Crystal Structure of Heparinase II from *Pedobacter heparinus* and Its Complex with a Disaccharide Product

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Heparinase II depolymerizes heparin and heparan sulfate glycosaminoglycans, yielding unsaturated oligosaccharide products through an elimination degradation mechanism. This enzyme cleaves the oligosaccharide chain on the nonreducing end of either glucuronic or iduronic acid, sharing this characteristic with a chondroitin ABC lyase. We have determined the first structure of a heparin-degrading lyase, that of heparinase II from *Pedobacter heparinus* (formerly *Flavobacterium heparinum*), in a ligand-free state at 2.15 Å resolution and in complex with a disaccharide product of heparin degradation at 2.30 Å resolution. The protein is composed of three domains: an N-terminal α-helical domain, a central two-layered β-sheet domain, and a C-terminal domain forming a two-layered β-sheet. Heparinase II shows overall structural similarities to the polysaccharide lyase family 8 (PL8) enzymes chondroitin AC lyase and hyaluronate lyase. In contrast to PL8 enzymes, however, heparinase II forms stable dimers, with the two active sites formed independently within each monomer. The structure of the N-terminal domain of heparinase II is also similar to that of alginate lyases from the PL5 family. A Zn²⁺ ion is bound within the central domain and plays an essential structural role in the stabilization of a loop forming one wall of the substrate-binding site. The disaccharide binds in a long, deep canyon formed at the top of the N-terminal domain and by loops extending from the central domain. Based on structural comparison with the lyases from the PL5 and PL8 families having bound substrates or products, the disaccharide found in heparinase II occupies the “+1” and “+2” subsites. The structure of the enzyme-product complex, combined with data from previously characterized mutations, allows us to propose a putative chemical mechanism of heparin and heparan-sulfate degradation.

Heparin and heparan sulfate glycosaminoglycans (HSGAGs) are major components of the extracellular matrix and are also found at the cell surface as part of proteoglycan cell surface receptors (1, 2). HSGAGs are negatively charged linear polysaccharides composed of repeating disaccharide units of hexopyranosuronic acid (uronic acid) and 2-amino-2-deoxy-α-D-glucopyranose (glucosamine, GlcNAc) (3). Heparan sulfate proteoglycans are abundant on the cell surface as receptors, where 2–5 polysaccharide chains are attached via a serine residue to a core protein (4). Heparin PGs are primarily synthesized and stored in mast cells. Consequently, short oligosaccharide sequences are released as bioactive heparin GAGs by the action of heparin hydrolase. These complex polysaccharides provide docking sites for numerous protein ligands and receptors involved in diverse biological processes, ranging from cancer (5, 6) to angiogenesis (7), anticoagulation (8), inflammatory processes, viral and microbial pathogenesis (9, 10), and multiple aspects of development (11).

HSGAGs are not homogeneous in their chemical structure but are subject to extensive modification of the linear chain during biosynthesis. These modifications are catalyzed through the sequential action of several enzymes and include epimerization of β-D-glucopyranosyluronic acid to α-L-idopyranosyluronic acid (IdoA), N-deacetylation coupled with N-sulfation of the glucosamine moiety, and O-sulfation of hydroxyl groups (2-O, 3-O, or 6-O). Heparin typically contains ~90% iduronic acid and 10% glucuronic acid, with predominant sulfation of iduronic acid at the 2-OH position. The hexosamine usually contains 3-O-sulfation and is predominantly N-sulfated (GlcNS). Occasionally, it is also present in the N-acetylated form (GlcNAc). Occurrence of a glucosamine unsubstituted at the 2-N position is rare. Heparan sulfate is a related polysaccharide with a higher percentage of GlcA and a lower percentage of 2-O- and N-sulfation within the polymer. Modification of HS is not uniform but rather is concentrated within specific regions of the polymer, giving rise to short sequence motifs responsible for the interactions between HS and a diverse repertoire of proteins leading to its multiple biological roles.

Two distinct chemical mechanisms are employed by proteins in order to break down HSGAGs: hydrolysis and β-elimination. Mammalian enzymes employ solely hydrolytic mechanisms to cleave the glycosyl-oxygen (C1–On) bond. The bacterial enzymes are either hydrolyses or lyases. Lyases act exclusively on the oxygen-aglycone (On–C4) bond adjacent to the uronic acid moiety at position C5 and result in a product with a C4–C5 double bond (Scheme 1). The mammalian enzyme heparanase, an endo-β-glucuronidase, cleaves HS utilizing a hydrolytic mechanism. It involves the addition of a water molecule to afford saturated oligosaccharide products (12, 13). Bacterial heparinas, on the other hand, cleave their polysaccharide substrates through an elimination mechanism (5). The proposed elimination mechanism involves neutralization of the negative charge of the uronic acid acidic group, presumably by interaction with a positively charged amino acid, thereby reducing the acidity of the C-5 proton (14). Next, a general base formation of one wall of the substrate-binding site.
Crystal Structure of Heparinase II

Heparin

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\text{[IdoA2S(1-4)GlcNS6S]-} \xrightarrow{+1} \text{[GlcA(1-4)GlcNAc]-} \xrightarrow{+2} \text{[GlcNH3, provided that the uronic acid is 2- or GlcNS. HepII will also degrade the rare disaccharide containing a glucosamine, reconstituting the OH function at the reducing end of the cleaved bond.} \\
\text{[22, 23]. The glucosamine in the substrate could be either GlcNAc or GlcNS. HepII will also degrade the rare disaccharide containing GlcNH3, provided that the uronic acid is 2-O-sulfated. Although HepII has greater affinity for heparin, its turnover rate for heparan sulfate is higher (16). Moreover, the enzyme displays preference toward degradation of glycosidic bonds containing GluA over ones containing IdoA. Heparinase II shows greater catalytic efficiency for longer rather than shorter oligosaccharide substrates (21). Previous chemical modification and site-directed mutagenesis studies suggested that cysteine and histidine residues are involved in catalysis (24, 25) and that the cleavage of the two substrates, heparin and heparan sulfate, occurs at separate but proximal active sites within a single HepII polypeptide chain (21). We report here the crystal structures of HepII as well as its complex with a heparin disaccharide reaction product. We have adopted the nomenclature of Davies et al. (26) for the sugar binding subsites, where “+ subsites” refer to those toward the reducing end of the substrate, and “− subsites” indicate those toward the nonreducing end of the polysaccharide chain. Cleavage occurs between sugars occupying the “−1” and “+1” subsites.

EXPERIMENTAL PROCEDURES

Protein Expression and Purification

Native HepII—Purification of HepII from P. heparinus was carried out as described previously (27). Briefly, P. heparinus cells overexpressing HepII (58) were cultivated in FH medium (58) supplemented with 10% (w/v) heparin (Dongying Hi-tech Chemical Industry Co., Dongying City, China) and 0.1 mg ml\(^{-1}\) trimethoprim antibiotic (Sigma). The cells were harvested, cells were disrupted using a French press, the lysate was clarified by ultracentrifugation (100,000 \(g\), 30 min, 4 °C), and the supernatant containing HepII was collected. HepII was purified using two chromatographic steps, SP-Sepharose (Amersham Biosciences) and hydroxylapatite (Bio-Gel HTP; Bio-Rad). HepII-containing fractions were concentrated to 40 mg ml\(^{-1}\) by ultrafiltration using a Centriprep-50 concentrator (Millipore Corp.), and the buffer was exchanged to 10 mM sodium phosphate, pH 7.5, 100 mM NaCl, 20 mM sodium formate, 5 mM dithiothreitol.

Cloning and Expression of HepII in Escherichia coli—P. heparinus was obtained as a lyophilized stock from the American Type Culture Collection (Manassas, VA), stock number 13125. Rehydrated stock was
used to seed a 100-ml culture, which was grown aerobically at 30 °C with moderate shaking to an optical density (A600) between 1.5 and 2 in defined medium containing 0.1% (v/v) heparin as described (28). Genomic DNA was isolated from the bacterial culture using the Qiagen DNeasy DNA purification kit according to the manufacturer's instructions for Gram-negative bacteria based on $\sim 2 \times 10^{5}$ cells/column. Purified genomic DNA was concentrated by ethanol precipitation and resuspended in Tris-EDTA, pH 7.5, at a concentration of 0.5 mg ml$^{-1}$. The quality of genomic DNA was assessed by UV absorbance at 260/280 nm and by electrophoresis on a 0.5% agarose gel and confirmed by PCR using P. heparinus-specific oligonucleotide primers.

The heparinase II gene lacking the signal sequence (residues 1–23) was directly amplified by PCR from P. heparinus genomic DNA using the forward primer 5′-CAT ATG TAT TCCCAA ACC AAG GCC GAT GTG GTT-3′ and reverse primer 5′-CTC GAG GAT CCT TAT CTC AAA AAA CGG TAG GTT CCT-3′ (Ndel and Xhol restriction sites are denoted in boldface type). Oligonucleotide primer sequences were based on the published hepB sequence annotated within the NCBI (GenBank$^{TM}$) database (accession number U27585). PCR conditions included 100 ng of genomic DNA and Phx DNA polymerase (Invitrogen) for 35 cycles using an annealing temperature of 61 °C and 3-min extensions at 68 °C. PCR products were purified by agarose gel electrophoresis, and 3′ dA overhangs were generated using 200 μM dATP and TaqDNA polymerase (New England Biolabs, Beverly, MA) for 10 min at 72 °C. Modified PCR products were ligated into the TOPO/TA PCR cloning vector 4.0 (Invitrogen) and used to transform One-shot TOP10 chemically competent cells (Invitrogen). Positive clones were verified by restriction enzyme analysis. The 2.2-kb HepII gene was subcloned into the T7-based expression plasmid pET28a (Novagen) as an Ndel-Xhol restriction site for expression using P. heparinus-specific oligonucleotide primers.

Sequence-verified plasmid pET28a/hepIIΔNterm was subsequently transformed into BL21(DE3) for expression. One-liter cultures were grown at room temperature (~20 °C) in LB medium supplemented with 40 μg ml$^{-1}$ kanamycin, and protein expression was induced with 500 μM isopropyl 1-thio-β-D-galactopyranoside added at an A600 of 1.0. Induced cultures were allowed to grow for 15–18 h at room temperature prior to harvesting.

Purification of Recombinant Heparinase II—Bacterial cells were harvested by centrifugation (6000 × g, 15 min, 4 °C) and resuspended in 30 ml of binding buffer (50 mM Na$_2$HPO$_4$, pH 7.9, 0.5 M NaCl, 5 mM imidazole). Lysis was performed by the addition of 0.1 mg ml$^{-1}$ lysozyme and incubation for 20 min at room temperature followed by intermittent sonication in an ice water bath using a Misonex XL sonicator at 40–50% output. This lysate was fractionated by centrifugation (20,000 × g, 30 min, 4 °C), and the supernatant was filtered through a 0.45-μm filter. HepII was purified by immobilized metal-chelate chromatography using a 5-ml Hi-Trap column (GE Healthcare) precharged with 200 mM NiSO$_4$ and subsequently equilibrated with binding buffer. The column was run at a flow rate of ~3 ml min$^{-1}$ that included a wash step with 50 mM imidazole. HepII was eluted in 5-ml fractions using imidazole elution buffer (50 mM Na$_2$HPO$_4$, pH 7.9, 0.5 M NaCl, 250 mM imidazole).

HepII from the IMAC step was further purified on a Sephadex G-25 column equilibrated with 20 mM Na$_2$HPO$_4$, pH 6.8, 150 mM NaCl. The His$_6$ tag was cleaved by the addition of biotinylated thrombin at 0.5 units/mg of protein and incubating overnight at 4 °C. Thrombin was removed by binding to streptavidin-agarose at 4 °C for 2 h using the thrombin capture kit (Novagen).

The enzyme was further purified on a Source 15S cation exchange column (GE Healthcare). Enzyme was diluted 1:4 (v/v) with 20 mM Na$_2$HPO$_4$, pH 6.8, and loaded onto the column, and a gradient from 0 to 1 M NaCl was applied over 10 column volumes. Protein concentration was determined by the Bio-Rad protein assay. Protein purity was assessed by SDS-PAGE followed by staining with Coomassie Brilliant Blue or Sypro Ruby Protein (Molecular Probes, Inc., Eugene, OR).

Expression and Purification of Selenomethionine-labeled HepII—Selenomethionine (SeMet) labeling of HepII by methionine inhibition was performed using a modified M9 medium (29). An overnight culture was used to inoculate 100 ml of LB supplemented with 400 mg/liter kanamycin and grown overnight at 37 °C. The culture was pelleted and resuspended in 8 ml of modified M9 medium supplemented with 20% (v/v) glucose, 100 μM CaCl$_2$, and 1 X BME vitamin solution (Sigma). A 1-ml volume of resuspended culture was used to inoculate each of 4×500 ml of M9 medium, and cells were cultured at 37 °C in 2-liter baffled flasks until an A$_{600}$ of 0.5–0.6. Solid amino acid supplement was added as a preweighed mixture, and SeMet (Sigma) presuspended in sterile water was added at a final concentration of 50 mg/liter. Cell growth was allowed to continue for ~30 min, isopropyl 1-thio-β-D-galactopyranoside was added to 0.5 mM, and the cells were cultured for 15 h at 30 °C. HepII was subsequently purified as described above.

The efficiency of SeMet incorporation was assessed by matrix-assisted laser desorption/ionization mass spectrometry using saturated sinapinic acid matrix solution in 50% (v/v) acetonitrile/water as a matrix. Molecular masses were calibrated using both bovine serum albumin and β-galactosidase as protein standards. The molecular mass difference between unlabeled and SeMet-labeled enzyme indicated greater than 90% incorporation.

Purified SeMet-labeled protein was concentrated to 6.2 mg ml$^{-1}$ by ultrafiltration using a Centricon YM-100 concentrator (Millipore Corp.) and the buffer was exchanged to 25 mM sodium phosphate, pH 6, 150 mM NaCl, 10 mM dithiothreitol, 0.2% (v/v) NaN$_3$.

Crystallization and Data Collection

Native HepII—Native HepII crystals were obtained from the protein overexpressed in P. haperinus, and diffraction data were collected as described previously (27). Briefly, initial crystals were obtained from 6.5 mg ml$^{-1}$ protein in buffer and a reservoir solution containing 17% (v/v) PEG 3350, 200 mM sodium phosphate pH 5. Crystals were optimized by microseeding. These crystals belong to space group P2$_1$2$_1$2$_1$, with unit cell parameters a = 70.0, b = 119.3, and c = 200.7 Å and two molecules in the asymmetric unit. For data collection, crystals were transferred briefly to a cryoprotectant solution (22.5% (v/v) PEG 3350, 0.07 M sodium phosphate, pH 5, 2.17 M sodium formate) and flash cooled in a nitrogen stream at 100 K (Oxford Cryosystems, Oxford, UK). Diffraction data were collected at beamline X25 (National Synchrotron Light Source, Brookhaven National Laboratory) on a Q-315 area detector (Area Detector Systems Corp., San Diego, CA) and processed to 2.15 Å resolution using HKL2000 (30). Data collection statistics are summarized in Table 1.

SeMet-labeled HepII—HepII overexpressed in E. coli did not yield crystals under the same conditions as for the protein produced in P. haperinus. Somewhat different conditions were found using the PEG Suite screen (Nextal Biotechnologies, Montreal, Canada) with the hanging drop vapor diffusion method at 20 °C. The protein crystallized only when it was supplemented with 3 mM low molecular weight heparin (Sigma). Cube-shaped crystals of SeMet-labeled HepII appeared in drops containing 20% (v/v) PEG 3350, 200 mM magnesium chloride within 3–5 days. These crystals belong to space group P2$_1_1_1_1$, with unit cell
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X-ray crystallographic data

| Parameters                  | Values               |
|-----------------------------|----------------------|
| Data collection             |                      |
| Space group                 | 2, 2, 2_1            |
| Cell dimensions             |                      |
| a (Å)                       | 70.0                 |
| b (Å)                       | 119.3                |
| c (Å)                       | 200.7                |
| α (degrees)                 | 90.0                 |
| β (degrees)                 | 90.0                 |
| γ (degrees)                 | 90.0                 |
| Wavelength (Å)              | 0.97940              |
| Resolution range (Å)        | 102.6–2.15 (2.21–2.15) |
| Observed/unique reflections |                      |
| Average redundancy          | 7.4 (7.3)            |
| Completeness (%)            | 98.7 (98.2)          |
| Root mean square deviation  |                      |
| Bond (Å)                    | 0.075 (0.226)        |
| Angles (degrees)            | 1.10                 |

Refinement statistics

- R-work (Å) 0.193
- R-free (Å) 0.232
- No. of atoms/average B-factor
  - Protein (main chain + side chain) 11,938/27.8
  - Disaccharide product 82/49.1
  - O-Linked glycan 19/37.2
  - Ligand (formate, phosphate) 928/31.1
  - Solvent 783/21.5
  - Ions (Zn²⁺) 2/26.7
- Ramachandran plot
  - Allowed (%) 99.0
  - Generously allowed (%) 0.7
  - Disallowed (%) 0.2
- Root mean square deviation
  - Bonds (Å) 0.008
  - Angles (degrees) 1.10

- R-value
- R-factor
- Average I/σ(I)
- Average redundancy
- Completeness (%)
- Root mean square deviation
- Bond (Å)
- Angles (degrees)

- All measured reflections with I > 1σ(I).
- The Friedel pairs were not merged.
- R-value = (Σ(|Ioobs| - |Iolab|)/Σ|Iolab|)
- R-factor = (Σ(|Iolab| - |Iolab|)/Σ|Iolab|)
- The two molecules in this crystal form had substantially different B-factors.

HepII Expressed in E. coli—Single-wavelength anomalous diffraction phasing was performed using the data collected for SeMet-labeled HepII at 0.97910-Å wavelengths. A total of 31 of 42 selenium sites were located using the program SOLVE (32). The heavy atom sites were refined using the program SHARP (33). A total of 11 additional selenium sites were located in the anomalous difference map. Inclusion of all of the identified selenium sites gave phases with an overall figure-of-merit of 0.35–2.5 Å resolution. Solvent flattening with RESOLVE (34) increased the figure-of-merit to 0.65 and automatically built a partial model consisting of 60% of main chain atoms and 45% of side chain atoms. The model was completed manually using the program O (35) and refined using the program REFMAC5 (36). Since all crystals showed some level of anisotropic diffraction, the refinement protocol included the TLS option with each of the three domains treated separately. The inclusion of TLS reduced the R-free and R-work by ~2%, but the average B-factor for the protein was very little affected. The analysis of TLS indicates the largest libration along the direction approximately parallel to the long axis of the molecule (supplementary material). Solvent molecules were added using ARP/wARP (37). Electron density maps showed the presence of a disaccharide within the substrate-binding site of each molecule, which was subsequently modeled as a trisulfated disaccharide corresponding to the heparin degradation product, DUAmp(1–4)GlcNS6S. A strong peak in the electron density surrounded by two histidines, aspartate, and water molecules was deemed to be a metal ion. This was assumed to be a Zn²⁺ ion based on the ligand geometry and on the fact that its refined B-factors were comparable with those of neighboring atoms. For other metals tested, the B-factors differed significantly from the neighbors. Refinement at 2.3 Å resolution converged with an R-factor of 0.176 and R-free of 0.221, with the model consisting of residues Asp-30 to Arg-772 of each monomer, each with a bound metal ion (Zn²⁺) and a total of 783 water molecules. The final refinement statistics are shown in Table 1.

HepII Expressed in P. heparinus—The HepII model was positioned within this unit cell using MolRep (38) and subsequently refined at 2.15 Å resolution using REFMAC5, to an R-factor of 0.193 and R-free of 0.232 (Table 1). The N-terminal residue was identified as a pyroglutamate, formed following cleavage of the signal sequence (20). The final model includes two molecules, each containing residues 26–772. A metal ion with octahedral coordination was identified in the electron parameters a = 52.0, b = 163.0, c = 95.2 Å, β = 105.4° and contain two molecules in the asymmetric unit. The Matthews coefficient V_M is 2.28 Å³ Da⁻¹ (31), and the solvent content is estimated to be 46%. Crystals were transferred briefly to a cryoprotectant solution (25% (w/v) PEG 3350, 200 mM magnesium chloride, 12% (v/v) glycerol) and flash cooled in the nitrogen stream at 100 K (Oxford Cryosystems). Diffraction data extending to 2.3 Å resolution were collected at beamline X8C (National Synchrotron Light Source, Brookhaven National Laboratory), using a Quantum-4 CCD area detector (Area Detector Systems Corp.) at a wavelength of 0.97910 Å, corresponding to the measured absorption inflection point, in order to optimize the f' component of the selenium anomalous scattering. Images were processed with the HKL2000 program package (30) (Table 1).
density map. Based on the nature of coordinating groups and their distances from the metal ion (2.2–2.3 Å), it was inferred to be a Zn$^{2+}$ ion. The electron density map showed that Thr-134 was O-glycosylated, and a tetrasaccharide corresponding to the known sequence of a typical O-glycosylation site found in *P. heparinus* (39) fitted well to the electron density. The model also contains 939 water molecules, two phosphates, and three formate molecules.

For all molecules within the different crystal forms, Asn-405 is in the disallowed region of the Ramachandran plot. This residue has well defined electron density and is involved in product binding. Each molecule contains four cis-peptides modeled adjacent to proline residues.

Coordinates of native HepII and the HepII-$\Delta$UAp2S(1–4)GlcNS6S complex have been deposited in the Protein Data Bank (40) with codes 2FUQ and 2FUT, respectively.
RESULTS AND DISCUSSION

Overall Monomer Architecture—The HepII crystal structure revealed a homodimer in the asymmetric unit. Each monomer has an α + β architecture consisting of three domains with an overall S-like shape with dimensions of 95 × 55 × 50 Å (Fig. 1A). The N-terminal domain (residues 26–356) contains 14 α-helices arranged in a double-layered (α/α)₆ toroid. The central domain (residues 357–676) contains 16 β-strands arranged in a two-layered stack of β-sheets. Several of the loops connecting the strands of this domain are long, contain short secondary structure elements, and extend away from the β-sheet. The C-terminal domain (residues 677–772) contains nine β-strands packed together in a manner resembling a β-barrel.

The N-terminal domain begins with two short β-strands in an antiparallel arrangement and continues with 13 α-helices forming a double-layered (α/α)₆ toroid (Fig. 1B). Helices a4–a13 (Fig. 1A) are arranged in six α-helical pairs into a toroid and are inclined by ~30–45° to the toroid axis. Five of them are hairpins formed by helices consecutive in sequence (α4–5, α6–7, α8–9, α10–11, and α12–13), whereas helices α2 and α14, distant in the linear sequence, form the sixth pair. The inner layer of the toroid is formed by helices α4, α6, α8, α10, α12, and α14, with their N termini at the top of the toroid, proximal to the central domain. The outer layer consists of helices α5, α7, α9, α11, α13, and α2, all oriented in the opposite direction to the inner helices.

The bottom of the toroid, opposite the central domain (Fig. 1A), is formed by short loops ranging from 3 to 9 residues connecting the helices within each hairpin. The first 60 N-terminal residues cover this face of the toroid, with Val-32 to Ile-43 forming a β-hairpin and Leu-51 to Tyr-52 protruding deep toward the center of the domain and contacting the inner helices α6, α8, α10, and α14 of the toroid. Loops connecting the consecutive hairpins are located at the top of the toroid, proximal to the C-terminal domain. They are substantially longer than the hairpin loops and extend away from the toroid. These loops create a deep canyon along the top of the toroid with two high rims at opposite sides (Fig. 1A). The long loop Arg-296 to Tyr-318, connecting the fourth and fifth helical hairpins, together with the side chains belonging to the N-terminal part of helix α10 forms the bottom of the canyon. This loop and the exterior of helices α9 and α11 provide the interface for packing against the C-terminal domain. Residues Gly-359 to Pro-369 comprise a surface-exposed loop connecting the N and central domains.

The central domain (residues Leu-370 to Asn-676) is composed of 20 antiparallel β-strands arranged in two β-sheets, a short distorted α-helix, and two short β-hairpins (Fig. 1C). The sheets are roughly parallel to each other, forming a two-layered β-sandwich (Fig. 1A). Both sheets are extensive, each composed of eight β-strands. These sheets are twisted, with a ~60° angle between the first and last strands. The first β-sheet starts with four long β-strands (β1–β4) that form the surface contacting the N-terminal domain. The fifth strand, β3, is composed of two segments (β5a and β5b, respectively, Fig. 1C) interrupted by a long Ω loop formed by Ile-423 to Asn-452. Two other long loops extend from this face of the first β-sheet extending the second rim of the canyon described above. These long loops contain the short secondary structure elements mentioned above. The tips of these loops (Leu-404 to His-406, Tyr-429 to Asn-437, Gly-467 to Ser-471, and Ile-491 to Ala-492) form a wall that extends the canyon located at the top of the N-terminal domain and contribute to the product binding site. The second β-sheet is composed of eight long β-strands, five of them over 12 residues long. There are four crossovers between the two β-sheets, all of them on the same side of the sheets (Fig. 1C).

The C-terminal domain, composed of residues His-682 to Arg-772, is connected to the central domain by a short linker. This domain is composed of two β-sheets with three strand crossovers between them (Fig. 1D). These sheets are smaller than those of the central domain and are composed of shorter strands. The first β-sheet is composed of five strands, whereas the second has four β-strands. This domain packs against the central domain, together forming a four-layered β-sheet stack.

Zinc Binding Site—The initial SAD electron density map showed a strong peak located near the interface of the N- and C-terminal domains. Asp-425, His-408, His-451, and three water molecules surround this peak with nearly perfect octahedral coordination by the nitrogen and oxygen atoms with distances of 2.1–2.4 Å (Fig. 2). Based on the type of coordinating atoms, distances, and coordination geometry, a Zn²⁺ atom was modeled into the electron density. The refined B-factor of this atom is similar to the B-factors of surrounding residues. Other plausible metal ions tested during the refinement resulted in unreasonable B-factors. The residues Asp-425 and His-451 coordinating the Zn²⁺ ion are located at the ends of the Ile-424 to His-451 Ω loop extending from the middle of strand β5. The zinc ion assists in holding these ends together. His-408 comes from the neighboring loop connecting strands β3 and β4. Both loops participate in interactions with the N-terminal domain and in particular with the tip of the Gln-301 to Arg-312 loop. Two of the water molecules coordinating the Zn²⁺ ion form hydrogen bonds to Gly-306 from this loop and participate in a hydrogen bond network bridging the two domains. The Zn²⁺ clearly plays a structural role and is not directly involved in catalysis, being located >15 Å from the substrate-binding site.

Glycosylation of Native HepII—P. heparius has the enzymatic machinery for the glycosylation of proteins that are directed to the periplasmic space (41). Of the five GAG lyases produced by this bacterium (heparinases I–III, chondroitinase AC, and chondroitinase ABC), all except for heparinase III are glycoproteins. The sequence of a branched heptasaccharide, attached to Ser-39, in heparinase I has been established using a combination of enzymatic digestion, NMR, and mass spectrometry.² This sequence is galactose-β(1–4)[galactose-α(1–3)[(2-O-Me)Fucose-β(1–4)xylose-β(1–4)glucuronic acid-α(1–2)]rhamnose-α(1–4)]mannose-α(1–O)Ser. The same oligosaccharide was identified previously in the structures of chondroitinase AC (39) and chondroitinase B (42). The structure of native HepII expressed and purified from P. heparius showed electron density extending from the side chain of Thr-134, indicating that this is a site of O-glycosylation. Four sugars linked to this threonine side chain, Man-(Rha)-GlcUA-Xyl, were fitted to the electron density. Weak density indicating the presence of two more sugars was visible but was too weak to reliably model. The branched rhamnose sugar

² B. Eggimann, personal communication.
fills a depression in the protein surface formed between the loop Asp-83 to Gly-103 and helix α6 and forms direct as well as water-mediated hydrogen bonds to the protein. The remaining sugars, in the main oligosaccharide branch, extend away from the surface of the protein.

**Dimer Formation**—Gel filtration and dynamic light scattering studies both indicate that HepII forms homodimers in solution. Consistent with the dimerization in solution, both HepII crystal forms showed the presence of homodimers (Fig. 1E). The dimers are formed from the two independent molecules in the asymmetric unit and are related by 2-fold noncrystallographic symmetry. The association of molecules into dimers is the same in all crystal forms; the different dimers superimpose with a root mean square deviation of 0.4 Å for all Ca atoms. The dimer has an elongated shape with an approximate size of 130 × 85 × 50 Å.

The surface area buried upon dimer formation calculated using the method of Lee and Richards (43) with a 1.4-Å probe radius is ~1300 Å² per molecule, corresponding to ~5% of the total surface area of each monomer. The dimer is formed by the outside-facing β-sheet of the C-terminal domain packing against the concave surface made of the helices α9, α11, and α13 of the N-terminal domain and the edge β-strands of the two β-sheets of the central domain of the other monomer (Fig. 1E). The intermolecular interactions include van der Waals contacts and hydrogen bonds, many of them bridged by over 70 water molecules and two phosphate ions within the interface. The dimer interface is located opposite to the substrate-binding canyon, suggesting that the active sites of each HepII monomer could function independently.

**Substrate Binding Site**—The structure of HepII crystallized in the presence of heparin included electron density that we have identified as a disaccharide, corresponding to the elimination degradation product of heparin, with an unsaturated uronic acid at the nonreducing end (Fig. 3A). The disaccharide product, ΔUApt2S(1–4)GlcNS6S, binds within a deep, elongated canyon that spans ~30–35 Å in length. This canyon is formed at the top of the N-terminal domain and is extended by loops from the central domain (Fig. 3B). It is clearly divided into two parts separated by a ridge: 1) the narrower less deep end where the plus sites are located and 2) the wider and deeper part in which the oligosaccharide on the nonreducing side of the cleaved bond resides. The disaccharide occupies the narrow section formed at the interface between the N-terminal and central domains, with its nonreducing end (ΔUApt2S) pointing toward the center of the canyon (Fig. 3B). This section is ~15 Å long, ~11 Å deep, and 7–8 Å wide.

Residues located in loops belonging to the N-terminal domain (in particularly loops connecting helices α7–α8, α9–α10, and α11–α12) form one rim of the canyon. The opposite rim of the canyon is composed of residues belonging to the loops of the C-terminal domain (the Ω loop connecting strand β5a to β5b, loops connecting strands β3b to β6 and β8 to β9). Elaborate hydrogen-bonding networks maintain the position and correct orientation of the side chains, creating the binding site (Fig. 3, C and D). One such network involves the side chains and some main chain atoms of Tyr-436, Asn-405, Arg-261, Glu-205, and Arg-148. The last three residues form salt bridges and make contacts to the carboxylic group of the uronic acid substrate. Another hydrogen-bonded network includes the side chains of His-406, Asp-307, Lys-446, and Tyr-468.

The disaccharide sugar rings are approximately parallel to the walls of the canyon. The carboxylic group of the uronic acid and the N-sulfate of the glucosamine point toward the bottom of the cleft (Fig. 3A), whereas the 6-O and 2-O sulfate groups of the heparin disaccharide ΔUApt2S(1–4)GlcNS6S point upward and are exposed to the solvent. The disaccharide forms intimate contacts with the protein through hydrogen bonds, stacking, and van der Waals interactions. All hydroxyl groups of the disaccharide are engaged in hydrogen bonds to protein side chains, one bridged by a water molecule (Fig. 3C, Table 2). The sugar ring of glucosamine is sandwiched by two aromatic rings, Tyr-436 on one side and His-202 on the other side. Such stacking interactions are typical for carbohydrate-protein interactions in general (44–46) and have been observed in other GAG lyases (47). The carboxylic group of the uronic acid is firmly held by interactions with four side chains: His-406, Arg-261, Glu-205, and Arg-148 (Fig. 3, A and C). Apart from His-406, the atoms hydrogen-bonded to the carboxylic group are nearly coplanar. The side chain of His-406 forms a second hydrogen bond to the carboxylic groups of Asp-307 and therefore has to be protonated. Furthermore, the very close distance of 2.4 Å between the carboxylic oxygen O6a of the uronic acid and the OE2 atom of Glu-205 clearly indicates that one of these two acidic groups is protonated. Based on the short distance between these two oxygens, it is plausible to suggest that this proton participates in a low barrier hydrogen bond (48).

As expected from the ability of the enzyme to cleave sulfated as well as nonsulfated polymers, the sulfate groups contribute little to substrate binding due to their limited contacts with the protein. Only three hydrogen bonds are formed, a direct hydrogen bond between 6-S and the backbone NH of Tyr-436 and two hydrogen bonds mediated through water molecules (Fig. 3B, Table 2). Nevertheless, even this small binding contribution by the sulfate groups may lead to the observed higher affinity of HepII toward heparin substrates (16).

Based on the proximity of the side chain of Tyr-257 to the unsaturated uronic acid (OH-Tyr-257-C-5UApt2S distance 3.8 Å) and its superposition on the catalytic tyrosine of chondroitin lyase and alginate lyase (see below), we propose that this residue plays a key catalytic role. Another potential candidate for a catalytic residue is His-202, which is on the opposite side of the uronic acid with the NE2His-202-C-5UApt2S distance of 4.2 Å.

The disaccharide product occupies the +1 and +2 subsites. This differentiates HepII from other lyases, where the disaccharide product binds more tightly to the minus subsites, as observed in the crystal structures of enzyme-product complexes (42, 49).

Previous investigations showed that HepII cleaves long oligosaccharides, preferably near their reducing ends, releasing di- or tetrasaccharides (21). This is consistent with the HepII structure presented here, which shows that the plus side (narrow end) of the canyon can accommodate most likely 3–4 subsites, whereas the minus half-site in the wider and longer part of the canyon could accommodate more subsites.

**Structural Similarities**—Although there is no detectable sequence similarity between HepII and other lyases, its structure shows clearly recognizable similarity to polysaccharide lyases from two unrelated sequence families, PL5 and PL8.

The PL5 family contains predominantly alginate lyases, ~370-amino acid-long single domain enzymes, although some of them have an additional ~250-amino acid-long C-terminal domain of presently unknown three-dimensional structure (CAZy; available on the World Wide Web at afmb.cnrs-mrs.fr/CAZY/PL.html). They have similar topology to the N-terminal domain of HepII and are represented by *Sphingomonas* sp. alginate lyase A1-III (Protein Data Bank codes 1QAZ and 1HV6 (50)). Like HepII, this alginate lyase has a toroidal shape made of five α-helical hairpins and a sixth pair formed by joining the N- and C-terminal helices. Its structure was also determined in complex with a trisaccharide reaction product located in subsites −1, −2, and −3. In the superposition of these two enzyme structures, the trisaccharide present in A1-III forms a continuation of the disaccharide located in HepII, supporting the notion that this disaccharide occupies the +1 and +2 subsites. Furthermore, the catalytic Tyr-246 of alginate lyase occupies the same position as...
FIGURE 3. Substrate binding site. A, disaccharide product ΔUAp2S(1–4)GlcNS6S with electron density ("omit map" calculated with phases derived from the model without the disaccharide) contoured at the 3σ level. B, surface representation of the binding site with a disaccharide product shown in a ball-and-stick representation. The N-terminal domain is shown in magenta, and the central domain is orange. The product occupies the plus sites, and the minus sites are empty. C, ball-and-stick representation of the disaccharide product bound to HepII. The disaccharide is shown in thicker lines, its carbon atoms are green, the surrounding HepII residues are shown in thin lines, and their carbon atoms are gray. The hydrogen bonds are shown by dashed lines and are colored yellow when between the disaccharide and protein residues and cyan between the protein residues. The red spheres represent water molecules participating in the hydrogen bonding network. D, schematic representation of interactions between the disaccharide product and HepII. The residues shown in gray approach the sugars from below. Three parallel lines indicate stacking of a side chain with the sugar ring. Substituents in positions 1, 2, and 3 of the uronic acid are in axial orientations in our structure.
Tyr-257 of HepII. There are differences in the loops surrounding the substrate-binding cleft, most likely related to the fact that alginate lyase does not possess another domain to aid its catalytic activity.

Topological similarity to lyases from family PL8 extends not only to the N-terminal domain but also to the other domains. This family includes chondroitinases AC, chondroitinase ABC, hyaluronate lyases, and xanthan lyases (CAZY; available on the World Wide Web at afmb.cnrs-mrs.fr/CAZY/PL.html). These enzymes are ~700–1000 amino acids long and contain several domains (39, 51). Like HepII, they have an N-terminal toroidal α-helical domain, and their superposition on the N-terminal domain of HepII shows that the toroid architecture (positions and orientations of helices) is well preserved, especially the five inner helices. The loops connecting the hairpins differ in length and conformation, but the common features are long canyons along the top of their N-terminal domains where the substrate-binding site is located. The shapes of these canyons are influenced by the loop conformations and differ between the aligned protein structures. The C-terminal helix α14 in HepII, which together with helix α2 forms the sixth hairpin, has its counterpart in PL8 family lyases displaced by ~10 Å and oriented nearly perpendicularly to the toroid axis, leaving the toroid partially open (54).

This structural alignment also resulted in a superposition of the HepII reaction product onto the +1 and +2 sugar-binding subsites of chondroitinases AC (47, 54). Moreover, this superposition also juxtaposes the catalytically important tyrosine side chains of chondroitinase AC (Tyr-234 in P. heparinum and Tyr-239 in Arthrobacter aurescens) and hyaluronan lyase (Tyr-408 in Streptococcus pneumoniae) with Tyr-257 of HepII and places His-230 in the proximity of His-406 of HepII.

The central domain and C-terminal domain of HepII form together a four-layered β-sheet stack, and its topology is similar to the C-terminal domain of PL8 family lyases. In the description of chondroitinase AC and hyaluronan lyase topology, the C-terminal half of the protein was described as a single domain (39, 51). Comparison with HepII indicates that a two-domain description of this region, like that adopted for HepII, better represents the actual topology. Superposition of the central and C-terminal domains of HepII onto the corresponding domain(s) of the other two proteins shows that their β-sheets overlay very well with a root mean square deviation in the range of 1.5–1.7 Å. However, the relative orientation of these two domains in HepII is different from that in chondroitinase AC and hyaluronan lyase (Fig. 4), justifying their assignment as separate domains.

The overall structure of HepII differs from that of other family PL8 enzymes not only in the relative orientation of the central and C-terminal domains but also in the relative orientation of the N-terminal and central domains (Fig. 4). This change in orientation of the central domain in HepII relative to that in other PL8 enzymes brings it more into contact with the substrate. Another difference between HepII and family PL8 proteins is their oligomeric state; whereas HepII form dimers, other PL8 enzymes exist in solution as monomers.

**Mutagenesis and Chemical Modification Studies**—Previous structure-function studies of *P. heparinus* HepII have been carried out through the complementary use of chemical modification and site-directed mutagenesis (21, 24, 25). In particular, these studies focused on the potential catalytic role of cysteine and histidine residues. The results from these mapping studies indicated that of the three cysteines, present in HepII, Cys-348 was uniquely susceptible to chemical modification by sulphydryl-reactive reagents. Mutation of this cysteine (C348A) resulted in a selective loss of activity toward heparin but not toward heparan-sulfate (24). Indeed, the HepII structure indicates that Cys-348 is the only cysteine with a surface-accessible sulphydryl group. It is located in the substrate-binding canyon at either the −2 or −3 subsite and could interact with the substrate. Indeed, preincubation of HepII with either heparin or heparan sulfate protected Cys-348 against this modification. The effect of the C348A mutations only on heparin substrate suggests differences in interactions at the minus subsites of an oligosaccharide containing iduronic acid versus glucuronic acid.

Using a similar chemical modification and site-directed mutagenesis approach, the influence of His-238, His-406, His-408, His-451, and His-579 on catalysis was investigated (25). Only His-238, His-451, and His-579 were accessible to chemical-modifying reagents. The results of these experiments are somewhat difficult to reconcile with the structure of HepII, since these three histidine side chains are not readily surface-accessible but rather buried within the protein. Modification could result from either flexibility of the molecule in solution, allowing access of diethylpyrocarbonate or partial unfolding of HepII in the presence of this molecule. On the other hand, the effect on activity of mutating these histidines to alanines agrees well with the structure reported here. His-238 is located within a bent helix, α9, where the disruption of the hydrogen bonding pattern occurs. This side chain provides support to the loop Glu-188 to Ala-203, which forms part of the substrate-binding site.
specifically making a hydrogen bond to the backbone carboxyl of Ala-203. Removing the His-238 side chain most likely affects the configuration of this loop and compromises substrate binding. Mutating His-408 and His-451 to alanines abolishes the activity of the enzyme toward both substrates. Since these two side chains are involved in coordination of the Zn\(^{2+}\) ion, their mutation would destroy the zinc binding site and affect local structure of the Ω loop, which contains residues interacting with the substrate (Tyr-429, Tyr-436, and Asn-437). Additionally, the neighboring His-406 and Asn-405 are part of the substrate-binding site and form hydrogen bonds with sugars in the +1 and +2 subsites. Finally, His-579, located at the end of the sixth strand of the first β-sheet in the central domain, is distant from the active site but close to the Zn\(^{2+}\) binding site and forms a hydrogen bond to Ile-449. Mutation of His-579 most likely affects the position of His-451 and leads to disruption of the Zn\(^{2+}\) binding site, which in turn alters the Ω loop conformation. Based on these data, the role of Zn\(^{2+}\) appears to be preservation of the structural integrity of one wall of the substrate binding site, whereas the rest of the residues pointed in the studies mentioned are either in direct contact with the substrate or have an effect in positioning important residues for substrate binding.

Putative Catalytic Mechanism—HepII has the ability to cleave the oxygen-aglycone between the uronic acid and hexosamine irrespective of the configuration at the C-5 atom (iduronic or glucuronic acid). It is in this respect similar to chondroitinase ABC but differs from the stereospecific hyaluronan lyases, chondroitinase AC, and chondroitinase B. The HepII-disaccharide reaction product complex provides the first insight into the active site and allows formulation of a possible mechanism. The β-elimination reaction mechanism, as proposed by Gacesa (14), requires neutralization of the negative charge of the carboxyl group of uronic acid in order to reduce the pK\(_a\) of the C-5 hydrogen. In other polysaccharide lyases, this is accomplished either by a Ca\(^{2+}\) ion ligand interacting with this group (chondroitinase B (55), lyase (56), pectin lyase (57)) or by the formation of a hydrogen bond between the asparagine oxygen atom and the acidic group of the uronic acid that requires its protonation, thus neutralizing the negative charge (chondroitinase AC (54)). HepII appears to utilize a strategy similar to that of chondroitinase AC. The carboxylic group of uronic acid makes a short contact (2.4 Å) with the side chain of Glu-205, which is flanked by Arg-261 and Arg-148 (Fig. 3, A and C). Such a short contact indicates formation of a hydrogen bond, requiring protonation of one of the acidic groups, and is characteristic of a low energy barrier hydrogen bond, where the proton is shared by the two oxygen atoms (48). The side chain of Tyr-257 is positioned in a manner similar to that of the catalytic tyrosine in chondroitin AC lyases, hyaluronate lyases, and alginate lyases and may function as a general base abstracting the C-5 proton. In the context of the present structure, the location of Tyr-257 on one side of the uronic acid ring is consistent with proton abstraction from the glucuronic acid. For abstraction of the proton from the C-5 of iduronic acid, this ring would have to be bound to the enzyme in an orientation that exposes the opposite side of the ring to Tyr-257. Although this may be possible, we cannot assert this from our present structure. However, the present structure indicates another possibility for proton abstraction from iduronic acid. The side chain of His-406 is located on the opposite side of the uronic acid ring to Tyr-257, and its NE2 atom is ~4 Å away from C-5. Therefore, this histidine could serve as a general base in case of iduronic acid containing substrate, without the need for a different binding mode. The previously shown preference of HepII for the cleavage of GlcA over IdoA (21) is in agreement with the structural view.

The best candidate for the role of general acid is Tyr-257. This residue is appropriately positioned to donate a proton to the glycosidic oxygen bridging hexosamine and uronic acid, concomitant with breaking the C-4–O-4 bond and formation of C-4–C-5 double bond.

Thus, based on the structure of the HepII with a disaccharide product, we propose that the elimination mechanism employed by HepII involves the following steps: 1) neutralization of the negative charge of the carboxylic group of the uronic acid by formation of a strong hydrogen bond with Glu-205 that reduces the pK\(_a\) of hydrogen bound to C-5; 2) proton abstraction by either Tyr-257 (GlcA) or His-202 (IdoA) acting as a general base; and 3) proton donation by Tyr-257 acting as a general acid to restore the OH functional group of the hexosamine product concomitant with breaking the C-4–O-4 bond and formation of the C-4–C-5 double bond.

We have mutated Tyr-257 to either Phe or Ala. The preliminary activity data show that both mutants are kinetically inactive relative to the wild type enzyme, but Y257F shows some minimal but discernible activity in an exhaustive digestion assay. Further study of these and other mutants to delineate the catalytic mechanism is in progress.

Conclusions—We describe here the structure of HepII, the first HSGAG-degrading enzyme, and its complex with a bound disaccharide degradation product. The structure shows that HepII has overall architecture similar to that of the enzymes from the PL8 lyase family but with a somewhat different relative disposition of domains, which leads to a significantly larger participation of the β-sheet domain in substrate binding and possibly catalysis. The shape of the substrate-binding site, located in a deep and long canyon formed at the interface between the N-terminal and middle domain, suggests that the protein binds tightly at least 4–6 sugars of the GAG chain. Based on the structure with a bound unsaturated disaccharide reaction product, we were able to identify residues forming the putative active site. Whereas the present structure shows a potential role for Tyr-257 in catalysis, the mechanism by which this enzyme is able to abstract the C-5 proton from either side of the uronic acid ring remains to be elucidated.
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