyan, SagDhuD; gray, Ga5DH (PDB code 3CXR). The residues Ile¹²¹ and Val¹²⁹ are indicated by stick models. *B*, part of helix 5 of SagDhuD (cyan) is loosened distinct from the long helix 5 of Ga5DH (gray). The residues Cys¹⁴⁹, Ala¹⁶⁶–Leu¹⁷⁰, and Ile¹⁹¹–Gly¹⁹⁴ are indicated by stick models. *C*, interaction between two monomeric subunits (chains A and B) (stereo diagram). The bent and shortened helices of SagDhuD are colored red and blue, respectively. *D*, subunit interaction of substrate/cofactor-bound Ga5DH (PDB code 3O03) (stereo diagram). Helices 4 and 5 are colored in red and blue, respectively. Substrate and cofactor are indicated by sphere models. The orientation of chain A is identical to that of chain A in *C*.

has a bent α -helix 4 (Fig. 8*A*) and a short α -helix 5 (Fig. 8*B*). Instead of the long α -helix 5 of Ga5DH, the N-terminal half portion of the helix is loosened to a loop and a short α -helix 5' (Fig. 8*B*). The loop between the β -strand 5 and α -helix 5', which is crucial for the formation of the substrate and cofactor-binding sites in most SDR enzymes, is subjected to conformational changes to cover the binding sites. This structural characteristic in the crystal of SagDhuD is probably unsuitable for binding substrate and/or cofactor. Generally, no conformational change occurs in the corresponding loop through substrate/cofactor binding in SDR enzymes, except in *E. coli* β -ketoacyl carrier protein reductase (FabG). A cofactor-induced con-

formational change has been observed in the loop of FabG (52), although this change is small compared with the drastic change in SagDhuD. The bending of the α -helix 4 is probably caused by the destruction of the hydrogen bonds in the main chain due to the presence of Pro¹²⁶ at the center of the helix (Fig. 8A). Two neighboring glycine residues (Gly¹⁶⁸-Gly¹⁶⁹) possibly contribute to the conformational change of the shorter α -helix 5 because the highly flexible glycine residue is entropically unsuitable for the construction of α -helices. The lack of the amino group in Pro¹⁹³ in the β -strand 6 causes no hydrogen bond formation with Cys¹⁴⁹ (Fig. 8B), thus resulting in no attendance of Cys¹⁴⁹ to the formation of the β -strand 5 and determining its high flexibility. These structural features were also confirmed in other wild-type and mutant SagDhuD enzymes crystallized under different conditions.

On the other hand, Ga5DH, which has a straight α -helix 4 and long α -helix 5, accepts the cofactor (51), although all of the above-mentioned SagDhuD residues, such as Pro¹²⁶, Cys¹⁴⁹, Gly¹⁶⁸, Gly¹⁶⁹, and Pro¹⁹³, are completely conserved in Ga5DH. In this study, SagDhuD was experimentally demonstrated to exhibit an NADH-dependent reductase/dehydrogenase activity, suggesting that SagDhuD also adopts a Ga5DH-like structure through conformational changes caused by the substrate/cofactor binding and that two structural conformations (*i.e.* active and inactive forms) are mutually converted through the binding and release of the substrate/cofactor. The quaternary structure of SagDhuD (Fig. 8C) is different from that of Ga5DH (Fig. 8D). The possible conformational change in SagDhuD may affect the assembly of two subunits in the tetramer (chains A and B) (Fig. 8C), distinct from FabG, in which a slight conformational change occurs in the tertiary structure but not in the quaternary structure. To demonstrate the conformational change in SagDhuD, we are attempting to obtain the substrate/cofactor-binding crystals of the enzyme.

In conclusion, the metabolic pathway of unsaturated glucuronic/iduronic acids from glycosaminoglycans and its relevant enzymes/genes were elucidated for the first time in streptococci. The isomerase DhuI and dehydrogenase DhuD were specific mainly to streptococci, although they enzymatically correspond to KduI and KduD, which are involved in the metabolism of unsaturated galacturonic acid from pectin. X-ray crystallography revealed that SagDhuI and SagDhuD include the structural characteristics essential for the enzyme reaction.

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Note Added in Proof—In the version of this article that was published as a Paper in Press on January 20, 2015, two identical descriptions for “CPF0404” were found in Fig. 2. “CPF0404” on hepC should have said “CPF0406”. In addition, the sequence for spr0290XhoI was missing from Table 1. The correct Fig. 2 and Table 1 are now shown.

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