Biochemical Characterization of the Chondroitinase B Active Site*

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Chondroitinase B from Flavobacterium heparinum is the only known lyase that cleaves the glycosaminoglycan, dermatan sulfate (DS), as its sole substrate. A recent co-crystal structure of chondroitinase B with a disaccharide product of DS depolymerization has provided some insight into the location of the active site and suggested potential roles of some active site residues in substrate binding and catalysis. However, this co-crystal structure was not representational of the actual enzyme-substrate complex, because the disaccharide product did not have the right length or the chemical structure of the minimal substrate (tetrasaccharide) involved in catalysis. Therefore, only a limited picture of the functional role of active site residues in DS depolymerization was presented in previous structural studies. In this study, by docking a DS tetrasaccharide into the proposed active site of the enzyme, we have identified novel roles of specific active site amino acids in the catalytic function of chondroitinase B. Our conformational analysis also revealed a unique, symmetrical arrangement of active site amino acids that may impinge on the catalytic mechanism of action of chondroitinase B. The catalytic residues Lys-250, Arg-271, His-272, and Glu-333 along with the substrate binding residues Arg-363 and Arg-364 were mutated using site-directed mutagenesis, and the kinetics and product profile of each mutant were compared with recombinant chondroitinase B. Mutating Lys-250 to alanine resulted in inactivation of the enzyme, potentially attributable to the role of the residue in stabilizing the carbanion intermediate formed during enzymatic catalysis. The His-272 and Glu-333 mutants showed diminished enzymatic activity that could be indicative of a possible role for one or both residues in the abstraction of the C-5 proton from the galactosamine. In addition, the Arg-364 mutant had an altered product profile after exhaustive digestion of DS, suggesting a role for this residue in defining the substrate specificity of chondroitinase B.

Dermatan sulfate (DS) and chondroitin sulfate (CS) are related glycosaminoglycans that are composed of a disaccharide repeat unit of uronic acid α/(1→3) linked to N-acetyl-D-galactosamine (GalNAc). These disaccharide repeats are β(1→4) linked to each other to form polymers of CS or DS. Epimerization at the C-5 position of the uronic acid moiety during the biosynthesis of DS leads to a mixture of L-iduronic and D-glucuronic acid epimers (1). In addition to C-5 epimerization, C-4 sulfation of GalNAc is another hallmark modification of the DS backbone. Rare sulfation at the 2-O and 3-O positions of the uronic acid moiety has also been reported (2, 3).

CS/DS polysaccharides have been implicated in a variety of biological phenomena ranging from anticoagulation to osteoarthritis (4–6). In fact, specific sequences of highly sulfated DS from a variety of invertebrate and mammalian sources are being pursued as pharmaceutically viable treatments for specific blood coagulation disorders (7–9). Changes in the DS side chain of the small proteoglycan, decorin, have been observed in human colon cancer (10), and modification of existing glycosaminoglycan sequences by chondroitinase B and chondroitinase AC may inhibit angiogenesis and tumor metastasis (11). Overall, the role of glycosaminoglycans as specific mediators of tumorigenesis and other biological events is an emerging field that offers great potential for the development of novel therapeutics (12, 13).

Flavobacterium heparinum is a common source for glycosaminoglycan-degrading lyases, producing both the extensively characterized heparin-degrading heparinases (14–16) and the DS/CS-degrading chondroitinases (17). Chondroitinase B is the only member of the chondroitinase family that degrades DS as its sole substrate (18, 19). We have recently developed a large scale recombinant expression and purification scheme for chondroitinase AC and B as a first step toward using these enzymes as tools for the characterization of CS/DS oligosaccharides (19).

Extensive biochemical characterization of the catalytic mechanism and substrate specificities of the heparinases enabled their application as tools to sequence biologically important heparin oligosaccharides (13, 20).

Chondroitinase B, like the other glycosaminoglycan-degrading lyases from F. heparinum, is thought to cleave its DS substrate through a concerted β-elimination mechanism originally proposed by Gerlt and Gassman (21). The first step in the proposed reaction is the abstraction of the C-5 proton on the uronic acid moiety by a basic amino acid forming an enolate intermediate. The enzyme stabilizes this carbanion intermediate usually via a positively charged, hydrophilic amino acid (21, 22). The final step of the reaction mechanism involves prototropy of the anomeric oxygen by an acidic residue with coupling.

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¶ The abbreviations used are: DS, dermatan sulfate; CS, chondroitin sulfate; GalNAc, N-acetylgalactosamine; ΔUA, uronic acid moiety with a 4,5 double bond; IdoUA, iduronic acid; MALDI-MS, matrix-assisted laser desorption/ionization mass spectrometry; 4S, sulfation at the 4-O position of galactosamine; 6S, sulfation at the 6-O position of galactosamine; 2S, sulfation at the 2-O position of a uronic acid; ΔDi4S, ΔUA-GalNAc-4S.
comitant β-elimination of the uronic acid, resulting in an unsaturated Δ4,5 bond (21, 22). A recently solved co-crystal structure of chondroitinase B with a disaccharide product of DS degradation, ΔUA-GalNAC-4S (23), provided the location of the active site and suggested residues that are potentially involved in substrate binding and catalysis based on their interactions with the disaccharide product. Although this structure is a good starting point to understand the location and topology of the active site, the functional role of the specific active site residues could not be directly ascertained. To begin with, the co-crystal structure represents an enzyme-product complex, not an enzyme-substrate complex. In fact, the minimum substrate length required for catalysis is a tetrasaccharide, as opposed to the disaccharide observed in the co-crystal structure. In addition, the ΔUA-containing disaccharide in the co-crystal structure has a unique planar carboxyl group not present in a viable substrate, thereby altering the interactions of active site residues with this disaccharide.

Therefore, we sought to address these issues through conformational studies with an actual substrate. A DS tetrasaccharide structure (obtained from the co-crystal structure with chondroitinase AC) was docked into the active site of chondroitinase B (24). This conformational analysis study uncovered several significant differences in the identification of specific roles for certain amino acids and identified a symmetrical distribution of active site residues that may impinge on the mechanism of action of chondroitinase B. Based on this analysis, we chose a subset of active site residues and by selectively mutating these amino acids to alanine using site-directed mutagenesis we provided evidence for the proposed roles of the catalytic and substrate binding residues. Our study provides the first molecular basis for understanding how chondroitinase B depolymerizes DS, a critical requirement for the future use of this enzyme in the sequencing and characterization of bioactive DS oligosaccharides.

MATERIALS AND METHODS

Materials—Porcine intestinal mucosa dermanan sulfate, chondroitin 4-sulfate, and chondroitin 6-sulfate were purchased from Sigma. The disaccharide standards were from Seikagaku/Associates of Cape Cod (Falmouth, MA). Oligonucleotide primers for PCR mutagenesis were from Invitrogen. All other reagents used are from common sources or are as noted under "Materials and Methods."

Docking of Dermatan Sulfate Tetrasaccharide into Chondroitinase B Active Site—The structure of the DS tetrasaccharide was obtained from a recently solved co-crystal structure of a chondroitinase AC mutant enzyme with a DS hexasaccharide (PDB, 1HM2). Only four of the sugar units in this hexasaccharide were defined in the co-crystal structure (24). Therefore, we used the defined tetrasaccharide region, ΔUA(1→3)GalNAC(4S)β1→4IdoUAβ1→3GalNAC4S, in our docking study. The initial orientation of this DS structure relative to chondroitinase B was obtained by superimposing the non-reducing end of the tetrasaccharide onto the disaccharide in the co-crystal structure. This preliminary orientation was modified by manually manipulating the tetrasaccharide structure to optimize favorable contacts between the active site amino acids and the tetrasaccharide. All the manipulations of the structures and docking were done using the viewer and docking modules of INSIGHT II.

The manually modified docked tetrasaccharide was subjected to an energy minimization process in which the potentials of the enzyme and the oligosaccharide were set using the AMBER force field and the oligosaccharide and the disaccharide were set using the AMBER force field modified to include carbohydrates (25) with sulfate and sulfamate groups (26). The enzyme-substrate complex was subjected to 300 steps of steepest gradient minimization without including charges, keeping most of the enzyme fixed and allowing only the regions close to the substrate to move. A force constant of 5,000 kcal was applied to each of the ring torsion angles, ensuring that the ring geometries of the sugar units in the tetrasaccharide were not significantly distorted. Each of the subsequent orientations of the tetrasaccharide substrate was evaluated for steric contacts and non-bonded interactions with the active site of the enzyme. The optimal orientation with reasonably low steric hindrance was selected for further energy minimization. The refined structure was further subjected to 300 steps of conjugate gradient minimization including charges. A distance-dependent dielectric with a scaling factor of 4.0 and a 1-4 non-bonded scaling factor of 0.5 was set while using the AMBER force field as recommended by the software manufacturer.

PCR Site-directed Mutagenesis of Chondroitinase B—Lys-250, Arg-271, Glu-333, Arg-363, and Arg-364 were mutated to alanine using overlap extension PCR for 15 cycles (16). The primer sequences for each of the mutants are listed below. The H272A mutant primers have the sequences 5′-AACCTTTCGTCGGCTATCAT-3′ and 5′-ATACCAAGCATGAC-3′. The E333A mutant primers have the sequences 5′-ATGGCTTCGCCGCACTGCTTT-3′ and 5′-AAGAGCATGCGCCGAAGCC-3′. The K250A mutant primers have the sequences 5′-ATCACCCCGGCTCGAAGGA-3′ and 5′-TCTCCTGGAAACGCTTGGAT-3′. The R271A mutant primers have the sequences 5′-ATGAACTTTGGTCACTGAT-3′ and 5′-ATCACCGGT-3′. The K267A mutant primers have the sequences 5′-ATATTTTTTGGCCCTCATC-3′. The N- and C- terminal primer sequences are as previously described (19).

The PCR reaction products were separated on an agarose gel, and the bands corresponding to the expected length were excised. DNA was extracted from the gel using a gel purification kit (Qiagen, Valencia, CA), the insert was subcloned into pCR7/NT (Invitrogen), and the plasmid was prepared using a miniprep kit (Qiagen). Each of the clones was sequenced to verify the presence of the individual alanine point mutations. Each chondroitinase B mutant was excised from pCR7/NT using an NdeI and BamHI (New England Biolabs, Beverly, MA) enzyme mixture and subcloned into a pCTU5b expression vector (Novagen, Madison, WI), which had been digested previously with these same enzymes. Recombinant chondroitinase B that had been cloned in a similar fashion was also expressed and compared with each of the alanine mutants.

Protein Expression and Purification—Recombinant chondroitinase B and the site-directed mutants were expressed and purified as previously described (19). The purity of the recombinant chondroitinase B and site-directed mutants was assessed by SDS-polyacrylamide gel electrophoresis analysis using precast 12% gels, the Mini-Protein II apparatus, and the silver stain-plus kit (Bio-Rad). A relative protein concentration was calculated using the Bradford assay (Bio-Rad) with bovine serum albumin as a standard.

Kinetic Analysis—The activity of chondroitinase B and various site-directed mutants was determined by adding 10–50 μl of the sample to a 1-ml cuvette containing 1 mg/ml DS in 50 mM Tris-HCl, pH 8.0, at 30 °C. Product formation was monitored at 232 nm as a function of time (19).

The kinetic parameters, K_m and V_max, were calculated for chondroitinase B and the site-directed mutants by obtaining the initial reaction rate at a range of substrate concentrations. The kinetic parameters, K_m and V_max, were calculated using the Michaelis-Menten equation: v = V_max [S] / (K_m + [S]). The k_cat (s−1) was calculated by dividing the V_max by the concentration of enzyme in the reaction.

Dermatan Sulfate Digestion and Capillary Electrophoresis—To examine changes in the product profile of each site-directed mutant compared with recombinant chondroitinase B (20 μg), digests of 1 mg/ml DS 50 mM Tris-HCl, pH 8.0, were performed for 12–14 h at 30 °C. The digests were analyzed using capillary electrophoresis as previously described (19). Briefly, the chondroitinase B and site-directed mutant digests were diluted 2-fold and analyzed with an extended path length cell and a voltage of 30 kV applied using reverse polarity. The running buffer consisted of 50 mM Tris, 10 mM EDTA, 1 mg/ml dextran sulfate that had been brought to a pH of 2.5 using phosphoric acid, and the saccharide peaks were detected by the runs performed at the regulated voltage.

The total peak area for the recombinant chondroitinase B and mutant digest profiles was calculated by adding the areas of the ΔUA2S-GalNAC-4S, ΔUA-GalNAC-4S,6S, and ΔUA-GalNAC-4S peaks. The total peak area for the R364A mutant also included the sum of the area of the three additional oligosaccharide peaks. The ratio of the ΔUA-GalNAC-4S peak area to the total peak area was then calculated for the
recombinant chondroitinase B and each mutant for a comparison of overall enzymatic activity.

**MALDI-Mass Spectrometry**—The reaction products from the R364A digest of DS were analyzed using MALDI-MS. Samples were prepared using the basic peptide (RG)15R as previously described (27). MALDI-MS spectra were acquired on a Voyager Elite system (PerSeptive Biosystems, Framingham, MA) in the linear mode with delayed extraction and similar instrument parameters to those described previously (27).

**Circular Dichroism**—Recombinantly expressed chondroitinase B and the inactive K250A mutant were concentrated and buffer-exchanged into 50 mM sodium phosphate, pH 7.0, using a Centricon 10 filter (Millipore, Watertown, MA). CD spectra were collected on an Aviv 62DS spectropolarimeter equipped with a thermostatic temperature control and interfaced to an IBM microcomputer. Measurements were performed in a quartz cell with a 1-mm path length. Spectra were recorded at 25 °C in an average of 10 scans between 205 and 270 nm with a 1.0-nm bandwidth and a scan rate of 3 nm/min. CD band intensities are expressed as molar ellipticities, [θ], in degrees cm² dmol⁻¹.

**RESULTS AND DISCUSSION**

**Interactions between Chondroitinase B and Dermatan Sulfate Substrate**—The structure of a previously crystallized DS tetrasaccharide was docked into the chondroitinase B active site. The direction of the tetrasaccharide relative to the enzyme was the same as the ΔUA-GalNAc-4S disaccharide product (23). Although the direction of both the disaccharide product and the tetrasaccharide is the same from non-reducing end (close to the C terminus above the active site) to reducing end (close to the N terminus below the active site), the tetrasaccharide is positioned to completely occupy the active site. The side chains of the residues (single letter code and number) of the protein interacting with the tetrasaccharide are shown. Basic residues (Lys, Arg, Asn, His) are blue, acidic residues (Glu) are red, and bulky aromatic residues (Phe, Trp) are purple. The subsite nomenclature is used to define the orientation of the tetrasaccharide from −2 (non-reducing end) to +2 (reducing end) in the active site. Cleavage occurs between the −1 and +1 site.

![Docking of the dermatan sulfate substrate in the active site of chondroitinase B.](image)

Fig. 1. Docking of the dermatan sulfate substrate in the active site of chondroitinase B. A, stereoview of a Connolly surface rendering of the active site of chondroitinase B with the docked dermatan sulfate tetrasaccharide (green) and disaccharide product (orange) with orientation replicated from the co-crystal structure (23). Although the direction of both the disaccharide product and the tetrasaccharide is the same from non-reducing end (close to the C terminus above the active site) to reducing end (close to the N terminus below the active site), the tetrasaccharide is positioned to completely occupy the active site. B, stick representation of the dermatan sulfate tetrasaccharide in the active site of chondroitinase B, colored according to the atoms (green, C; blue, N; red, O; and yellow, S) (left) and the two-dimensional schematic distribution of the active site residues (right). The side chains of the residues (single letter code and number) of the protein interacting with the tetrasaccharide are shown. Basic residues (Lys, Arg, Asn, His) are blue, acidic residues (Glu) are red, and bulky aromatic residues (Phe, Trp) are purple. The subsite nomenclature is used to define the orientation of the tetrasaccharide from −2 (non-reducing end) to +2 (reducing end) in the active site. Cleavage occurs between the −1 and +1 site.
from the basic cluster of residues His-116, Arg-184, and Arg-219, previously implicated to provide a binding site for an additional 4-O-sulfate group located at the reducing end of GalNAc (24). Our docking and energy minimization resulted in repositioning of the tetrasaccharide substrate to achieve maximum contact with the active site cleft of the enzyme (Fig. 1A). In the final orientation, the tetrasaccharide completely occupied the −2, −1, +1, and +2 subsites of the active site of chondroitinase B.

Active Site Residues—Because the docked tetrasaccharide occupied all of the chondroitinase subsites, our theoretical enzyme-substrate complex provided a better picture of the interaction between the DS substrate and the active site residues compared with what was observed in the co-crystal structure (23). Glu-333, Lys-250, Arg-271, and His-272 were identified as key residues involved in catalysis based on proximity to the −1 and +1 subsites containing cleavable −GalNAC4Sβ(1→4)IdoUA linkage (Fig. 1B). This cluster of charged residues in the catalytic site suggests that there may be more than the prototypical triad of residues that are involved in the proton abstraction and donation mechanism resulting in the β-elimination cleavage. Glu-333 is positioned proximal to the O-1 of GalNAC-4S in such a way that it could potentially mediate proton abstraction via a water molecule. This interaction is consistent with the earlier observation from the co-crystal structure that implicated Glu-333 as a general base for proton abstraction based on the distance from its OE1 to the reducing end O-1 (4.4 Å) of the ΔUA-GalNAC-4S (24). The proximity of His-272 and Lys-250 to the C-5 proton (Fig. 1B) indicates that these residues are also positioned to act as a general base for proton abstraction. However, Lys-250 is the only residue in proximity to the carboxylate moiety of the IdoUA monosaccharide. This strongly supports its involvement in neutralizing the charge of the carboxylate group, which is a key step required for β-elimination (21). Arg-271 is proximal to both the ring oxygen and O-1 of the GalNAc residue and thus is positioned to protonate the leaving O-1 atom of the GalNAc after cleavage.

Because the co-crystal structure did not contain any monosaccharide units in the +1 and +2 subsites, the authors could only speculate on the roles of most of the above residues from the co-crystal structure (23). For instance, Lys-250 was suggested as a likely candidate for charge neutralization. However, its role was not definitive from the co-crystal structure because its only interaction was with the reducing end O-1 of the disaccharide via a water molecule. In addition, His-272 was described as an unlikely candidate in proton abstraction because it was not close enough to the reducing end of the disaccharide to act as a general base, although our analysis indicates that this may not be the case.

Substrate Binding Residues—Several residues involved in substrate binding were identified from our theoretical chondroitinase B-tetrasaccharide complex. These include basic residues Arg-318, -363, and -364 and pyranose ring stacking aromatic residues Phe-296 and Trp-298. Phe-296 provides a parallel stacking interaction with the IdoUA in the −2 subsite, and Trp-298 stacks perpendicularly with the IdoUA and GalNAc in subsites −2 and −1, respectively (Fig. 1B). Arg-364 is positioned to interact with both the 4-O-sulfate of the GalNAC-4S and the carboxylate group of the non-reducing end IdoUA (Fig. 1B), consistent with what was observed in the co-crystal structure (23). Because the 4-O-sulfate group of GalNAC-4S and IdoUA represents hallmark modifications of DS, the Arg-364 residue is most likely to be involved in substrate specificity of the enzyme. Arg-318 interacts with the IdoUA in the −2 site, and Arg-363 is positioned to interact with an additional GalNAC-4S moiety on the non-reducing end in what would potentially be subsite −3. Finally, Asn-213 interacts with the N-acetyl group of GalNAc in the −1 subsite (Fig. 1B).

In the product release site (subsites +1 and +2), the side chains of Arg-184 and His-116 are oriented to provide favorable ionic interactions with the GalNAC-4S residue at the reducing end of the DS tetrasaccharide (Fig. 1B). These interactions provide a more definitive meaning to the speculated role of these two basic residues in binding to the 4-O-sulfate group at the reducing end of the DS substrate. Taken together, our enzyme-substrate complex, when compared with the earlier co-crystal structure, provides a clearer framework of the various residues involved in substrate binding and product release.

Active Site Symmetry—In addition to providing further insight into the exact role of each residue in the chondroitinase B active site, our conformational study has also uncovered a chemical symmetry of amino acid side chains in this region. In fact, there appears to be an internal 2-fold symmetry of the positively charged, negatively charged, and hydrophobic residues in the active site about an axis passing through the cleavage site (−1 and +1) and perpendicular to the axis of the β-helix (Fig. 2). Specifically the proposed residues that are involved in the substrate binding site (−2 and −1), including Phe-296, Arg-318, and Arg-364, seem to have corresponding residues in the product release site (+1 and +2), including Tyr-222, Arg-184, and Arg-219 that are related by this symmetry. In addition, Glu-245 is in proximity to the catalytic site and...
appears to be related to the Glu-333 residue by the same 2-fold symmetry (Fig. 2).

Understanding the significance of the active site symmetry will provide valuable insights into the mechanism by which chondroitinase B depolymerizes its DS substrate. Based on our current observations, we can offer several plausible explanations regarding the importance of this active site symmetry. To begin with, the distance between the carbonyl oxygens of both Glu-245 and Glu-333 is about 9.5 Å, a distance comparable with the diameter of the structure of the DS substrate projected along the helical axis. Thus, if both of these negatively charged glutamic acids are involved in catalysis, their symmetrical arrangement would facilitate the translation of the substrate through the active site cleft without the need for its rotation, leading to more efficient DS depolymerization. In addition, this active site symmetry may be involved in accommodating the

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**Fig. 3.** Capillary electrophoretic analysis of the dermatan sulfate reaction products for the catalytic mutations. A, recombinant chondroitinase B (20 μg), B, H272A, C, E333A, and D, K250A were incubated with 1 mg/ml dermatan sulfate for 12 h at 30 °C. Capillary electrophoretic analysis was performed using an extended path length cell and a voltage of 30 kV applied using reverse polarity. Saccharides were injected into the capillary using hydrodynamic pressure and were detected using an ultraviolet detector set at 232 nm. The running buffer consisted of 50 mM Tris, 10 mM dextran sulfate that had been brought to a pH of 2.5 using phosphoric acid. The disulfated disaccharides, ΔUA-GalNAC-4S, ΔUA-GalNAC-4S, ΔUA-GalNAC-4S, and ΔUA-GalNAC-4S, are indicated by one asterisk and two asterisks, respectively. Inset, electropherogram of the ΔUA-GalNAC-4S (ΔDi4S) disaccharide standard.
perturbations in the DS chain caused by the conformational flexibility of iduronic acid, a common component of DS (28). The symmetry of the active site may also be involved in defining the direction in which the substrate is processed through the active site. Interestingly, the DS-derived disaccharide in the co-crystal structure that is an actual product of chondroitinase B action is in the substrate binding site, not the product release site. This observation, coupled with the active site symmetry, raises the issue that the directionality of the active site might be more complex than originally thought. In fact, the reducing end of a genuine substrate may be potentially oriented toward the C-terminal end of an enzyme (a pattern of binding common among other polysaccharide lyases, Refs. 29 and 30) and not toward the N-terminal end as seen in the co-crystal structure (23). The directionality of substrate binding within the active site of polysaccharide lyases is usually unambiguously defined by a structural feature similar to the presence of a Ca\(^{2+}\) ion at one end of the cleft, as is the case with pectate lyase C from Erwinia chrysanthemi (30). This underscores the uniqueness of the chondroitinase B active site symmetry and the need for further characterization.

**Mutagenesis and Active Site Characterization**—Having identified the key substrate binding and catalytic residues using our theoretical enzyme-substrate complex, we sought to establish their functional roles using site-directed mutagenesis. The basic residues, Lys-250, Arg-271, and His-272, were chosen based on their location in the active site of chondroitinase B. In addition, the acidic residue Glu-333 was chosen because of its possible role in proton abstraction. We also mutated two of the residues implicated in substrate binding and specificity, namely Arg-363 and Arg-364, to alanine. These site-directed mutants were cloned into pET15b and expressed alongside the recombinant chondroitinase B.

**Table II**

| Enzyme                  | \(\Delta D_4S/\text{Total peak area}\) |
|-------------------------|-----------------------------------------|
| Chondroitinase B        | 0.93                                    |
| K250A                   | n.d.*                                   |
| H272A                   | 0.94                                    |
| E333A                   | 0.93                                    |
| R363A                   | 0.93                                    |
| R364A                   | 0.39                                    |

* No peaks were observed for the K250A digest.

In addition to kinetic analysis, each of the mutant enzymes and the recombinant chondroitinase B was allowed to exhaustively digest DS to determine changes in product profile that may belie alterations in substrate specificity. These digests were diluted and analyzed using capillary electrophoresis. Complete digestion of the dermatan substrate was seen with the chondroitinase B reaction, as indicated by a major disaccharide peak (Fig. 3). This prominent disaccharide peak in all of the electropherograms was identified as \(\text{UA-GalNAC-4S} \) (referred to as \(\Delta D_4S\)) through co-migration of the known DS disaccharide standards. The two minor peaks that elute around the chondroitinase B reaction, as indicated by a major disaccharide peak (Fig. 3). This prominent disaccharide peak in all of the electropherograms was identified as \(\text{UA-GalNAC-4S} \) (referred to as \(\Delta D_4S\)) through co-migration of the known DS disaccharide standards. The two minor peaks that elute around

**FIG. 4.** Capillary electrophoretic analysis of the reaction products for the substrate binding mutations. A, R363A and B, R364A were incubated with 1 mg/ml dermatan sulfate for 12 h at 30 \(^\circ\)C and analyzed by capillary electrophoresis. The length and sulfate composition of the additional peaks in the R364A digest (B) were determined using MALDI-MS. Peak 1 is an octasaccharide (1922.4 Da) with five sulfates. Peak 2 is a hexasaccharide (1539.7 Da) with five sulfates. Peak 3 is a tetrasaccharide (999.2 Da) with three sulfates. The disulfated disaccharides, \(\text{UA2S-GalNAC-4S} \) and \(\text{UA-GalNAC-4S,6S} \), are indicated by one asterisk and two asterisks, respectively.
function without one of them, albeit at a much slower catalytic rate.

In contrast, changing Lys-250 to alanine completely ablated the activity of chondroitinase B (Table I and Fig. 3). To ensure that the mutating Lys-250 did not influence the overall stability of the protein, the CD spectrum of K250A was compared with the spectrum of recombinant chondroitinase B. Although the virtual identity of the CD profiles does not preclude the possibility that there are perturbations in the local environment surrounding Lys-250 that are not represented in the CD profile, it does suggest there are no gross conformational changes induced in chondroitinase B by mutating Lys-250 to alanine (Fig. 5). Therefore, Lys-250 is essential for the catalytic activity of chondroitinase B.

Along with the active site residues discussed above, we mutated Arg-271 to alanine. Interestingly, the R271A mutant was expressed at comparable levels to the recombinant chondroitinase B but was completely insoluble. Several attempts to denature and refold the mutant using different methods including a strong chaotropic agent (4 M guanidinium HCl) proved unsuccessful (data not shown). The insolubility of the R271A mutant could implicate this residue in the active site chemistry of chondroitinase B. Another possibility is that removing the side chain of Arg-271 somehow interferes with the hydrophobic interactions of Phe-296 and Trp-298, leading to a side chain of Arg-271 somehow interferes with the hydrophobic interactions of chondroitinase B. Another possibility is that removing the mutant could implicate this residue in the active site chemistry of chondroitinase B. The R363A mutant produced a similar profile to chondroitinase B after exhaustive digestion of DS (Fig. 4).

In contrast to the R363A results, mutating Arg-364 to alanine led to a complete loss of activity in the real time kinetic assay and an altered product profile after exhaustive digestion of DS (Table I and Fig. 4). In fact, the ratio of the ∆UA-GalNAC-4S peak area to the total peak area was only 0.39, significantly lower than the ratio for the recombinant chondroitinase B (Table II). In addition, the ∆UA-GalNAC-4S peak was not the only prominent peak in the electropherogram (Fig. 4).

To further characterize the novel peaks seen in the R364A digest of DS, the sample was analyzed using MALDI-MS. Peak 3 had a mass of 999.2 Da, which identifies it as a tetrasaccharide containing three sulfates. Peak 2 had a mass of 1539.7 Da, which identifies it as hexasaccharide containing five sulfates. Finally, peak 1 had a mass of 1922.4 Da, which classifies it as an octasaccharide, also containing five sulfates. Adding more of the R364A mutant enzyme to the sample did not result in a significant decrease of these higher order peaks, suggesting that these oligosaccharides are the end products of the reaction. As suggested by our structural analysis, Arg-364 is critical for proper substrate binding and digestion of DS by chondroitinase B.

Compositional analysis of the DS starting material revealed that the ∆UA2S-GalNAC-4S and ∆UA-GalNAC-4S,6S disaccharides are 2.3 and 4.6% of the total disaccharide content (data not shown). Interestingly, there is a shift in the percentages to 5.5 and 2.3% for the ∆UA2S-GalNAC-4S and ∆UA-GalNAC-4S,6S disaccharides, respectively, when DS is digested by the R364A mutant, suggesting that the oversulfation of the higher order oligosaccharides is at the 6-O position (data not shown). Therefore, it appears that Arg-364 is involved in the ability of chondroitinase B to recognize and cleave regions containing ∆UA-GalNAC-4S,6S in DS. This interesting insight into the specificity of chondroitinase B is currently being pursued and will prove useful in the generation of biologically important DS oligosaccharides.

Taken together, these results for the first time directly implicate Lys-250, His-272, Glu-333, and possibly Arg-271 in the catalytic degradation of DS by chondroitinase B. Because the H272A mutation shows a 6.5-fold decrease in $k_{cat}$, this residue can potentially be involved in proton abstraction (Table I). Histidine has been implicated in the enzymatic degradation of other glycosaminoglycan-degrading enzymes, including Group B streptococcal hyaluronate lyase and heparinases I, II, and III (16, 31, 32). However, because the enzyme activity is not completely ablated, another residue may also be involved in abstraction of the C-5 proton. Glu-333, another candidate for C-5 proton abstraction, showed a nearly 40-fold decrease in $k_{cat}/K_m$ when mutated to alanine (Table I). Nevertheless, because the enzyme still retains close to full activity over a 12-h period (Fig. 3), Glu-333 may not be the sole residue involved in the C-5 proton abstraction. One possibility is that Glu-333 and His-272 work in concert to lower the $pK_a$ of the C-5 proton and to abstract it. A mutant chondroitinase B in which both residues are mutated will help further elucidate the roles of these residues. Another possibility is that Glu-245, the symmetrical active site residue to Glu-333, may also play a part in proton abstraction (Fig. 1B).

Mutating Lys-250 to alanine led to a complete loss of enzymatic activity of chondroitinase B toward the DS substrate. Because the ε-NH$_2$ of the lysine ($pK_a$ of 10.5) is mostly protonated in the reaction buffer (pH 8.0), it seems unlikely that this residue would be involved in proton abstraction. Also, our conformational study points to the involvement of Lys-250 in stabilizing the charge of the carboxylate moiety. This charge stabilization is required in the proposed β-elimination mechanism to lower the $pK_a$ of the C-5 proton for base abstraction (21). Therefore, the complete loss of enzymatic activity in the K250A...
mutant is most likely due to this lack of stabilization of the carboxylate group (and the carbanion intermediate), effectively preventing abstraction of the C-5 proton.

CONCLUSIONS

Biochemical characterization of polysaccharide lyases is a challenging task because of the complex steps involved in their catalytic process. In addition, the wide range of pH optima for many of these enzymes complicates the determination of the precise role of active site residues. Several x-ray co-crystal structures of polysaccharide lyases with their respective substrates or products have been solved (23, 24, 30). These structures provide static descriptions of inert enzyme-substrate complexes that are potentially valuable for identifying active site residues. However, because the co-crystallized substrate is not a native substrate for the enzyme because it would be degraded during the crystallization, these crystal structures do not provide sufficient information for definitively establishing the role of these residues in activity. For example, even after obtaining several crystal structures of active site chondroitinase AC mutants with different substrates, three different scenarios were proposed for the specific role of active site residues in catalysis (24). In the case of the chondroitinase B, there is even less information on the functional roles of the active site residues, because it was co-crystallized with a disaccharide product that is chemically different from the DS substrate and does not have the minimum substrate length required for catalysis.

Our study provides a first step toward defining the substrate binding and catalytic functions of the active site residues in chondroitinase B. Based on the interactions with the DS tetrasaccharide and the kinetics of the alanine mutants, we have provided substantial evidence on the involvement of Lys-250, His-272, and Glu-333 in catalysis. Lys-250 is a critical residue most likely involved in stabilizing the carboxylate moiety allowing for proton abstraction. In contrast to the previous suggestion of the involvement of a single Glu-333 residue in proton abstraction, our results demonstrate that both His-272 and Glu-333 could potentially be involved in the proton abstraction process. In addition to defining the roles of the catalytic residues, we have also used a battery of biochemical studies to define the role of Arg-364 in conferring substrate specificity. Mutating Arg-364 to alanine produced an altered product profile after exhaustive digestion of DS.

Unlike the typical situation for lyases, there appears to be more than a triad of residues involved in the degradation of DS by chondroitinase B. In addition, we have observed a 2-fold symmetry in the distribution of the active site residues with similar chemical properties. This symmetry has not been observed in other polysaccharide lyases, and we are currently investigating the significance of the symmetry in the recognition and mechanistic processing of substrate.

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