Structures of *Streptococcus pneumoniae* Hyaluronate Lyase in Complex with Chondroitin and Chondroitin Sulfate Disaccharides

INSIGHTS INTO SPECIFICITY AND MECHANISM OF ACTION*

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*Streptococcus pneumoniae* hyaluronate lyase is a surface enzyme of this Gram-positive bacterium. The enzyme degrades hyaluronan and chondroitin/chondroitin sulfates by cleaving the β,4-glycosidic linkage between the glycan units of these polymeric substrates. This degradation helps spreading of this bacterial organism throughout the host tissues and facilitates the disease process caused by pneumococci. The mechanism of this degradative process is based on β-elimination, is termed proton acceptance and donation, and involves selected residues of a well defined catalytic site of the enzyme. The degradation of hyaluronan alone is thought to proceed through a processive mode of action. The structures of complexes between the enzyme and chondroitin as well as chondroitin sulfate disaccharides allowed for the first detailed insights into these interactions and the mechanism of action on chondroitins. This degradation of chondroitin/chondroitin sulfates is non-processive and is selective for the chondroitin sulfates only with certain sulfation patterns. Chondroitin sulfation at the 4-position on the nonreducing site of the linkage to be cleaved or 2-sulfation prevent degradation due to steric clashes with the enzyme. Evolutionary studies suggest that hyaluronate lyases evolved from chondroitin lyases and still retained chondroitin/chondroitin sulfate degradation abilities while being specialized in the degradation of hyaluronan. The more efficient processive degradation mechanism has come to be preferred for the unsulfated substrate hyaluronan.

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small all-β domain, which probably acts as a spacer, and then the catalytic and C-terminal domains. Although both streptococcal HLs and *P. vulgaris* CL contain N-terminal presumed carbohydrate-binding domains, there appears to be a key difference in their relationships to the rest of the molecule. In *P. vulgaris*, there is a large interface between the presumed carbohydrate binding domains and the catalytic domain (22). In streptococcal HLs, the presence of the spacer domain suggests that the presumed carbohydrate-binding domain is placed further from the catalytic domain. The lack of success in crystallizing full-length streptococcal HL is also suggestive of flexibility between carbohydrate binding and catalytic domains (i.e. a dynamic independence with possible implications for function) (7).

Functionally, the hyaluronate lyase enzyme degrades primarily hyaluronan and also certain chondroitin/chondroitin sulfates (1, 23). These polymeric glycans are widely distributed among various host tissues (24–26) and are involved in numerous physiological processes. The functional/physiological role of hyaluronan has been described in our earlier work (3, 4, 27, 28) and by others (24–26, 29). Chondroitin (Ch) and chondroitin sulfates (ChS) are built from repeats of a disaccharide composed of N-glucuronate connected through a β1,3 linkage to N-acetyl-D-galactosamine. The disaccharides are connected through β1,4-glycosidic linkages (Fig. 1). They differ from hyaluronan (polymeric β-glucuronate-β1,3-N-acetyl-D-glucosamine connected by β1,4-glycosidic linkages) only in the anomeric configuration at the C-4 carbon of the N-acetyl-D-glucosamine. The predominant chondroitins isolated from natural tissues are unsulfated chondroitin, chondroitin 4-sulfate, and chondroitin 6-sulfate. 4- and 6-sulfation of chondroitin occur at the C-4 and C-6 positions of the GalNAc (e.g. see Ref. 30). Chondroitin-2-sulfate has also been identified, but in this case the 2-sulfation occurs at the C-2 carbon of the GlcA (N-glucuronate) ring (31). The existence of ChS 3-sulfated on the GlcA residue (32) and a fucosylated ChS form (33) has been demonstrated.

Ch and ChS are abundantly distributed in nature, being found in the extracellular matrix, cell surfaces (34), and neural tissues (35) as well as in invertebrates (33, 36, 37). In addition to normal physical properties expected from being a part of the extracellular matrix, especially cartilage, chondroitins have multiple other functions such as macromolecular interactions and cytoadherence (38).

Information regarding the structures of hyaluronan, chondroitin, and chondroitin sulfate structures has been obtained from x-ray crystallography, NMR, and other biophysical and computational methods. However, whereas crystallography reveals a variety of structures (3- and 4-fold helices for short chain hyaluronan (39) and 2-fold helices for 4-sulfated chondroitin (40)), some NMR studies in D₂O support a 2-fold helical conformation for hyaluronan, chondroitin, and chondroitin sulfate in solution (41–44). The presence of such a 2-fold helical structure could explain the ability of these polymers to form higher order structures, stabilized by hydrogen bonds and hydrophobic interactions, such as through antiparallel association of chains for hyaluronan (41–44). These studies suggest that the ability of chondroitin and its sulfates to form higher order structures depends on the presence and position of sulfate group. Full sulfation at the 4-position, but not at the 6-position, impedes association (45). The bound conformations of these polymers revealed by complexes to lyases (15, 19) are also 2-fold helices, although with small scale bending and twisting. The binding of substrate in conformations close to those found in solution presumably enhances binding.

However, many other NMR studies do not support such a structure for, at least, hyaluronan in aqueous solution, whereas it may exist in Me₂SO or possibly in the solid phase at low pH (46–50). The dynamic/flexible character of hyaluronan seems to emerge from this work. Furthermore, molecular dynamic simulations have provided considerable insight into this question, suggesting that hyaluronan is highly dynamic in solution (51–53). In addition, recent studies have found no evidence for chain-chain association in concentrated hyaluronan solutions (54). More studies are clearly needed to elucidate structural properties of hyaluronan and chondroitins.

In this work, we report the results of cocrystallization of *S. pneumoniae* hyaluronate lyase with unsulfated and 2-, 4-, and 6-sulfated chondroitin disaccharides. This information is related to biochemical knowledge regarding the ability of the enzyme to cleave different chondroitins. Further modeling and structural comparisons shed light on the structure-function relationships of HL and the related enzyme CL and help to understand the adaptation of each to their respective favored substrates.

**EXPERIMENTAL PROCEDURES**

**Materials**—Chondroitin and chondroitin sulfates used in this study were purchased from Sigma. All other chemicals were purchased either from Fisher or Sigma.

**Complex Formation, Crystallization, and Diffraction Data Collection**—The wild-type, H399A, and Y408F mutant forms of *S. pneumoniae* HL were produced as previously reported (11, 55). The protein was concentrated to 5 mg/ml in 10 mM Tris–HCl buffer, pH 7.4, 150 mM EDTA, 2 mM EDTA. The unsulfated chondroitin (α-DUA-[β3GalNAc]2) and chondroitin sulfates with various sulfation patterns (chondroitin-2-sulfate (α-DUA-2S-[β3GalNAc]2), chondroitin-4-sulfate (α-DUA-4S-[β3GalNAc]4), chondroitin-6-sulfate (α-DUA-6S-[β3GalNAc]6), and chondroitin-6-sulfate (α-DUA-6S-[β3GalNAc]6) were purchased from Sigma. The crystals of the complexes with these various chondroitin
and chondroitin sulfates were obtained by co-crystallization. The hanging drop vapor diffusion method was employed (56), and equal volumes (1 μl each) of the enzyme, 50 mM chondroitin solution (in 10 mM Tris-HCl), and reservoir solution were used. The crystallization conditions were essentially identical to those of the wild type enzyme as previously reported (55). These crystals grew within 2 weeks and were of similar size and shape of those of the wild-type enzyme.

The crystals were cryoprotected and frozen as previously described for the native crystals (8, 55). The diffraction data were collected using a synchrotron source, beamline 19ID of the Structural Biology Center, Advanced Light Source, Argonne National Laboratory or beamline x25 of the National Synchrotron Light Source, Brookhaven National Laboratory. 3x3 Oxford or Brandeis-4 CCD detectors were used, and the data were collected in oscillation mode (57) as reported (8, 55). The crystals of the complexes were isomorphous to those of the wild-type enzyme (8, 55).

Structure Solution and Refinement—Of the various combinations of wild-type and mutant enzymes with different sulfated and unsulfated chondroitin disaccharides, full refinement was only carried out for the best diffraction crystal for each ligand. An exception was made for ΔD6S, for which both wild-type and Y408F protein complexes were refined in order to verify that the introduction of the mutation had no effect on the mode of ligand binding. The structures of crystals grown in the presence of ΔD6S-UA-2S were also not refined, since it was clear from the outset that ligand was not bound.

The structures were solved by rigid body refinement in CNS (58) using the 1.56-Å crystal structure of S. pneumoniae HL (PDB code 1egu) (8) as a search model. Refinement proceeded by alternating rounds of computational refinement using CNS (58) and manual rebuilding with O (59). All data were used throughout with no intensity- or ω-based cut-offs applied. SigmaA-weighted map coefficients (60) were used throughout. An R<sub>free</sub> value (61), calculated from 5% of reflections set aside at the outset, was used to monitor the progress of refinement. Water molecules were placed into 3σ positive peaks in F<sub>c</sub>–F<sub>c</sub> maps when density was also evident in 2Fo–Fc maps and suitable hydrogen bonding partners were available. Strong difference density at the catalytic site corresponding to the chondroitin disaccharides was evident. Subsites from the crystallographic solution were modeled into suitably shaped regions of electron density. Final statistics for the three complex structures are shown in Table I.

Programs of the CCP4 package (62) were used for manipulations and structural superpositions made with LSQMAN (63). Topology and parameter files were made with the help of the HICUP data base (64). Visualization of structures and manual modeling of chondroitin sulfation were carried out with O (59). Possible modes of ligand binding toward the nonreducing end of substrates were explored by manual positioning of bound or unbound (40) ligand structures with the constraint that binding in the –1-position, adjacent to the site of cleavage, must remain the same as seen crystallographically for complexes with smaller oligosaccharides (reported here and in Refs. 13 and 15).

Other Methods—The enzyme concentration was determined by the UV absorption at 280 nm using the molar extinction coefficient calculated based on the native or mutant S. pneumoniae hyaluronate lyase amino acid residue sequence data (12, 55). The calculated molar extinction coefficients were 127,090 for the native enzyme

RESULTS AND DISCUSSION

Structural Description—The overall protein structures of the three complexes described here, wild type enzyme with ΔDi0S (WT-ΔDi0S), Y408F mutant with ΔDi4S (Y408F-ΔDi4S), and Y408F mutant with ΔDi6S (Y408F-ΔDi6S), are essentially superimposable on previous S. pneumoniae HL structures (8, 13, 15) (Fig. 2). The enzyme molecule is composed of two domains, the catalytic domain having a α/α barrel structure and the other composed mainly of an antiparallel, three-layer β-sandwich (Fig. 2). The function of the β-sheet domain is the modulation of polymeric substrate access to the catalytic cleft present in the mostly α-helical domain. The catalytic cleft transverses the α-helical domain. So far three substrate disaccharide binding sites have been visualized crystallographically using a hyaluronan hexasaccharide (15). The active site of the enzyme is also located within the barrel domain on one side of the cleft area (15, 27, 28, 65). The recombinant protein crystallized contains residues Ala<sup>168</sup>–Glu<sup>391</sup> of the full-length enzyme (7) with an added His<sub>6</sub> tag at the C terminus (12, 55). The
full-length enzyme contains additional residues at the N-terminal arranged in two domains (7). The first of these is clearly a carbohydrate/hyaluronan binding module, which presumably acts to enhance the overall affinity of HL for substrates (for a full discussion, see Refs. 5 and 7 and see below). The second, small domain has no obvious function and may act simply as a spacer to distance the catalytic domain from the hyaluronan-binding domain at the extreme N terminus of the full-length protein (7).

The use of mutant Y408F enzyme for the two complexes with sulfated chondroitins leads to no discernible alteration in protein or ligand conformations upon comparison with previous complex structures (15). The validation of the use of mutant enzyme through the comparison of ΔD6S structures was particularly important, since in the related CL, the corresponding mutation did lead to altered ligand binding (22). The mutant enzymes afforded crystals diffracting x-rays to significantly higher resolution than the corresponding wild-type enzyme crystals (Table I). Therefore, for the complexes with ΔDi4S and ΔDi6S, the structures obtained with the mutant enzyme will be employed in the discussion that follows. As previously observed, different crystals enable definition of slightly different numbers of residues at the termini of the main protein chain (8, 13, 15). Thus, for WT-ΔDi0S, Y408F-ΔDi4S, and Y408F-ΔDi6S, respectively, Ala160-Leu890, Val170-Glu891, and Ser168-Glu991 were included in the model. Similarly, 12, 23, and 20 residues were modeled with two alternate conformations in these three structures. In corresponding positions of each structure, four sulfate ions, deriving from the crystallization solution (55), were bound. All lie distant from the catalytic site and appear to have no functional implications.

The disaccharide ligands in the three complexes are all very well defined by density (Fig. 3) and have real space correlation coefficients in the range 0.89—0.94. Structural superposition of the proteins leads to superposition of the ΔDi0S, ΔDi4S, and ΔDi6S disaccharides placed essentially identically to the first, HA1, of the two hyaluronan (HA) disaccharides previously visualized HA1 and HA2 (13). Crystal structures of carbohydrate active enzymes in complex with substrates often reveal varying numbers of subsites into which individual polymeric carbohydrate units bind. These may be on either side of the bond to be cleaved. In order to facilitate their description, Davies et al. (66) have introduced a standard methodology in which negatively numbered subsites −1, −2, −3, etc., bind substrate to the nonreducing side of the bond to be cleaved, with site −1 closest to the cleaved bond. In the same way, sites +1, +2, +3, etc. lead away from the cleavage site toward the reducing end of the polysaccharide substrate. The two positions occupied in the complexes reported here, according to the nomenclature of Davies et al. (66), are subsites −1 and −2. The different anomericity at the C-4 position of the N-acetyl-D-glucosamine in chondroitin, compared with the N-acetyl-D-glucosamine of hyaluronan, is well defined by electron density as exemplified by the WT-ΔDi0S complex (Fig. 3c). This difference leads to slight reorganization of the water network in the vicinity of this binding site but not to any changes in the protein structure of the entire enzyme.

The structure of Y408F-ΔDi4S shows that at the occupied subsites −1 and −2, the addition of the sulfate group at the 4-position of N-acetyl-D-galactosamine ring causes no change at all in the binding mode of this disaccharide or in the local protein structure. The 4-sulfate group makes no direct interactions with the protein. In contrast, the addition of a sulfate group at position 6 of the bound conformation of ΔDi0S disaccharide without any conformational changes of this chondroitin molecule would lead to steric clashes with Aan890. Accommodation of the sulfate group requires rotation about the C-5–C-6 bond of around 135° (Fig. 3c). Once again the 6-sulfate group only makes through-water interactions with the protein residues.

The interactions of the bound disaccharides are summarized in Table II. The distances associated with each individual

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**Table I**

Crystallographic and refinement statistics

|                      | WT-ΔDi0S | Y408F-ΔDi4S | Y408F-ΔDi6S | WT-ΔDi6S |
|----------------------|----------|-------------|-------------|----------|
| **Unit cell**        |          |             |             |          |
| a (Å)                | 83.66    | 83.74       | 83.91       | 83.70    |
| b (Å)                | 104.27   | 103.78      | 104.20      | 101.01   |
| c (Å)                | 98.79    | 101.15      | 101.50      | 101.16   |
| Low resolution diffraction limit (Å) | 50.0    | 50.0        | 50.0        | 50.0     |
| High resolution diffraction limit (Å) | 1.78    | 1.75        | 1.60        | 1.90     |
| Completeness (%)     | 84.2 (76.5) | 100.0 (100.0) | 99.5 (96.4) | 92.1 (83.3) |
| I/σ(I)               | 17.1 (4.6) | 25.0 (2.2)  | 31.2 (1.7)  | 8.8 (0.9) |
| Multiplicity (%)     | 2.5 (2.2) | 7.9 (7.4)   | 7.2 (3.8)   | 4.0 (3.7) |
| Rmerge (%)           | 7.2 (15.4) | 8.9 (75.7)  | 8.5 (50.3)  | 7.6 (78.6) |
| Nonhydrogen protein atoms | 5915     | 5995        | 5984        | 5818     |
| Sulfate atoms        | 20       | 20          | 20          | 20       |
| Nonhydrogen chondroitin (sulfate) disaccharide atoms | 26       | 30          | 30          | 30       |
| Nonhydrogen solvent atoms | 573      | 537         | 638         | 604      |
| Number of reflections | 72,283   | 90,026      | 117,091     | 63,869   |
| R (%)                | 20.3 (23.4) | 20.2 (31.0) | 19.4 (31.9) | 19.6 (32.8) |
| Rmerge (%)           | 22.4 (26.2) | 21.9 (28.9) | 20.9 (31.1) | 22.2 (35.4) |
| Mean temperature factor B (Å²) |          |             |             |          |
| All atoms            | 26.0     | 25.7        | 24.4        | 31.4     |
| Protein              | 25.2     | 24.8        | 23.2        | 27.4     |
| Protein main chain   | 23.9     | 23.2        | 21.6        | 26.8     |
| Protein side chain   | 26.7     | 26.6        | 25.9        | 28.5     |
| Sulfate              | 48.7     | 49.5        | 51.5        | 57.8     |
| Chondroitin (sulfate) disaccharide | 22.4     | 30.1        | 34.2        | 50.3     |
| Solvent              | 32.1     | 32.3        | 32.4        | 35.8     |
| Root mean square deviation |          |             |             |          |
| Bond lengths (Å)     | 0.006    | 0.005       | 0.005       | 0.005    |
| Bond angles (degrees) | 1.3      | 1.3         | 1.3         | 1.2      |

a Values in parentheses are for the highest resolution shell (WT-ΔDi0S, 1.84—1.78 Å; Y408F-ΔDi4S, 1.81–1.75 Å; Y408F-ΔDi6S, 1.66–1.60 Å; WT-ΔDi6S, 1.97–1.90 Å).
FIG. 3. Stereo images showing electron density associated with ligands, in final \(\alpha A\)-weighted \(2F_o - F\), maps contoured at 1.3 \(\sigma\), in the complexes. In a, C-4 of the N-acetyl-D-galactosamine unit is labeled. a, wild type enzyme-\(\Delta Di_0S\) disaccharide; b, Y408F mutant-\(\Delta Di_4S\) disaccharide; c, Y408F mutant-\(\Delta Di_6S\) disaccharide.
**Table II**

Contacts of bound chondroitin (sulfate) disaccharides to hyaluronate lyase

| GlcUA atom | Protein atom | Distance in WT-ΔDi0S (Å) | Distance in Y408F-ΔDi0S (Å) | Distance in Y408F-ΔDi6S (Å) |
|------------|--------------|--------------------------|----------------------------|-----------------------------|
| O-2        | Arg⁴⁶⁶ Nη1   | 2.73                     | 2.77                       | 2.71                       |
| O-3        | Arg⁴⁶⁶ Nη2   | 2.59                     | 2.55                       | 2.58                       |
| O-6A       | Glu⁴⁷⁷ Oη2   | 2.73                     | 2.68                       | 2.79                       |
| O-6B       | Arg²⁴³ Nη1   | 2.59                     | 2.56                       | 2.63                       |

| GalNAc (sulfate) atom | Protein atom | Distance in WT-ΔDi0S (Å) | Distance in Y408F-ΔDi0S (Å) | Distance in Y408F-ΔDi6S (Å) |
|-----------------------|--------------|--------------------------|----------------------------|-----------------------------|
| O-1                   | Tyr⁴⁰⁶ Oη    | 2.59                     | NA*                       | NA*                         |
| O-1                   | Tyr⁴⁰⁶ Nη2   | 3.01                     | 3.01                       | 3.05                       |
| C-1                   | Trp²⁹² Nη1  | 3.98                     | 3.95                       | 3.88                       |
| C-5                   | Trp²⁹² Cη2  | 3.50                     | 3.45                       | 3.43                       |
| C-7                   | Tyr⁴⁰⁶ Cζ    | 3.52                     | 3.69                       | 3.74                       |
| C-8                   | Tyr⁴⁰⁶ Cε1   | 3.40                     | 3.74                       | 3.74                       |

* NA, these distances not available for the Y408F mutant enzyme.

atomic interaction in the three complexes are very similar. Both the GlcUA and GalNAc (sulfate) units make hydrogen bonds with the enzyme. Only the GalNAc unit additionally has hydrophobic interactions. The interacting residues of the enzyme include both those assigned as catalytic (Tyr⁴⁰⁶) and those contributing to the "hydrophobic patch" (Trp²⁹²).

**Substrate Specificity of S. pneumoniae Hyaluronate Lyase and Related Enzymes—**Different HLs and CLs enzymes have different specificities for their polysaccharide substrates. At one extreme, the phage H4489A and Streptomyces hyalurolytocos HLs have strict specificity for hyaluronan, not cleaving chondroitins of any kind (67, 68). At the other end of the spectrum, P. vulgaris chondroitin sulfate ABC lyase can cleave unsulfated and sulfated chondroitins as well as dermatan sulfate (69). S. pneumoniae HL lies between these two extremes. The experimental characterization of the substrate specificity of S. pneumoniae HL for chondroitin and chondroitin sulfates is limited to the results reported here, although the S. agalactiae enzyme has been characterized. For example, the S. agalactiae enzyme has been shown to process HA preferentially, but also unsulfated and some 4- and 6-sulfated chondroitins (70–72). As mentioned in the Introduction, the predominant chondroitins isolated from tissues are exactly these: unsulfated chondroitin, chondroitin 4-sulfate, and chondroitin 6-sulfate. Chondroitin 2-sulfate has also been identified, with unsulfated chondroitin, chondroitin 4-sulfate, and chondroitin 6-sulfate (70–72). In contrast, the ΔDi4S disaccharide binds well to subsites −1 and −2 and the ΔDi2S (Table I) that would be indicative of disorder and negligible alterations in disaccharide positioning and protein structure. This is in excellent agreement with the biochemical results obtained for the HL enzyme from S. agalactiae (72), showing that 4-sulfation of the nonreducing end disaccharide is tolerated, whereas 4-sulfation of the reducing end disaccharide impedes processing. A doubly 6-sulfated chondroitin tetrasaccharide was processed (72). Given the 55% overall sequence identity between S. pneumoniae and S. agalactiae enzymes and their essentially identical three-dimensional crystal structures and catalytic cleft residues (8, 65), it is reasonable to assume that their substrate specificities are the same or at least similar.

Detailed substrate specificity information is also available for the structurally homologous CL from Flavobacterium hepari

nunum (73) and its component the chondroitin AC lyase (22). Although sharing only around 20% sequence identity with S. pneumoniae HL, it too can cleave HA, unsulfated, and 6-sulfated chondroitins. However, its preferred substrate is 4-sulfated chondroitin. Structures of chondroitin AC lyase in complex with hyaluronan, chondroitin, and dermatan sulfate oligosaccharides have been determined. The entire oligosaccharide was not always visualized, so that for hyaluronan the binding mode for a disaccharide only, bound as here in subsites −1 and −2, was obtained. In contrast, corestabilization of dermatan 4-sulfate and 6-sulfated chondroitin (the latter to a mutant enzyme) revealed tetrasaccharides bound in subsites −2 to +2.

**Results with Chondroitin 2-Sulfate—**The complete absence of electron density for bound ligand in crystals soaked with ΔDi-UA-2S suggests that the inability of S. pneumoniae HL to process 2-sulfated chondroitin lies, at least partly, in the structure of subsite −2. Indeed, attempts to model ΔDi-UA-2S in the HL catalytic site, based on the present structure of ΔDi0S, show that no space exists for the 2-sulfate group. Modeling shows that the bound conformation of the disaccharide severely restricts the conformational possibilities open to the 2-sulfate group, since most conformations clash either with the 3-hydroxyl group of the D-glucuronic acid in subsite −2 or with the acetamido group of the sugar derivative in subsite −1 (Fig. 4). The remaining conformational possibilities are disallowed by the positioning of Arg⁴⁶⁶. This residue forms hydrogen bonds with both O₂ of the glucuronic acid in subsite −2 and with the amide group of the N-acetyl-D-galactosamine in subsite −1 and is held in position by twin hydrogen bonds to Glu⁴⁰⁶ (Fig. 4).

**Complex with Chondroitin 4-Sulfate—**In contrast, the ΔDi4S disaccharide binds well to subsites −1 and −2 of S. pneumoniae HL (Fig. 3b) with no significant elevation of B-factor compared with ΔDi0S (Table I) that would be indicative of disorder and negligible alterations in disaccharide positioning and protein structure. This is in excellent agreement with the biochemical results obtained for the HL enzyme from S. agalactiae (72), showing that 4-sulfation of the nonreducing end disaccharide (binding to subsites −2 and −1) of tetrasaccharides substrates is tolerated by HL. In contrast, reducing end disaccharide sulfation (binding to subsites +1 and +2) leads to compounds that may not be cleaved by HL. The factors responsible may be identified using the complex of S. pneumoniae HL with a hyaluronan hexasaccharide (15) as a basis, since the results presented here and elsewhere (22) show that different polysaccharides adopt broadly similar binding modes to lyases. Modeling of the 4-phosphorylation at the +2 site, in the favorable extended conformation that avoids clashes with other ring constituents, led to steric clashes with residues Trp²⁹³, Phe²⁴⁰, and Asn²⁴⁹. However, the substrate-binding cleft broadens at this point, so the possibility of a relocation of the ring should be considered. Indeed, such a relocation is visible in the structure of F. hepari

nunum CL complexed to 4-sulfated dermatan sulfate (Fig. 5). The residues corresponding to Trp²⁹³ and Asn²⁴⁹ are conserved in the CL, whereas the hydrophobic portion of the
Lys\textsuperscript{171} side chain occupies the same space as the Phe\textsuperscript{240} side chain in HL. The CL complex with 4-sulfated dermatan shows a significant reorientation of the ring bound at the +2 subsite compared with the same position in the \textit{S. pneumoniae} HL complex with hyaluronan hexasaccharide. The plane of the ring rotates about \(60^\circ\) around the axis of the bound oligosaccharide, relieving the clashes of the 4-sulfate that would otherwise occur. Instead, the hydrophobic face of the sugar in subsite +2 lies flat against the side chain of Trp\textsuperscript{427} in the kind of interaction commonly observed between carbohydrate binding proteins and their ligands (74). The 4-sulfate group interacts with His\textsuperscript{225} and Asn\textsuperscript{170} (corresponding to HL His\textsuperscript{399} and Asn\textsuperscript{446}). Significantly, Trp\textsuperscript{427} of CL lies on an insertion relative to HL. Modeling the same sugar position into the HL structure shows that instead of the favorable interaction with Trp, only a single hydrophobic interaction with Met\textsuperscript{427}, located in a different region of the protein chain, would be available. Thus, it may be suggested that the presence of Trp\textsuperscript{427} in CL provides an alternative favorable binding position for subsite +2 sugar in which unfavorable interactions of a 4-phosphate group with catalytic and hydrophobic patch residues may be avoided. This alternative position simply does not exist in HL so that 4-sulfation will lead to significant loss of affinity compared with unsulfated oligosaccharides.

At the −3 subsite, 4-sulfation also appears to be incompatible with the bound conformation of chondroitin, if assumed to resemble that of bound hexasaccharide hyaluronan, since all conformations lead to protein clashes. Such clashes are particularly numerous with Arg\textsuperscript{255}, which seems to lack the space to reorient. Thus, in order for 4-sulfated chondroitin to bind to subsite −3 of HL, disruption of the naturally favored binding modes at least at subsite −3 and probably at both subsites −3 and −4 would be required. In fact, the binding in subsites −3 and −4 might not be essential for enzyme activity for the following reasons. HL is known to act initially through random endolytic cleavage (72) for all substrates, but, with the exception of the case of substrates with exactly four saccharide units on the nonreducing side, binding in subsites −3 and −4 would be impossible, since the ligand in subsite −4 occupies a surface indentation with its nonreducing end in contact with protein. In other words, with subsite −4 occupied, further saccharide units at the nonreducing end could not be accommodated. After this initial endolytic cleavage, the enzyme acts exolytically and processively from the nonreducing to the reducing end of the substrate chain, producing disaccharide product (15, 27, 28). Such a process would not involve binding to subsites −3 and −4. It is also worth noting that the substrate in subsites −3 and −4 was significantly less well defined than the remainder (15). In summary, structural features of HL at the −3 and +2 subsites are not conducive to binding of 4-sulfated chondroitin, although, as explained, the impediments to binding at subsite −3 may not in fact be of physiological relevance in either endolytic or exolytic cleavage phases.

**Complex with Chondroitin 6-Sulfate**—The ability of HL to freely cleave doubly 6-sulfated chondroitin tetrasaccharide implies that 6-sulfation at subsites +2 and −1 provides no impediment to binding. Binding of 6-sulfated chondroitin disaccharide in subsites −1 and −2 is observed crystallographically (Fig. 3c). Although 6-sulfation of ligand in subsite −1 leads to no additional favorable interactions with the protein, it is well accommodated without significant alterations to the structures of the disaccharide rings or the protein. The consequences of 6-sulfation of chondroitin at subsites +2 were again explored by modeling. At subsite +2, the 6-sulfate may be reasonably well accommodated without steric clashes, although in unfavorably close proximity to Glu\textsuperscript{388}. However, the 6-hydroxyl group of bound hyaluronan hexasaccharide makes no direct contacts with the enzyme and appears free to rotate to place the 6-sulfate in a more solvent-exposed position (15). Unexpectedly, 6-sulfation at subsite −3 leads to severe steric clashes, since the 6-hydroxyl group binds into a surface indentation. This suggests that 6-sulfated chondroitin cannot bind in exactly the same way as observed for hyaluronan hexasaccharide. Once again, however, binding at these subsites is unlikely to be relevant to either the initial endolytic cleavage (except in the special case in which exactly four saccharide units lie between the nonreducing end and the cleavage site) or to the rapid exolytic processive phase of substrate cleavage, as argued above.

**Chondroitin Sulfates Substituted at the 3-Position**—The existence of ChS 3-sulfated on the GlcA residue (32) and a fucosylated ChS form, also modified at the 3-position of the GlcA residue (33), has been demonstrated. Modeling based on the structure of HL in complex with ΔDi0S suggests that neither of these modifications will be accepted in subsite −2. In fact, although the 3-hydroxyl group makes a hydrogen bond with Glu\textsuperscript{477}, it points out into solution, so that a sulfate group could be accommodated without steric clashes. Nevertheless, the sulfate group would be in unfavorably close proximity to Glu\textsuperscript{477}, resulting in electrostatic repulsion. In the case of the much larger fucosyl modifications, it is clear that sufficient space does not exist for their accommodation in the catalytic site. It is possible that further impediments to binding of 3-substituted chondroitins exist in other subsites of the catalytic cleft, but the properties of subsite −2 are sufficient to indicate that these modified chondroitins will not be bound by HL.

**Mechanism of \textit{S. pneumoniae} Hyaluronate Lyase Catalysis and Action on Chondroitins**—The structures of the Ch and ChS complexes with the pneumococcal HL enzyme fully support the notion that the degradation of Ch and ChS proceeds via the same mechanism as proposed for the degradation of hyaluronan and termed the proton acceptance and donation mechanism (see Introduction) (11, 27, 28). The interactions of the Ch/ChS substrates mirror the essential interactions of the enzyme with HA, whose degradation was studied in more detail. Briefly, the reaction is initiated after HA/Ch/ChS binding in the enzyme cleft and by acidification of the C-5 hydrogen of HA/Ch/ChS by Asn\textsuperscript{389} that acts as an electron sink (16). Then His\textsuperscript{399} withdraws this hydrogen, and subsequently a Tyr\textsuperscript{409} provides a hydrogen atom to the glycosidic oxygen of the β1,4
linkage, a process resulting in the formation of a double bond between the C-4 and C-5 carbons of the substrate(s) and the cleavage of the glycosidic linkage. Finally, the enzyme exchanges hydrogens with the water microenvironment reading the enzyme for the next round of catalysis.

Other possible schemes for this mechanism have been described. These proposed mechanisms differ primarily in the identity of the proposed acid and base for the reactions degrading polymeric glycans HA/Ch/ChS or alginate (22, 75). The amino acids most commonly implicated in other schemes are Arg^{462} or Tyr^{408} (acting also as a base). In our structures, His^{399} and Arg^{462} share significant electrostatic interactions with Glu^{577}. His^{399} and Tyr^{408} also share interactions; therefore, all of these implicated residues have significant influence on their conformations and, as a consequence, influence on the catalytic activity. Also, the relatively longer distance between the C-5 carbon and His^{399} as observed in the structure of the complex with HA (15) drastically shortens when the flexibility of the enzyme is included in this evaluation. Therefore, no clear evidence exists at this time that would allow for the modification of the proton acceptance and donation mechanism proposed by us earlier for hyaluronate lyase.

**Implications for Mode of Action**—The structural differences between HL and CL may be correlated to their modes of action, as revealed by kinetic analysis with a variety of substrates (16, 71–73, 76). As mentioned, biochemical data show that HL cleavage of HA, its primary substrate, proceeds via an initial endolytic cleavage, which is followed by rapid exolytic and processive activity, producing solely an unsaturated disaccharide unit as the end degradation product (3, 15, 27, 28).

In contrast, *F. heparinum* CL cleaves chondroitin and chondroitin sulfates and, to a lesser extent, dermatan sulfate, producing a mixture of variously sized oligosaccharide products containing even numbers of saccharide units (73). In the cases of 4- and 6-sulfated chondroitin, but not in the case of dermatan sulfate, cleavage continues to form disaccharides (73). Cleavage occurs solely in a random, endolytic manner (76). The 2-fold helical crystal structure of 4-sulfated chondroitin (40) was used in our modeling studies to explore the potential of CL to bind long (sulfated) chondroitin oligosaccharides. This structure closely resembles both the recently obtained solution structures observed by NMR in D_{2}O (41) and the CL-bound oligosaccharide conformations (22). The modeling shows that the

**FIG. 5.** Stereo view of comparison of hyaluronan hexasaccharide binding with *S. pneumoniae* hyaluronate lyase (green) (15, 22) and binding of dermatan sulfate tetrasaccharide to *F. heparinum* chondroitin AC lyase (cyan) (15, 22). Binding occurs in subsites +2 to −2, left to right in the orientation shown. The N-acetyl-D-glucosamine hyaluronan unit in subsite +2 of the hexasaccharide is not shown. Instead, a superposed 4-sulfated N-acetyl-D-galactosamine chondroitin unit is pictured (purple). Residues are numbered as in *S. pneumoniae* HL except for nonconserved CL residues, which are numbered in italic type. The potential catalytic residues Asn^{349}, His^{399}, Tyr^{408}, and Arg^{462} (*S. pneumoniae* HL numbering) superimpose very well and are shown for HL alone. They are distinguished by gray bonds and ball-and-stick representation.

**FIG. 6.** Binding of longer oligosaccharides in the entire length of the active site clefts of lyases. Binding modes in subsites −2, −1, +1, and +2 (a) and subsites −1, +1, and +2 (b) are those observed crystallographically (15, 22). The remaining oligosaccharides are modeled, as detailed under “Experimental Procedures.” Key catalytic residues are labeled in both cases. In a, the two large insertions in HL, relative to CL, which prevent extended binding to the former in the favorable 2-fold helical conformation, are shown as magenta loops. a, *F. heparinum* chondroitin AC lyase; b, *S. pneumoniae* hyaluronate lyase.
extensive, unimpeded tunnel that forms the CL binding site (see Introduction) can accommodate long polymeric Ch and ChS oligosaccharides, with few steric clashes, so as to cut in the middle of the bound glycan chain (Fig. 6a). Complex crystal structures of CL have only visualized subsites –2 to +2. Nevertheless, the existence of a putative subsite –3 can be hypothesized, since the 4-sulfate of the corresponding saccharide unit is well placed to favorably interact with Arg<sup>304</sup>. However, the comparison with HL reveals a dramatic structural difference (Fig. 6a). Two large amino acid insertions, numbered 186–203 and 508–539, in HL relative to CL form a blockage at one end of the binding site canyon (Fig. 6a). A long oligosaccharide may not therefore be accommodated in the HL binding site in the most favorable 2-fold helical conformation (see Introduction). This steric restriction lies in the region containing the negatively numbered subsites (i.e., in the region binding to the nonreducing end of the oligosaccharide). Since exolytic cleavage of terminal disaccharide units involves only two negatively numbered subsites (–1 and –2), these steric restrictions have no impact on the exolytic mode of action. It is known, however, that HL can make initial endolytic cuts in larger substrates (72), albeit more slowly than it cleaves exolytically. Modeling shows how this might be achieved. The experiments were carried out with the bound conformation of HA hexasaccharide ligand (15), which contains a distinct bend between the saccharide units in subsites –2 and –1 as compared with the ideal 2-fold helical conformation of HA. The hexasaccharide ligand was shifted with respect to the enzyme so that the N-acetyl-glactosamine unit originally occupying subsite +2 in the crystal structure was made to occupy subsite –1. The resulting hexasaccharide chain position is shown in Fig. 6. In this position, only relatively insignificant steric clashes between bound ligand and the side chains of Glu<sup>204</sup> and Lys<sup>318</sup> are observed. Similar experiments with an ideal 2-fold helical conformation of HA showed that productive binding in subsite +1 inevitably leads to serious steric clashes of the remainder of the substrate with the blocked end of the substrate binding canyon of HL. Thus, deformation away from the straight, elongated conformation is a prerequisite for the binding of longer oligosaccharides so as to enable endolytic cleavage. In the binding mode shown in Fig. 6b, subsite –1 is occupied in exactly the same way as seen crystallographically in the various HL complexes. However, the bend in the bound hexasaccharide conformation leads to a displacement at subsite –2 relative to the crystallographically observed positions. This displacement seems sufficient to perturb the interactions between substrate and enzyme. Thus, it may be proposed that HA has sufficient intrinsic flexibility to enable binding of HL in the middle of long polysaccharide chains but that the distortion introduced, relative to the ideal 2-fold helical HA conformation, leads to suboptimal binding at subsite –2 and no binding to subsites –3 and –4. In summary, rapid exolytic cleavage only requires binding to subsites –2 to +2. Such binding occurs in the optimal way observed crystallographically with multiple hydrogen bonding, electrostatic, and hydrophobic interactions. In contrast, in order to cleave endolytically in the middle of the chain, HL, even with a steric blockage at one end of the substrate binding canyon, must accommodate the oligosaccharide chain in the region beyond subsite –2. This interaction may be satisfactorily modeled with the bent hexasaccharide conformation but seems to involve the energetic cost of less than ideal interaction at subsite –2, as well as any energetic cost associated with the bending of substrate. The mechanism by which HL achieves processive cleavage of HA has previously been studied through structural and dynamic analysis (3, 14, 15, 27, 28). Some structural features such as extensive use of hydrophobic interactions with substrate, interactions that generally lack directionality in their strength, are also seen in other processive enzymes (14, 77). Lack of directionality in the interactions will aid the slippage of product along the active site cleft without dissociation. Twisting motions between the two principal domains, revealed by simulated dynamics, also seem to play a role (15, 19). Why, therefore, given the overall structural similarity of HL and CL (see Introduction), expected to lead to similar dynamic behavior (15, 19, 78), and the conserved hydrophobic interactions with substrate does CL not exhibit processive substrate cleavage (76)? One likely explanation lies in the added substrate bulk caused by sulfation of chondroitin. HL and CL share a constricted in the active site cleft near the catalytic residues (Fig. 2a). For processivity to occur, the chain undergoing cleavage must remain bound to the enzyme. Thus, after cleavage of the substrate, the unsaturated disaccharide product bound in subsites –2 and –1 must dissociate, and the shortened chain bound, with its nonreducing end in subsites +1 and +2, must translate the equivalent of one disaccharide to occupy once more subsites –2 to +2. This translation places the next glycosidic bond for cleavage in the catalytic site. As well as this significant translation, the approximate 2-fold symmetry of the bond substrate helix means that a rotation about the helical axis of around 180° must occur. It seems likely that these significant reorientations occur with greater difficulty for a sulfated substrate than for unsulfated hyaluronan. This must not be the only factor determining the inability of CL to carry out processive cleavage, since CL action unsulfated chondroitin is nonprocessive (76). Nevertheless, by analogy, it seems probably that substrate sulfation will hamper or eliminate processive cleavage in HL. Data regarding the issue would provide a useful test of our hypothesis.

The inability of CL to carry out processive cleavage would be an additional factor favoring endolytic activity over exolytic cleavage. If both products must dissociate before the next productive binding, then for larger chains, there will effectively be competition between different modes of binding. Occupation of the whole active site cleft (predicted to be feasible by modeling (Fig. 6a) and leading to endolytic cleavage) would be tighter than binding of substrates, irrespective of length, with their nonreducing end in subsite –2. This latter, less energetically favorable binding would lead to occupation of only four subsites, –2 to +2, and would result in exolytic cleavage.

Degradation of HA and Ch/ChS in Their Physiological, Aggregated States—It seems that degradation of hyaluronan, chondroitin, or chondroitin sulfates by bacterial hyaluronate lyases enzymes, including <i>S. pneumoniae</i> HL, is a complex phenomenon. In addition to the proposed multistep catalytic mechanism (8), the issue of the processive versus nonprocessive degradation needs to be considered. The evidence suggests that degradation of HA by <i>S. pneumoniae</i> HL is a processive event, as shown previously (3, 14–16, 19, 27, 28). It is initiated by a random endolytic “initial bite” by the enzyme on polymeric HA, resulting in cleavage of the polymeric HA chain into two parts. This process is then followed by processive, exolytic cleavage of one HA disaccharide at a time until the entire chain is degraded. The same process could be applied to degrading of Ch and ChS. However, the presence of different sulfation patterns in naturally occurring chondroitin/chondroitin sulfates probably prevents their processive cleavage as argued above. The structural evidence provided here correlates perfectly with biochemical data showing that <i>S. pneumoniae</i> HL can only degrade Ch/ChS at the β1,4 linkage when the disaccharide on the nonreducing side of the cleaved bond is unsulfated or 6-sulfated; 4-sulfation is not tolerated on the nonreducing side, al-
though it is accepted on the reducing side of the bond to be cleaved. 2-Sulfated chondroitin is not cleaved (72) (this study). The reason for this specificity is directly related to the steric clashes between the enzyme and the substrate in the later sequence. Therefore, the degradation of Ch/ChS proceeds, as a consequence, primarily by the endolytic “random bite” mechanism.

In addition, hyaluronan and chondroitins possess specific, relatively well defined structures. Some studies suggest that these structures depend on the environment, such as the presence of NaCl and divalent cations (Ca$^{2+}$, Mg$^{2+}$). For example, recent NMR study shows that high molecular mass (over 300 kDa) HA assumes a 2-fold helix structure that is arranged into antiparallel β-sheet structures stabilized by hydrogen bonds between the HA chains (43, 44). This structural property of HA is still under discussion, but it might suggest a better picture of HA and chondroitin degradation. One such proposed picture is described below. However, more studies are needed to validate such proposal. Assuming aggregation of HA, as a consequence, the initial degradation of high molecular weight HA chains probably proceeds through a random endolytic cleavage only at the sites where such chains expose the β1,4 linkage in a proper conformation for degradation to HL. Due to the argued 2-fold helical conformation of HA, the next β1,4 linkage will be rotated by –180° and as such probably not accessible for HL enzyme due to the β-sheet mesh like structures. As the size of HA chains decreases due to the HL action their ability to aggregate will also decrease. At hyaluronan molecular mass below 300 kDa, the ability of HA to aggregate was indeed shown by electron microscopy-rotary shadowing to decrease (79). At the same time, HA chains below ~50 disaccharides in length (~40 kDa) as shown by light scattering evidence do not aggregate in salt solutions (80). These still largely arguable properties of HA indicate that somewhere below 300 kDa the molecule can be degraded by bacterial hyaluronate lyases using a processive mechanism due to smaller aggregation. In this model, as the average size of HA chain decreases, the processive mechanism takes over the random cleavage leading to exponential HA degradation.

The structure of Ch/ChS was proposed to be similar to that of the proposed HA 2-fold helices (45), and indeed chondroitins have been shown to assume in D$_2$O solution a 2-fold helical structure with suggested various patterns of aggregation dependent on patterns of sulfation (45). 6-Sulfated chondroitin, but not 4-sulfated chondroitin, molecules could also form mainly duplex structures. The unsulfated Ch was shown to form similar higher aggregated structures as HA (45). Therefore, degradation of these polymeric molecules, just as for high molecular weight HA, should proceed via an endolytic, random bite type mechanism. In this model, due to selectivity of HL for different sulfation patterns within ChS substrate, endolytic cleavage will predominate until the entire polymer is degraded. This differs from the situation with HA substrate, where exolytic cleavage takes over as the substrate molecular weight reduces. The final degradation product of (sulfated) chondroitin is also a disaccharide unit with the limitation of the selectivity due to the sulfation patterns described above.

Interestingly, the N-terminal portion of S. pneumoniae HL, not visualized crystallographically due to autoprocessing, has been recently shown to bear homology to carbohydrate binding domains (7). It is proposed that this domain helps to co-localize enzyme and substrate, thereby enhancing catalytic efficiency. A further possible function would be disruption of the higher order aggregated substrate conformations, spreading the individual helical strands of these polymeric glycans apart to facilitate the degradative process. It may also be that the N-terminal domain, after binding to HA/Ch/ChS, serves to feed these chains to the catalytic domain. HL would then be able to degrade the HA chains, at least, through primarily processive degradation, bypassing the initial nonprocessive action pattern (7). A role for the N-terminal domain in feeding substrate into the active site, perhaps helping to retain the product that must undergo translation in order to reposition for the next processive cleavage step, would help explain the lack of processivity observed for CL, even when acting upon unsulfated chondroitin.

**Evolutionary Perspective**—Evolutionary studies suggest that heparin-like polymers were the first ancestral glucosaminoglycan in metazoan life (reviewed in Ref. 81). The second glucosaminoglycan polymer to evolve was chondroitin, with hyaluronan appearing significantly later. The first organism to produce chondroitin or the first to produce HA is still largely unknown. The recent availability of genomic sequences of many organisms allows for the inference, from their genetic content, of which glucosaminoglycans they contain. These studies show, for example, that the worm *Caenorhabditis elegans* (Nematoda) and the fruit fly *Drosophila melanogaster* (Insecta) both have heparin, heparan sulfate, and chondroitin (the unsulfated form only in the worm) but that neither contains hyaluronan (82).

The appearance of HA later in evolution than chondroitin suggests that hyaluronate lyase enzymes possibly developed from chondroitin lyases and that the original/ancestral HL was actually a CL. Over time in the higher organisms with HA present and essential for their survival, some of the CLs acquired specificity for hyaluronan and lost the efficiency to break down Ch/ChS. The residual ability of HL to cleave Ch/ChS is an indication of this ancestral origin. Since HA does not have any sulfation patterns that affect catalysis, the HL enzyme developed a processive mechanism of action allowing for free sliding down the HA polymer chain with exolytic cleavage of one HA disaccharide at a time (3, 15, 19, 27, 28). Evidence suggests that sulfation of chondroitin is (partly) responsible for the apparent inability of known lyases to processively cleave the chondroitin chain. Thus, processive cleavage of ChS is not possible by either HL or CL, and both enzymes cleave ChS by the same random endolytic process. It is tempting to speculate that HLs later acquired the N-terminal carbohydrate binding domain as a means to enhance the processive cleavage that became possible with the advent of HA. This domain would function to liberate individual chains from higher order HA aggregates and/or to help feed them into the catalytic site. Either role would improve the efficiency of processive cleavage.

**Conclusions**—We have studied the structural basis of cleavage of chondroitin and chondroitin sulfates by x-ray crystallography and molecular modeling. Chondroitins bind in subsites –2 and –1 in exactly the same way as the favored substrate hyaluronan, and the same probably holds for other subsites. Structural characteristics of the relevant subsites can be convincingly related to the known inability of HL to process 2-sulfated chondroitin, its limited ability to cleave the 4-sulfated form, and its indifference to chondroitin sulfation at the 6-position. The comparison of biochemical and structural data for *S. pneumoniae* HL and F. heparinum CL proved highly informative. Since chondroitin proceeded hyaluronan in evolutionary terms, the latter probably more closely resembles the common ancestor of the two than the HL. The CL, probably in common with the shared ancestor, has an unimpeded substrate binding cleft correlated with a random endolytic mode of action. In contrast, endolytic activity in HL is hampered by obstruction of one end of the substrate binding cleft, so that cleavage in the middle of long substrates may only occur with unfavorable
distortion of substrate and its interactions with enzyme. The more efficient processive exolytic mode of action, viable for the unsulphated HA substrate, is thereby favored. The acquisition of a carbohydrate binding domain by HMs, likely to have dynamic independence from the catalytic domain, may represent a second adaptation to the processively cleavable HA substrate, by exerting roles in either disentanglement of strands from higher order HA aggregates or in feeding those strands processively into the catalytic domain.

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