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Catalytic Mechanism of Heparinase II Investigated by Site-directed Mutagenesis and the Crystal Structure with Its Substrate

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Heparinase II (HepII) is an 85-kDa dimeric enzyme that depolymerizes both heparin and heparan sulfate glycosaminoglycans through a β-elimination mechanism. Recently, we determined the crystal structure of HepII from Pedobacter heparinus (previously known as Flavobacterium heparinum) in complex with a heparan disaccharide product, and identified the location of its active site. Here we present the structure of HepII complexed with a heparan sulfate disaccharide product, proving that the same binding/active site is responsible for the degradation of both uronic acid epimers containing substrates. The key enzymatic step involves removal of a proton from the C5 carbon (a chiral center) of the uronic acid, posing a topological challenge to abstract the proton from either side of the ring in a single active site. We have identified three potential active site residues equidistant from C5 and located on both sides of the uronate product and determined their role in catalysis using a set of defined tetrasaccharide substrates. HepII H202A/Y257A mutant lost activity for both substrates and we determined its crystal structure complexed with a heparan sulfate-derived tetrasaccharide. Based on kinetic characterization of various mutants and the structure of the enzyme-substrate complex we propose residues participating in catalysis and their specific roles.

Heparin and heparan sulfate (HS)3 glycosaminoglycans (GAGs) are negatively charged, linear polysaccharides composed of repeating disaccharide units of uronic acid and glucosamine residues (GlcN, 2-amino-2-deoxy-α-D-glucopyranosanone) (1). Heparin typically contains ~90% iduronic acid (IdoA, α-L-idopyranosyluronic acid) and 10% glucuronic acid (GlcA, β-D glucopyranosyluronic acid), with a high content of 2-O-sulfono groups on the IdoA residue. The glucosamine residue in heparin is predominantly substituted with N-sulfo groups (GlcNS, where S is sulfo) and 6-O-sulfo groups with a small number of N-acetyl groups and much less frequently with 3-O-sulfo groups. In contrast, HS is somewhat more diverse in its primary structure and characterized by a higher percentage of the GlcA epimer, N-acetyl-substituted GlcN (GlcNAc) and a lower percentage of 2-O-sulfono, 6-sulfono, and N-sulfo groups. The modifications in HS are not uniform; rather, they are concentrated within specific regions of the polysaccharide, giving rise to a short, sulfo group containing sequence motifs responsible for the interactions between HS and a diverse repertoire of proteins leading to its multiple biological roles. These complex polysaccharides provide docking sites for numerous protein ligands involved in diverse biological processes, ranging from cancer and angiogenesis, anticoagulation, inflammatory processes, viral and microbial pathogenesis to multiple aspects of development (2–9). Moreover, HS-GAGs are abundant at the cell surface as part of the proteoglycan cell surface receptors (3, 4).

Specialized microorganisms express GAG-degrading lyases serving nutritional purposes of both themselves and their vertebrate hosts. The lyases depolymerize GAGs through a β-elimination mechanism characterized by the release of an unsaturated product (10). The efficiency of the elimination relies on the stereospecificity of the active site amino acids participating in the various mechanistic steps of elimination, namely charge neutralization of the uronic acid carboxyl group, proton abstraction by a general base, and protonation of the leaving group by a general acid. Heparinase II (heparin lyase II; heparitinase II, HepII; EC number 4.2.2.7) from Pedobacter heparinus (previously known as Flavobacterium heparinum) with a molecular mass of 84.5 kDa and a pI of 8.9, is unique in its ability to cleave both heparin and HS-like regions of GAGs (Fig. 1) regardless of their sulfation patterns (11). The mature protein consists of 747 residues starting with pyroglutamate 26 and ending in Arg772, with a molecular mass of 84,545 Da (12) and is...
the prototype of a new polysaccharide lyases family PL21 (CAZy (37)).

HepII acts in an endolytic manner (13) and displays broad selectivity, catalyzing the cleavage of linkages adjacent not only to IdoA and GlcA but also to the rare \( \alpha-L-\)galacturonic acid residues (14, 15). At the glucosamine positions HepII will degrade either GlcNAc or GlcNS and even the rare GlcN, provided that uronic acid contains a 2-O-sulfo group. Although HepII has greater affinity for heparin, its turnover rate for HS is higher (16). Moreover, the enzyme displays preference toward degradation of glycosidic bonds containing GlcA over ones containing IdoA. HepII shows greater catalytic efficiency for longer rather than shorter oligosaccharide substrates (13). Previously, we have solved the structure of native HepII (overexpressed in its original host \( P. \) heparinus; PDB accession code 2FUQ (12, 17, 18) and the structure of rHepII (overexpressed in \( E. \) coli) in complex with a disaccharide product (PDB accession code 2FUT (17)).

The crystal structure combined with solution studies showed that the biological unit of the enzyme is a dimer with two active sites located at opposite sides of the dimer and separated by \( \sim 80 \) Å (17). Each monomer of HepII has an \( \alpha+\beta \) lyase-fold similar to that of the enzymes from the PL8 family (CAZy).

Marked differences in the relative disposition of the structural subdomains in HepII, as compared with the other PL8 family proteins with known structures, lead to a significantly larger participation of the central \( \beta \)-sheet domain in substrate binding.

The HepII-disaccharide complex provided the first insight into the active site and allowed us to speculate on possible roles of several amino acids in performing the \( \beta \)-elimination. Based on the structure, three residues, Tyr\(^{257}\), His\(^{202}\), and His\(^{406}\), were proposed to form the putative active site. Here we investigate further the role of these residues in the catalytic mechanism of HepII employing site-directed mutagenesis, enzymatic characterization of the mutants, crystallography, and molecular simulations.

**EXPERIMENTAL PROCEDURES**

*Preparation of the HS Tetrasaccharide Substrates*

Heparin and HS sodium salts were obtained from porcine intestinal mucosa (Celsus Laboratories, Cincinnati, OH). Recombinant \( P. \) heparinus heparinases I and III expressed in \( E. \) coli and used in depolymerization were a gift from Dr. J. Liu. HS tetrasaccharides were prepared as previously described (19). Briefly, the HS was partially digested (30% reaction completion) with heparinase III and fractionated by gel-permeation chromatography using a Bio-Gel P-10 column to obtain uniform sized oligosaccharides. The fraction containing tetrasaccharides was desalted on a Bio-Gel P-2 column (Sigma). The tetrasaccharide mixture was then concentrated and fractionated on a semi-preparative HPLC column (Shimadzu, Columbia, MD). After collection of individual peaks from the HPLC, the peaks were re-separated (Spherisorb S5 SAX 20\( \times \)250 mm semi-preparative column, Waters, Milford, MA) to obtain high purity oligosaccharides. Fifteen peaks, corresponding primarily to tetrasaccharides, were obtained in the initial semi-preparative HPLC. Chromatographic purification of these peaks were analyzed by LC-MS (Agilent 1100 LC/MSD, Agilent Technologies, Inc., Wilmington, DE) under conditions previously described (20). Generally, 5 \( \mu \)g of each sample was loaded through a 5-\( \mu \)m Agilent Zorbax SB-C18 (0.5 \( \times \)250 mm) column. Eluent A was water/acetonitrile (85:15, v/v) and eluent B was water/acetonitrile (35:65, v/v). Both eluents contained 12 m\( \mu \)m tributylamine and 38 m\( \mu \)M ammonium acetate and their pH was adjusted to 6.5 with acetic acid. A gradient of 0% B for 15 min, and 0% to 100% B over 85 min was used at a flow rate of 10 \( \mu \)l/min. The electrospray interface was set in negative ionization mode with the skimmer potential at \(-40.0 \)V, capillary exit...
previously described (19). Basically, 0.9 mg of tetrasaccharide was concentrated and then desalted by a Bio-Rad Bio-Beads Sx-2 column (1 × 50 cm) and the product was lyophilized and its structure was confirmed by LC-MS.

**Site-directed Mutagenesis**

HepII was cloned as described earlier (17). HepII mutants were constructed using the QuikChange Site-directed Mutagenesis Kit (Stratagene), according to the manufacturer’s protocol and their sequences confirmed by DNA sequencing. The mutants were expressed in *E. coli* BL21(DE3).

**Activity Analysis of Mutant Heparinase II on Polysaccharides and Structure-defined Tetrasaccharides as Substrate**

Activity studies were performed on both polysaccharide and structurally defined tetrasaccharide substrates using WT and mutated heparinase II. In the polysaccharide assays, each reaction used 2 μg/μl of heparin or HS treated with 0.5 μg/μl of either WT or mutated enzyme in 50 mM sodium phosphate buffer, pH 7.0, at 37 °C. Reactions were terminated after 20 h by adding 30 mM hydrochloric acid until the pH reached 3.0. Aliquots were taken from the mixture for the measurement of absorbance change at 232 nm. PAGE has also been performed to qualitatively analyze the degree of reaction completed in addition to photometric measurement (see supplementary data for details).

Activity assay of WT and mutated heparinase II on tetrasaccharide substrate was performed each using 0.5 μg/μl of enzyme reacting with 0.1 μg/μl of tetrasaccharide in 20 mM sodium phosphate buffer containing 0.1% bovine serum albumin, pH 7.0, at 37 °C. Control reactions were prepared using the same amount of substrate with no enzyme. The reaction was run for 12 h and was terminated by boiling the mixtures for 10 min followed by centrifugation at 10,000 × g to remove the inactivated enzymes. The supernatants containing the reaction products were separated and analyzed by LC-MS.

**HepII Expression, Purification, and Crystallization**

Native HepII—Purification of HepII from *P. heparinus* was carried out as described previously (17, 18). Briefly, *P. heparinus* cells overexpressing HepII (21) were cultivated in FH medium (21) supplemented with 10% (w/v) heparin (Dongying Hi-tech Chemical Industry Co., Dongying City, China) and 0.1 mg/ml of trimethoprim antibiotic (Sigma). The cells were harvested and disrupted using a French press. The crude 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 6.5 mg/ml 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, 5 mM dithiothreitol.

The native protein was crystallized and diffraction data were collected as described previously (17, 18). Briefly, initial crystals were obtained from 6.5 mg/ml of protein in 10 mM sodium phosphate, pH 7.5, 100 mM NaCl, 5 mM dithiothreitol and a reservoir solution containing 17% (w/v) PEG 3350, 200 mM sodium phosphate, pH 5. The initial, needle-like crystals were crushed manually and used for microseeding over the same
reservoir solution. Elongated bar-shaped crystals grew overnight at room temperature.

rHepII Mutants—Expression, purification, and crystallization of rHepII from *P. heparinus* were preformed as described earlier (17). Briefly, recombinant, histidine-tagged rHepII was expressed in BL21(DE3), grown in room temperature (20 °C) in LB medium supplemented with ampicillin. The protein was purified by immobilized metal-chelate chromatography (Qiagen) and the histidine tag was removed using thrombin (Sigma). The enzyme was further purified on a Source 15S cation exchange column (GE Healthcare). The purified protein was concentrated to ~7 mg/ml by ultrafiltration using a Centricron YM-100 concentrator (Millipore Corp.) in 25 mM HEPES, pH 6.9, 150 mM NaCl, 5 mM diithiothreitol. The protein was crystallized at room temperature using the hanging drop vapor diffusion method in 24-well Linbro plates (Hampton Research). One microliter of protein was mixed with 1 μl of reservoir solution containing 25–26% PEG 3350, 200 mM ammonium acetate or 200 mM MgCl₂ or 200 mM Mg-formate, 100 mM Tris, pH 8–8.5, set over 1 ml of reservoir solution.

**Data Collection Structure Determination and Refinement**

Native HepII crystals were soaked briefly in reservoir solution supplemented with 20% glycerol and 3 mM HS tetrasaccharide substrate and flash frozen under nitrogen stream at 100 K (Oxford Cryosystems). Diffraction data extending to 2.1-Å resolution were collected at beamline X8C (NSLS, Brookhaven National Laboratory), using a Quantum-4 CCD area detector (ADSC, San Diego, CA) and processed using HKL2000 (22). Data collection statistics are summarized in Table 1.

The structures were determined by molecular replacement using the program MolRep (23) with the PDB deposited coordinates of nHepII and rHepII as a search models (PDB codes 2FUQ and 2FUT) (17). The structures were refined using the program REFMAC5 (24). The refined structure of the nHepII-HS tetrasaccharide complex contains two protein molecules in the asymmetric unit. The model also contains 3 Zn²⁺ ions, 511 water molecules, 10 phosphate molecules, 3 glycosylation sites containing 4-membered glycans, and 3 disaccharide products bound to each monomer of HepII. The refined structure of the rHepII-HS tetrasaccharide complex contains two protein molecules in the asymmetric unit, each comprised of Ala²⁹–Arg⁷⁷² and contains 3 protein molecules in the asymmetric unit. The model also contains 3 Zn²⁺ ions, 511 water molecules, 10 phosphate molecules, 3 glycosylation sites containing 4-membered glycans, and 3 disaccharide products bound to each monomer of HepII. The refined structure of the rHepII-HS tetrasaccharide complex contains two protein molecules in the asymmetric unit, each comprised of Ala²⁹–Arg⁷⁷², 2 Zn²⁺ ions, 784 water molecules, 4 acetate molecules, and two tetrasaccharide molecules. The refinement statistics for both structures are summarized in Table 1.

**Molecular Dynamics of HS and Heparin Tetrasaccharides Bound to HepII**—The starting model for calculations was the WT HepII with superimposed tetrasaccharide 3 determined here. An IdoA-containing tetrasaccharide analog was constructed with IdoA in a 1C₄ chair or in the 2S₉ twist-boat conformation. The protonation state at physiological pH was adopted, with the exception of several His residues for which tautomeric and ionization states were decided upon visual examination of their structural contexts. The Glu²⁰⁵ residue

**TABLE 1**

| X-ray crystallographic data | Data collection | Structure Determination and Refinement | Refinement statistics | Ramachandran plot |
|-----------------------------|-----------------|----------------------------------------|-----------------------|-------------------|
| **Protein**                 | HepII HS-ΔUAp   | H202A/Y257A-tetra                      | 2P2, 2                | 8.3 (4.4)         |
| **Space group**             | P2₂, 2         |                                        | 0.066                 | 15.5 (3.1)        |
| **a, b, c (Å), β (°)**      | 200.7, 209.6, 58.9, 90 |                                        | 3.3 (1.7)             |                   |
| **Wavelength (Å)**          | 1.5418         |                                        | 82.9 (34.0)           |                   |
| **Resolution range (Å)**    | 37.6–2.35 (2.43–2.35) |                                        | 1.1000                |                   |
| **Observed/unique reflections** | 477,225/103,320(6,464) |                                        | 45.5–2.10 (2.15–2.10) |                   |
| **Average redundancy**      | 4.6 (3.8)      |                                        | 2230,689/68,316(2,115)|                   |
| **Completeness (%)**        | 98.1 (90.9)    |                                        | 782/47.7              |                   |
| **R<sub>free</sub> (%)**    | 0.134 (0.302)  |                                        | 8.3 (4.4)             |                   |
| **Average I/σ(I)**         | 8.3 (4.4)      |                                        | 11,876/50.0           |                   |
| **Number of atoms/average B-factor** | 17,931/24.1 | 104/68.7                              | 7.9 (4.4)             |                   |
| **Disaccharide/Tetrasaccharide** | 78/30.7       |                                        | 22/64.6               |                   |
| **O-linked glycan**         | 123/35.0       |                                        | 782/47.7              |                   |
| **Ligand (phosphate/acetate/Zn²⁺)** | 48/43.2      |                                        | 8.3 (4.4)             |                   |
| **Solvent**                 | 536/21.2       |                                        | 8.3 (4.4)             |                   |
| **Number of atoms/average B-factor** | 17,931/24.1 | 104/68.7                              | 7.9 (4.4)             |                   |
| **Disaccharide/Tetrasaccharide** | 78/30.7       |                                        | 22/64.6               |                   |
| **O-linked glycan**         | 123/35.0       |                                        | 782/47.7              |                   |
| **Ligand (phosphate/acetate/Zn²⁺)** | 48/43.2      |                                        | 8.3 (4.4)             |                   |
| **Solvent**                 | 536/21.2       |                                        | 8.3 (4.4)             |                   |
| **R<sub>work</sub> (%)**   | 0.229/0.267    |                                        | 0.199/0.234           |                   |
| **ramachandran plot**       | Allowed (%)     |                                        | 8.3 (4.4)             |                   |
| **Generously allowed (%)**  | 98.9           |                                        | 8.3 (4.4)             |                   |
| **Disallowed (%)**          | 0.2            |                                        | 8.3 (4.4)             |                   |
| **Root mean square deviation from ideal values** | 0.010          |                                        | 0.007                 |                   |
| **Bonds (Å)**               | 1.554          |                                        | 1.0                   |                   |
| **Angles (°)**              | 8.3 (4.4)      |                                        | 3E80                  |                   |
| **Space group**             | 2P2, 2         |                                        | 3E7J                  |                   |

*All measured reflections with I > 2σ(I).*

*Ramachandran plot*:

- Allowed (%): 98.9
- Generously allowed (%): 0.9
- Disallowed (%): 0.2

*Root mean square deviation from ideal values*:

- Bonds (Å): 0.010
- Angles (°): 1.554
- Space group: 2P2, 2
- Average redundancy: 4.6 (3.8)
- Completeness (%): 98.1 (90.0)
- R<sub>work</sub> (%): 0.134 (0.302)
- Average I/σ(I): 8.3 (4.4)

**Bound to HepII**—The starting model for calculations was the H202A/Y257A-tetra structure deposited with IdoA in a 1C₄ chair or in the 2S₉ twist-boat conformation. The protonation state at physiological pH was adopted, with the exception of several His residues for which tautomeric and ionization states were decided upon visual examination of their structural contexts. The Glu²⁰⁵ residue

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Supplemental Material can be found at:

http://www.jbc.org/content/suppl/2010/04/19/M110.101071.DC1.html
was separately treated in the neutral and ionized states. Each HepII-tetrasaccharide complex was sampled by a 5-ns constrained MD simulation in which only the substrate, select protein side chains in the binding site, and the solvent molecules were allowed to move. The AMBER10 (25, 26) suite of programs was used to perform the MD simulations and trajectory analysis. Details of the methodology are provided under supplementary data.

**Protein Data Bank Accession Codes**—The coordinates and structure factors have been deposited in the Protein Data Bank Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ, as codes 3E7J and 3E80.

**RESULTS AND DISCUSSION**

**Activity of HepII Mutants**

*Heparin and HS as Substrates*—Three amino acids, His<sup>202</sup>, Tyr<sup>257</sup>, and His<sup>406</sup>, in the vicinity of the uronic acid C5 atom, the chiral center of the reaction, were previously identified as potential catalytic residues (17). The side chains of Tyr<sup>257</sup> and His<sup>406</sup> stack parallel to each other on one side of the sugar ring (a general direction of the C5 proton in GlcA in HS substrate). The third residue, His<sup>202</sup>, approaches the C5 from the opposite side of the ring than His<sup>406</sup> (a general direction of C5 proton in IdoA in heparin substrate). The Tyr<sup>257</sup>–His<sup>406</sup> arrangement is similar to that found in the active site of other PL8 enzymes, namely ChonAC (27, 28), ChonABC (29), hyaluronan lyases (30), and alginate lyase (31). These enzymes utilize the tyrosine side chain in a deprotonated state as a general base to abstract the C5 proton, triggering the elimination (27). To investigate the roles of these residues in catalysis we constructed Y257F, Y257A, H406A, and H202A mutants. They were expressed and purified to homogeneity. The mutants showed no measurable activity in a real time assay at 37 °C that measured the initial velocity of double bond formation (monitoring the absorbance at 232 nm).

To evaluate if these mutants displayed a low level of residual activity, we mixed each single mutant with either heparin or HS for a prolonged period of time (~12 h) and analyzed the banding patterns of the oligosaccharides distribution by PAGE (supplemental Fig. S1). UV detection was used to quantify roughly the amount of oligosaccharide degradation products. Parallel experiments were done with all the mutants using WT HepII as control (Table 2). In these assays the Y257F mutant showed the highest residual activity, whereas the H406A mutant showed the lowest residual activity toward both heparin and HS substrates. The products were measurable only when a high concentration of protein and prolonged incubation period were used. H202A also displays low residual activity, in particular toward heparin.

To exclude the possibility that the diminished activity is due to effect of these mutations on substrate binding rather than catalysis we measured the binding kinetics of WT and mutant HepII to heparin using surface plasmon resonance (see supplemental data for experimental details). The obtained $K_{on}$, $k_{off}$, and $K_D$ showed that the mutations had only a small effect on binding, with $K_D$ changing by no more than a factor of 3 (Table 3).

**Defined Tetrasaccharide Substrates**—To better characterize the specificity of these mutants and their effects on catalysis we have prepared small amounts of defined tetrasaccharides as shown in Fig. 2. Pairs of epimeric tetrasaccharides were prepared containing either iduronic acid or glucuronic acid at their +1 site and differing in their sulfation pattern. The structures of tetrasaccharide 1 (0.9 mg), tetrasaccharide 2 (1.0 mg), tetrasaccharide 3 (1.1 mg), tetrasaccharide 4 (2.0 mg), and 5 (3.0 mg) were confirmed by LC-MS, <sup>1</sup>H NMR, and two-dimensional COSY NMR as follows: 1, ΔUAp(1→4)-β-d-GlcNpS(1→4)-α-l-IdoAp(1→4)-β-d-GlcNpAc; 2, ΔUAp(1→4)-β-d-GlcNpS(1→4)-α-d-GlcAp(1→4)-β-d-GlcNpAc; 3, ΔUAp(1→4)-β-d-GlcNpAc(1→4)-α-d-GlcAp(1→4)-β-d-GlcNpAc; 4, ΔUAp(1→4)-β-d-GlcNp(1→4)-α-d-GlcAp(1→4)-β-d-GlcNpAc; and 5, ΔUA2S(1→4)-β-d-GlcNpS6S(1→4)-α-l-IdoA2S(1→4)-β-d-GlcNS6S (Fig. 2).

The activity of HepII and its mutants was assessed over a prolonged incubation time. The degree of tetrasaccharide digestion using WT HepII is presented in Table 4. The first two entries in Table 4 and Fig. 3 show the results on 1 and 2, substrates that only differ in the chirality at the C5 position in their internal uronic acid residue. Tetrasaccharide 2 has an internal GlcA, the most common uronic acid unit in HS and tetrasaccharide 1 has an internal IdoA, the most common uronic acid in heparin. Y257F reached 100% digestion on tetrasaccharide 2 but only ~90% conversion on tetrasaccharide 1. H406A had trouble digesting both tetrasaccharides 1 and 2, giving an

### Table 2

|        | WT     | Y257F | H406A | H202A | Y429A | Y429F |
|--------|--------|-------|-------|-------|-------|-------|
| HP     | 3.71   | 4.28  | ND<sup>a</sup> | 0.03  | 0.12  | 0.49  |
| HS     | 4.28   | 0.05  | 0.2   | 10    | 0.35  | 0.87  |
| HP     | ND<sup>a</sup> | ND<sup>a</sup> | 0.03  | 31    | 0.5   | 62    |
| HS     | ND<sup>a</sup> | ND<sup>a</sup> | 0.2   | 10    | 0.5   | 53    |
| HP     | ND<sup>a</sup> | ND<sup>a</sup> | 1.2   | 53    | 1.2   | 53    |
| HS     | ND<sup>a</sup> | ND<sup>a</sup> | 1.2   | 53    | 1.2   | 53    |

### Table 3

|        | $K_{on}$ | $k_{off}$ | $K_D$ |
|--------|----------|-----------|-------|
| (WT)-heparin | 3.65 × 10<sup>4</sup> | 2.69 × 10<sup>-3</sup> | 7.36 × 10<sup>-8</sup> |
| (Y257F)-heparin | 4.05 × 10<sup>4</sup> | 2.54 × 10<sup>-3</sup> | 6.27 × 10<sup>-8</sup> |
| (H406A)-heparin | 5.42 × 10<sup>4</sup> | 1.16 × 10<sup>-3</sup> | 2.14 × 10<sup>-8</sup> |
| (H202A)-heparin | 3.99 × 10<sup>4</sup> | 1.83 × 10<sup>-3</sup> | 4.59 × 10<sup>-8</sup> |
| (Y429A)-heparin | 4.79 × 10<sup>4</sup> | 9.54 × 10<sup>-4</sup> | 1.39 × 10<sup>-7</sup> |
| (Y429F)-heparin | 8.72 × 10<sup>4</sup> | 1.46 × 10<sup>-3</sup> | 1.67 × 10<sup>-7</sup> |


Table 4

| Conversion percentage of WT and MT heparinase II (0.5 μg/μl) acting on structure defined tetrasaccharides (Tetra) (0.1 μg/μl) after a 12-h incubation at 37 °C |
|---------------------------------|-----------------|------------------|-----------------|-----------------|
| WT                             | Y257F           | H406A            | H202A           | Y429A           | Y429F           |
| Tetra 1                         | 100*            | 100              | 92              | 0               | 95              | 96              |
| Tetra 2                         | 100             | 93               | 92              | 100             | 95              | 94              |
| Tetra 3                         | 10              | 38               | 53              | 3               | ND              | ND              |
| Tetra 4                         | 55              | ND               | 18              | ND              | ND              | ND              |
| Tetra 5                         | 100             | ND               | 100             | 100             | 100             | 100             |

* Conversion percentage = [product peak area/product peak area + substrate peak area] \times 100 (from Fig. 3 and supplemental Fig. S2).
* ND, not determined.

Catalytic Mechanism of Heparinase II

Incomplete conversion of both. The GlcA containing tetrasaccharide 2 was completely converted to disaccharide products, whereas the IdoA containing tetrasaccharide 1 was not a substrate for H202A. The WT HepII served as a positive control, giving 100% conversion of both 1 and 2. Both polysaccharide and tetrasaccharide studies show that Y257F acts less efficiently on GlcA containing substrates than IdoA containing substrates. H202A is shown in both polysaccharide and tetrasaccharide studies to prefer to act on GlcA containing sites, indicating a role of the His\(^{202}\) in the HepII digestion of IdoA-containing substrates such as heparin. When His\(^{406}\) is mutated into Ala, cleavage of both GlcA and IdoA epimers are inhibited.

The mutants were next examined for their activities on tetrasaccharides 3, 4, and 5 (the last three entries in Table 4 and supplemental Fig. S2). Tetrasaccharide 3 is the least digested, whereas tetrasaccharide 5 showed full conversion. Structural differences between tetrasaccharides 3, 4, and 5 can be seen in the substitution of the GlcN residue at the −1 site. Tetrasaccharide 3 contains a GlcNAc, tetrasaccharide 4 contains an unsubstituted GlcN, and tetrasaccharide 5 a GlcNS. These data suggests that the activities of rHepII H202A, H406A, and WT HepII on these tetrasaccharide substrates are significantly impacted by the polarity of the C2 amino group in the GlcN residue at the −1 subsite. The highly polar GlcNS residue in tetrasaccharide 5 makes it the best substrate, whereas the low polarity GlcNAc residue in tetrasaccharide 3 makes it the poorest substrate.

We crystallized HepII(Y257F), HepII(Y257A), and HepII(H202A) in the presence of tetrasaccharide substrate 3 and solved the structures of these complexes (data not shown). The structure of each complex showed only the presence of a disaccharide degradation product bound in the active site, confirming residual activity of these mutants.

The observation that all single mutants showed some residual activity made us look for other residues with a potential role in catalysis. Only one such residue was apparent, Tyr\(^{429}\). Therefore, we constructed Y429A and Y429F mutants and assayed its properties (Table 4). Whereas this mutation affects specific activity, the effect was much less dramatic, indicating that this side chain is likely involved in substrate binding but not directly in catalysis.

Next, we prepared three double mutants, H202A/Y257A, Y257A/H406A, and H202A/H406A, to test if they still display any residual activity. Overnight incubation with excess heparin or HS substrates showed no appearance of disaccharides indicating that these double mutants are completely inactive. We have crystallized one of these mutants, H202A/Y257A, with HS tetrasaccharide 3 and determined the structure of the enzyme-substrate complex. The entire tetrasaccharide was observed in the electron density map.

Structure of HepII-HS Disaccharide Degradation Product

Previously we have determined the structure of HepII complexed with a heparin disaccharide degradation product (ΔUA-GlcNS6S-GlcA2S-GlcNS6S) and postulated that both heparin and HS bind HepII in the same site and with only one active site present in the enzyme (17). To validate this hypothesis we soaked native crystals of HepII with a representative HS tetrasaccharide 3 (ΔUA-GlcNAC-GlcA-GlcNAC), and solved the structure of this complex (Table 1). Only the density for a disaccharide degradation product (ΔUA-GlcNAC) was observed in the crystal indicating that the enzyme retained activity in the crystal. The disaccharide was bound at “+1” and “+2” subsites (Fig. 44a), in a very similar way as observed for a disaccharide product of heparin degradation in HepII co-
crystallized with heparin (17). Indeed, the superposition of the two complexes based on the Cα atoms of HepII shows that the two disaccharides overlay each other. The disaccharide was found in a deep, elongated, positively charged cleft formed between the N-terminal and the central subdomains of HepII. Both sugar rings were stacked parallel to the rims of the cleft and the N-acetyl/N-sulfo group points toward the bottom of the cleft. The binding site is clearly divided into two parts, a narrow and less deep part where the “+” subsites are located (toward the reducing end of the substrate (32)) and a wider and deeper part containing the “−” subsites, in which the sugars on the non-reducing side of the cleaved bond are bound. The products occupy +1 and +2 subsites differentiating HepII from other GAG lyases, where the disaccharide product binds tighter to the − subsites (31, 33). Elaborate hydrogen-bonding networks maintain the position and correct orientation of side chains lining the binding site. The disaccharide forms intimate contacts with the protein through hydrogen bonds, aromatic stacking, and van der Waals interactions (Fig. 4a).

Structure of HepII(H202A/Y257A)-HS Tetrasaccharide

The recombinant HepII H202A/Y257A double mutant was crystallized and the crystals were soaked with the HS representative tetrasaccharide 3, ∆UA-GlcNAC-GlcA-GlcNAC. The omit electron density map showed clearly the entire substrate (Fig. 4b). This structure allowed us to map the full binding/catalytic subsite from “−2” to +2 (PDB accession number 3E7J). No major change in the structure as compared with the WT enzyme was observed. The − subsites displayed fewer direct contacts between the protein and the oligosaccharide than the “+” sites, representing weaker and less specific binding (Fig. 4c).

The GlcNAC in +2 subsite adopts a similar conformation and keeps the same interactions with Asn405, Gly470, Asn437, and His202 as GlcNS6S (Fig. 4d). Moreover, Tyr436 side chain stacks parallel against the GlcNAC sugar ring in the +2 subsite increasing its stability and contributing to the binding. At the +1 subsite the tetrasaccharide contains a GlcA rather than a ∆UA, present in the product, which is present in a different
conformation and shows slightly different binding to the protein. This is in part due to the replacement of Tyr<sup>257</sup> by an alanine in the H202A/Y257A double mutant, which created a void that is occupied by the carboxylate group of GlcA. The GlcA moiety assumes the energetically favorable chair conformation. Indeed, modeling the side chain of Tyr<sup>257</sup> showed that the carboxylic oxygen of GlcA would be only 0.8 Å from the hydroxyl group of Tyr<sup>257</sup>. The carboxylic oxygen atoms in the GlcA of the substrate are hydrogen bonded to NE<sup>His-406</sup> and OH<sup>Tyr-429</sup> and through a water molecule to NH<sub>2</sub><sup>Arg-261</sup>, whereas the carboxylic oxygen atoms of the product bind directly to OE<sub>2</sub><sup>Glu-205</sup>, NE<sup>His-406</sup>, and NH<sub>2</sub><sup>Arg-261</sup>. As expected, the – subsites maintain fewer direct interactions with HepII. The GlcNAc in the –1 subsite binds with its acetyl group pointing toward the opening of the cleft. Only two arginine side chains, Arg<sup>96</sup> and Arg<sup>484</sup>, are involved in direct interactions with GlcNAc in –1 subsite (Fig. 4e). The NH<sub>2</sub><sup>Arg-96</sup> group is hydrogen bond to the O<sub>3</sub><sup>GlcNAc</sup> and the NH<sub>1</sub><sup>Arg-148</sup> to O<sub>5</sub><sup>GlcNAc</sup> and O<sub>6</sub><sup>GlcNAc</sup>. The interactions are completed by two water molecules linking O<sub>3</sub><sup>GlcNAc</sup> to OD<sub>2</sub><sup>asp-309</sup> and the N-acytetyl O atom to the N<sup>Tyr-430</sup> atom. The GlcA at –2 subsite forms one direct hydrogen bond between the carboxylic group of GlcA and NH<sub>2</sub><sup>Arg-96</sup> and indirect bonds via water molecules connecting O<sub>3</sub><sup>GlcA</sup> with NZ<sub>Lys-316</sub> and ND<sub>2</sub><sup>Asn-259</sup> and O<sub>2</sub><sup>GlcA</sup> with O<sup>asp-307</sup>. The bound tetrasaccharide is bent between the sugar rings bound in the – subsites and the + subsites (Fig. 4c). A similar bending of the polysaccharide backbone around the glycosidic sessile bond was shown in the enzyme-substrate complex of chondroitin lyase AC from <i>Arthrobacter aurescens</i> (27).

The structures of the representative HS tetrasaccharide (ΔUA-GlcNAc-GlcA-GlcNAc) and heparin or HS disaccharide product (ΔUA-GlcNS6S/GlcNAc) provide the basis for understanding the broad substrate specificity of this enzyme. As mentioned above, sulfation of the +2 GlcA does not affect substrate binding as extrapolated from the +1, +2 subsites occupied by the products (Fig. 4d). The structures also provide an explanation for the inability of HepII to cleave the polysaccharide when GlcNAc in the +2 subsite is sulfated at position 3. The 3-OH group points toward the bottom of the substrate binding cavity and is in proximity to Arg<sup>261</sup>, Asn<sup>405</sup>, and His<sup>406</sup>. The sulfate attached at this position would collide with these side chains preventing proper positioning of the +1 uronic acid for catalysis. The – subsites are located in the more open part of the HepII binding cleft. Only few interactions were found and substrates seem to have some degree of freedom with and do not discriminate against any specific substitutions. The observed dependence of catalytic efficiency on the polarity of the C2 amino group in the GlcN residue at the –1 subsite is likely related to subtle effects as it cannot be simply explained by the structure alone.

**Molecular Dynamics of HS and Heparin Tetrasaccharides Bound to HepII**

Comparison of all the HepII structures we have determined here and previously (17) showed that oligosaccharide binding causes only small changes in the enzyme, localized to the substrate binding site. This behavior parallels what we have observed for chondroitinase AC (27, 34) and chondroitinase B (35). Therefore we asked the question: what conformation of the tetrasaccharide is compatible with the structure of the wild type HepII in the ground state? To answer this question for the GlcA- and IdoA-containing substrates we resorted to constrained molecular dynamics (MD) simulations. During the simulations only the substrate molecule and select protein side chains lining the binding site were allowed to move in the explicit solvent. This conservative substrate modeling assured that the protein structure was prevented from opening or distortion of the active site during the simulation and that the substrate had to adapt to a relatively rigid binding site. The starting conformation of tetrasaccharide 3 (ΔUA-GlcNAc-GlcA-GlcNAc) in the WT HepII was modeled based on its structure bound to the (H202A/Y257A) HepII mutant, with GlcA in the +1 subsite. An analog of tetrasaccharide 3 with the GlcA substituted by IdoA was modeled in WT HepII with IdoA in the +1 subsite. The sugar residues in the +1 subsite were built in their respective stable conformations. In case of GlcA the C<sub>1</sub>C<sub>2</sub> chair was modeled, whereas for IdoA two conformations were investigated, C<sub>1</sub>C<sub>2</sub> chair and C<sub>3</sub>S<sub>2</sub> twist-boat. The initial enzyme-substrate binding mode and conformation of sugar units in +2, −1, and −2 subsites were taken from the crystal structure of tetrasaccharide 3 bound to the mutated HepII (supplemental data). Each of these models was subjected to 5 ns of constrained molecular dynamics simulation.

The obtained MD models for GlcA- and IdoA-containing tetrasaccharides bound to WT HepII are depicted in Fig. 5, a and b, respectively. In both cases, the modeled position of the uronic acid carboxylate group corresponds to that observed for the bound product (Fig. 5c) as determined previously (17) and in this study. Accordingly, the carboxylic group is in a different position than observed in the tetrasaccharide 3 bound to the HepII(H202A/Y257A) mutant (Fig. 4c) and does not collide with Tyr<sup>257</sup>. Other enzyme-substrate interactions in the models are largely preserved as in the crystal structure of tetrasaccharide 3 bound to the mutant enzyme, particularly in the +2 and −1 subsites.

In the modeled substrates bound to WT HepII, neutralization of the carboxylic moiety of GlcA or IdoA is predicted to be achieved primarily by three enzyme side chains: Glu<sup>205</sup>, Arg<sup>261</sup>, and His<sup>406</sup>, including a short H-bond to the Glu<sup>205</sup> carboxylate group, similarly to that determined experimentally for the bound product (Fig. 5c). Protonation of the Glu<sup>205</sup> side chain was critical to preserve this mode of uronic acid neutralization during the MD simulations consistent with that observed in all HepII crystal structures, i.e. there was a requirement for a proton sharing between the carboxylate groups of the uronic acid and of the Glu<sup>205</sup> side chain, supporting our proposition for the crucial role of Glu<sup>205</sup> in charge neutralization of the carboxylate. During the MD simulation of the tetrasaccharide substrate, the GlcA sugar ring in the +1 subsite transitioned to a boat conformation (Fig. 5a). This is a less stable conformation than the C<sub>1</sub>C<sub>2</sub> chair conformation observed for the GlcA ring in the tetrasaccharide 3 bound in a more relaxed state to the HepII(H202A/Y257A) mutant due to the space created by the Y257A mutation. Apparently, the steric hindrance in the WT
Catalytic Mechanism of Heparinase II

In the case of the IdoA residue bound in the +1 subsite, both the stable $^1C_4$ chair and $^2S_0$ twist-boat ring conformations of this sugar allowed carboxylate neutralization modes consistent with that observed in the enzyme-product complex. However, an analysis of MD trajectories indicate that only in the model of the tetrasaccharide with the IdoA residue in the twist-boat conformation is the phenolic hydroxyl of the Tyr$^{257}$ residue within H-bonding distance from the leaving group oxygen in the $-1$ subsite able to act as a general acid (2.9 Å in $^2S_0$ versus 3.4 Å in $^1C_4$). We thus propose that in a catalytically competent binding mode, the IdoA residue will be accommodated in the $+1$ subsite in a twist-boat conformation (Fig. 5, b and c).

The substrate-enzyme ground state models obtained by constrained MD simulation indicate the conformation of the tetrasaccharide substrates compatible with the reasonably rigid substrate binding site of HepII. They are informative for surveying the position of putative catalytic residues around the C5 atom of the uronic acid in the $+1$ subsite. Comparison of GlcA and IdoA in substrates with $\Delta U$ in the product shows that the $sp^3$ C5 atoms of GlcA and IdoA are located on the opposite sides of the plane defined by the substituents of the $sp^2$ C3 atom of the $\Delta U$ product (Fig. 5c). The H5 proton of GlcA points toward the Tyr$^{257}$ hydroxyl group (C5$^{GlcA}$-OH$^{Tyr-257}$ distance of 3.6 Å, Fig. 5a), whereas the H5 proton of IdoA points toward the His$^{202}$ ring (C5$^{IdoA}$-NE$^{His-202}$ distance of 3.7 Å) (Fig. 5b). Tyr$^{257}$ is within H-bonding to the O1 atom of the residue in the $-1$ subsite (O1$^{GlcNAc}$-OH$^{Tyr-257}$ distances of 2.8 and 2.9 Å for GlcA- and IdoA-containing substrates, respectively).
Proposed Catalytic Mechanism

In the context of the structures of HepII product and substrate complexes and MD simulations we propose that Glu\textsubscript{205}-His\textsubscript{406}-Arg\textsubscript{261} is responsible for charge neutralization of the uronic acid. The neutralization of the negative charge on the carboxylic group at position H11001 is achieved by formation of a low energy barrier hydrogen bond with a protonated Glu\textsubscript{205}. Glu\textsubscript{205} is assisted by His\textsubscript{406} and Arg\textsubscript{261}, which form hydrogen bonds to the second oxygen atom of the carboxylate group (Fig. 6). This charge neutralization reduces the $pK_a$ of the hydrogen bound to C5, priming it for abstraction by a basic side chain during the second step of the $\beta$-elimination. In GlcA-containing substrate (HS) we propose that Tyr\textsubscript{257} serves a dual function: in a deprotonated form it could serve as the catalytic base abstracting the C5 proton from GlcA (Fig. 6, top). In the MD model the $\text{OH}^{\text{Tyr-257}}-\text{O}^{1}\text{GlcNAc}$ distance is $\sim$2.6 Å. Following the elimination of the glycosidic bond, Tyr\textsuperscript{257} could serve as a general acid, donating a proton to the non-reducing end of the GlcNAC leaving group (OH\textsuperscript{Tyr-257}-O\textsuperscript{1}\text{GlcNAC} distance of 2.8 Å), restoring the -OH functional group prior to product release. This mechanism is similar to that proposed for chondroitinase AC (27). During degradation of IdoA-containing substrate (heparin), His\textsubscript{202}, located on the opposite side of C5 to Tyr\textsubscript{257}, is proposed to serve as a general base removing the proton from the IdoA epimer (NE\textsuperscript{His-202}-H5\textsuperscript{IdoA} = $\sim$2.6 Å) (Fig. 6, bottom). Because the intermediates from heparin and HS degradation are the same (after the formation of the planar C4-C5 double bond at the uronic acid) Tyr\textsuperscript{257} is proposed to serve as the general acid during both heparin and HS degradation.

The proposed role of His\textsubscript{406} is the neutralization of the uronic acid carboxylate, and not a general base for abstraction of the H5 proton from the GlcA residue. Mutation of His\textsubscript{202} into alanine severely impaired degradation of the IdoA-containing sulfated substrate relative to the GlcA-containing sulfated substrate. Accordingly, the structural model is consistent with a role of His\textsubscript{202} as a general base for abstraction of the H5 proton.

![Proposed mechanism of HepII-catalyzed hydrolysis of heparin and HS substrates based on structural and mutagenesis studies.](http://www.jbc.org/content/suppl/2010/04/19/M110.101071.DC1.html)
only from the IdoA residue. In the case of degradation of GlcA-containing substrates, the structural model is consistent with Tyr fulfilling roles as both general base and acid successively.

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