Hyaluronan Binding and Degradation by Streptococcus agalactiae
Hyaluronate Lyase*

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Streptococcus agalactiae hyaluronate lyase is a virulence factor that helps this pathogen to break through the biophysical barrier of the host tissues by the enzymatic degradation of hyaluronan and certain chondroitin sulfates at β-1,4 glycosidic linkages. Crystal structures of the native enzyme and the enzyme-product complex were determined at 2.1- and 2.2-Å resolutions, respectively. An elongated cleft transversing the middle of the molecule has been identified as the substrate-binding place. Two product molecules of hyaluronan of the molecule has been identified as the substrate-respectively. An elongated cleft transversing the middle of the molecule has been identified as the substrate-binding place. Two product molecules of hyaluronan degradation were observed bound to the cleft. The enzyme catalytic site was identified to comprise three residues: His779, Tyr488, and Asn429. The highly positively charged cleft facilitates the binding of the negatively charged polymeric substrate chain. The matching between the aromatic patch of the enzyme and the hydrophobic patch of the substrate chain anchors the substrate chain into degradation position. A pair of proton exchanges between the enzyme and the substrate results in the cleavage of the β-1,4 glycosidic linkage of the substrate chain and the unsaturation of the product. Phe429 likely determines the size of the product at the product release side of the catalytic region. Hyaluronan chain is processively degraded from the reducing end toward the nonreducing end. The unsulfated or 6-sulfated regions of chondroitin sulfate can also be degraded in the same manner as hyaluronan.

Streptococcus agalactiae (group B streptococci, GBS) is the predominant cause of meningitis and septicemia in humans, often leading to the death in neonates (1, 2). The knowledge about the molecular basis that leads to the development of these invasive diseases is still limited. Several putative pathogenic factors were identified, including the polysaccharide capsule, hemolysin, C5a peptidase, C-proteins, and hyaluronate lyase (3). High levels of hyaluronate lyase (SagHL) were found in the extracellular cultures of the disease-causing strains of serotype III GBS (4–6). Clinical studies indicated that strains of type III GBS that produce more extracellular hyaluronate lyase (division I S. agalactiae) are apparently more virulent than those strains producing less (division II S. agalactiae) (5, 6). Division I S. agalactiae usually causes invasive infections, whereas division II bacteria cause diseases much less frequently and are usually found in asymptotically colonized infants. The molecular function of SagHL is to degrade hyaluronan (HA) and certain chondroitin sulfates (CS). The enzymatic degradation of these polysaccharides breaks down the biophysical barrier of the host connective tissues, therefore facilitating the invasion and spreading of this pathogen.

Structurally, HA is a high molecular weight polysaccharide built by linear repeats of disaccharide units, (β-1,4-D-glucuronic acid-β-1,3-N-acetyl-β-D-glucosamine)n. It is produced by almost all members of the animal kingdom, as well as by certain members of Streptococci (7), and is widely distributed in various connective tissues and the nervous system. The human tissues known to contain HA include synovial fluid, the vitreous humor of the eye, umbilical tissue, amniotic fluid, aorta, leukocytes, plasma, blood platelets, brain, articular cartilage, liver, prostate, skin, teeth, and urine (8). Through inter- and intramolecular interactions, HA forms strikingly viscous solutions, which provides not only a soft and elastic “bed” for tissue cells to grow on, but also a medium for the intercellular communication. Various kinds of cell surface receptors are secreted into the extracellular matrix for the purpose of communication between cells. Many of them, such as CD44 and RHAMM, are known to bind to HA (9) and be involved in signal transduction processes.

Bacteria usually secrete hyaluronate lyases for the purpose of HA degradation, and the enzyme produces unsaturated products, whereas animals employ hyaluronidases, a group of hydrolases, for the HA degradation without unsaturation of the products (10). Various Gram-positive microorganisms including species of Streptococcus, Clostridium, Propionibacterium, Pseudostreptococcus, and Streptomycetes produce hyaluronate lyase (11). All known bacterial hyaluronate lyases were found to share substantial sequence identity and thus likely to use the similar mechanism for the catalytic action (12).

The degradation of HA by bacterial hyaluronate lyases occurs at the β-1,4 glycosidic linkage between disaccharide units and produces C4–C5 double bond on the glucuronic residue of its products. The mechanism of the unsaturation in the HA degradation was recently revealed (12). This depolymerization of HA in vivo causes the destruction of the intracellular matrix and exposes the host cells to various bacterial toxins (13). Significant additive attenuation in virulence was observed for streptococci with hyaluronate lyase and pneumolysin double mutations (13). Therefore, the HA degradation is an important step in bacterial invasion and spreading. Because sequences
and the mechanisms of HA degradation are different for bacteria and animals, it suggests that these two groups of enzymes are significantly different. Therefore, hyaluronate lyase may be targeted to inhibit bacterial invasion (14).

As part of our effort to elucidate the molecular basis of action of this enzyme, *S. agalactiae* hyaluronate lyase (SagHL) was overexpressed, purified, and crystallized as reported previously (15–17). Here we present the SagHL native crystal structure at 2.1 Å resolution and a complex structure with the 4,5-unsaturated disaccharide, the degradation product of this enzyme, at 2.2 Å resolution. The complete active site residues were identified. The mechanisms of HA and CS degradation were proposed based on the x-ray crystallography studies of SagHL native and disaccharide complex structures, the substrate binding modeling studies, and the known mutations of the active site residues (18). The structure basis of substrate binding, cleavage site selection, substrate degradation, product unsaturation and release, product size determination, and the direction of HA chain processive degradation are discussed.

### EXPERIMENTAL PROCEDURES

**SagHL Structure Determination**—SagHL expression (gene *hylB*<sub>3502</sub>), purification, crystallization, and data collection have been reported previously (16). SagHL crystal structure was determined at 2.1 Å resolution by the molecular replacement (MR) method using a poly(A)LA model derived from the *S. pneumoniae* hyaluronate lyase (SphHL) crystal structure (12). The sequence homology of SagHL to SphHL is 53%. MR calculation was performed using the program AMoRe (19). The two domains of the SphHL structure (α-domain and the β-domains) were treated as two separate molecules. The MR calculation provided one solution that was about 10 times higher than the other peaks. No impossibly structural conflicts were observed among symmetry-related molecules on graphics. The initial R factor was 0.502 and R<sub>free</sub> 0.497. The SagHL enzyme has additional 82 residues at the N terminus and six places of insertions as compared with the SphHL enzyme. The insertions were treated as chain breaks in the next step of the model building on graphics using program O (20). The structural refinement was performed using program X-PLOR (21) against 53,312 reflections at 2.1 Å resolution range. The R<sub>free</sub> flag was assigned to 2% (1,074) reflections for the validation of the refinement progress. The inserted residues, side chains, and the N terminus were built gradually on graphics using O (20) between each round of refinement until the R factor dropped to 0.282 and R<sub>free</sub> to 0.340. Then, the B-factor refinement was introduced and waters were added following standard criteria. Statistics for the SagHL crystal data collection and structural refinement were summarized in Table I.

**SagHL-ΔDi-HA Complex Structure Determination**—The unsaturated HA disaccharide degradation product, 2-acetamido-2-deoxy-3-O-β-D-glucopyranosyluronic acid)-n-glucose (ΔDi-HA), was obtained as described previously (17). Native SagHL crystals were soaked with 50 mM ΔDi-HA for elongated period of time. The crystals were cryoprotected using the same condition as for the native crystals (16), and the diffraction data set at 2.2 Å resolution was collected. The diffraction data were processed using the HKL2000 package (22) with R<sub>merge</sub> of 0.101 and the completeness of 98.1%. The native SagHL structure without water molecules was used as the initial model for the complex structure determination and refinement using program X-PLOR protocols (21). The structure was manually fitted on graphics using program O (20), and was refined against 45,510 reflections at 50–2.2 Å resolution range. Only 2% (860) reflections were randomly selected and assigned R<sub>free</sub> flags for monitoring and validation of the progress of the structural refinement. Two ΔDi-HA molecules and 256 waters were incorporated into the final structure model.

**Modeling of Tetrasaccharide into the Active Site**—The model of tetrasaccharide bound in the active site of SagHL was obtained to investigate binding of longer HA substrates in the active site cleft of the enzyme. The tetrasaccharide coordinates were taken from the crystal structure of SagHL disaccharide HA complex consisting of two disaccharide units, HA1 and HA2 (see below). The HA1 disaccharide molecule was kept unmoved, whereas the HA2 disaccharide was rotated and moved to form a linear tetrasaccharide with HA1. Then, the coordinates of the derived tetrasaccharide were optimized against the protein part using energy dynamic minimization in X-PLOR (21) with the protein coordinates fixed initially and released in the second round of the refinements.

### RESULTS AND DISCUSSION

**Activation of SagHL**—The native SagHL undergoes extensive proteolysis after its synthesis, resulting in a mixture of at least three molecular mass products: 118, 111, and 92 kDa (18, 23). The 118-kDa form is enzymatically inactive and the 111- and 92-kDa forms are active (18). It suggests that SagHL is synthesized as an inactive 118-kDa form inside the bacteria (23). When the enzyme is secreted into the extracellular matrix, it is activated by the cutting off of the signal segment at the N terminus, leaving an active 111-kDa form (23). The enzyme is then further degraded into a more stable 92-kDa form (16). This last process is likely to be caused by autodegradation in the presence of calcium ions (16). The SagHL structure determination work was performed on the autodegradation product of *hylB*<sub>3502</sub>, which is the 92-kDa form of SagHL.
and its sequence was determined to be from Ser^{171} to Ile^{984} (16).

**Overall Structure**—All 814 residues were modeled in the 2.1-Å resolution SagHL crystal structure. A total of 538 waters were incorporated into the final SagHL model. The final $R_{cryst}$ is 0.191 and $R_{free}$, 0.253. In the final structure, 99.6% of the residues fall in the energetically favored regions in the Ramachandran plot (24). Two residues, Thr^{400} and Asn^{769}, are in disallowed regions. Both residues are located on the surface loop regions, and their electron densities are plausible. The side chains of Glu^{468} and Tyr^{635} form several hydrogen bonds with the main chain atoms of these two residues, respectively. These interactions likely displace Thr^{480} and Asn^{769} to the energetically unfavorable regions.

The SagHL crystal structure is constructed from three structural domains (Fig. 1A), the N-terminal β-sheet domain (βI-domain, containing residues from Ala^{171} to Val^{244}), the middle α-helical domain (α-domain, residues from Phe^{252} to Asn^{611}), and the C-terminal β-sheet domain (βII-domain, residues from Lys^{621} to Ile^{984}). Two peptide linkers connect these three domains. Linker I (residues from Thr^{245} to Asn^{252}) connects the βI-domain and the α-domain, and linker II (residues from Asp^{612} to Leu^{620}) connects the α-domain and the βII-domain. The α-domain is primarily composed of 13 α-helices that are named sequentially from helix 1 to helix 13 from the N terminus to the C terminus (Fig. 1B). Ten of them (helix 3 to helix 12) are arranged into an incomplete $α_2/α_4$ barrel structure, and helix 2 and helix 13 are in the position of blocking the incomplete barrel opening. The two ends of this barrel structure are not of the same size. The larger end encompasses the predominant cleft, which is located between the α- and the βI-domains. The smaller end is blocked by helix 1. The N-terminal βI-domain is primarily composed of seven β-strands arranged into two anti-parallel β-sheets. The C-terminal βII-domain is mainly composed of 25 β-strands arranged into five anti-parallel β-sheets.

The orientation of the N-terminal segment from Ser^{171} to Glu^{182} extends out of the βI-domain along the surface of the α-domain toward the cleft region (Fig. 1A). Such orientation of this segment of the enzyme suggests that this part might be a linker to yet another structural domain at the N terminus. It is possible that the unknown part of the structure, the first 212 residues in the sequence of SagHL at the N terminus, might form at least one structural domain near the cleft region. The function of the βI-domain is still unclear, but it might possibly serve as a connection of the catalytic α-domain (see below) to this unknown structural domain.

The cleft in the α-domain of SagHL is located in the middle of the SagHL structure, close to the interface between the α-domain and the βII-domain (Fig. 1). The dimensions of this cleft are about 30 Å in length, 10 Å in width, and 10 Å in depth. A total of 28 charged residues, 22 from the α-domain and 6 from the βII-domain, are accumulated along the edges of the cleft. These residues are Lys^{778}, Lys^{292}, Lys^{299}, Lys^{305}, Asp^{306}, Arg^{321}, Arg^{380}, Arg^{416}, Asp^{422}, Lys^{468}, Glu^{468}, Asp^{478}, Lys^{479}, Asp^{484}, Arg^{540}, Arg^{542}, Lys^{546}, Glu^{557}, Arg^{560}, Arg^{564}, and Lys^{586} from the α-domain; and Glu^{667}, Glu^{668}, Lys^{712}, Lys^{720}, Lys^{725}, and Lys^{868} from the βII-domain. Most of these residues are positively charged, including 10 lysines and 9 arginines. These charged residues are distributed along both sides and the bottom of the cleft and likely contribute to the attraction and binding of the negatively charged, tape-shaped substrates, HA or CS. This charged region of the enzyme is by far its most positively charged segment overall.

**SagHL-ΔDi-HA Complex Structure and Substrate Binding**—To confirm that the cleft is the place where the substrate/product bind to pursue the enzymatic action catalyzed by SagHL, the product of this enzyme, 4,5-unsaturated disaccharide, ΔDi-HA, was soaked into the SagHL crystal. The refined structure of native SagHL without water molecules was used as primary model for the SagHL-ΔDi-HA complex structure solution and refinements against 45,510 reflections. The final $R_{cryst}$ was 0.182, and the $R_{free}$ was 0.254; 332 water molecules were incorporated into the electron density as described under “Experimental Procedures.”

The protein part of the complex structure is similar to the native SagHL structure with the root mean square deviation of 0.89 Å. Similarly to the native enzyme, it consists of three structural domains: βI, α, and βII. The orientation of the residues in the cleft is virtually the same as in the native enzyme structure. The structure of HA substrate has two distinct features that facilitate its binding to the enzyme cleft. One is the negative charges due to the carboxylate groups along the HA chain, and the hydrophobic patches generated by the sugar rings of the tape-shaped HA chain. The arrangement of these hydrophobic patches is ambidextrous (25). Two ΔDi-HA mole-
cules were observed binding to the cleft region in the SagHL-

Di-HA complex structure and were named as the HA1 and

HA2 position Di-HA, respectively (Fig. 2). HA1 is in contact

with six protein residues from the α-domain only (Fig. 3A), and

HA2 is in contact with another group of six residues from both

the α- and the βII-domains (Fig. 3B). Enzyme residues inter-

acting with these two Di-HA molecules and the hydrogen

bonds at their interfaces were listed in Table II. As expected,

the sugar rings of the N-acetyl-β-D-glucosamines of HA1 and

HA2 are in a chair conformation, whereas the sugar rings of the

unsaturated β-D-glucuronate are in a half-chair conformation.

The SagHL-Di-HA complex structure confirmed that the cleft

is the region where the substrates are bound and degraded.

**Modeling of the Substrate Binding and the Active Site Iden-

tification**—The two Di-HA molecules, HA1 and HA2, observed

in the SagHL-Di-HA complex structure were not connected
together as it would be in the case of a tetrasaccharide, al-

though their positions are close to such arrangement. To ana-

lyze more precisely the substrate binding pattern, a tetrasac-

charide hyaluronan was modeled into the HA binding cleft of

the enzyme based on the two Di-HA positions in the SagHL-

Di-HA complex structure (HA1 and HA2; as described under

“Experimental Procedures”) (Fig. 4). The two-disaccharide

building blocks of the tetrasaccharide were also named HA1

and HA2 because the displacement from their corresponding

positions in the complex structure is small. HA1 is located at

the reducing end of the HA substrate chain, and HA2 is at the

nonreducing end. HA1 disaccharide was almost unmoved, and

the HA2 disaccharide needed to be slightly rotated to obtain

the tetrasaccharide arrangement. HA2 was rotated so that the

N-acetamino-2-deoxy-D-glucose residue of HA2 is facing the

D-glucuronic acid residue of HA1 and to allow for the β-1,4

FIG. 2. Electron density maps for two Di-HA molecules in the SagHL-Di-HA complex structure. The figure was made using O (20)

and Ribbons software (40). The figure shows the 2Fo − Fc electron density maps at 1σ level for HA1 Di-HA (A) and HA2 Di-HA (B).
glycosidic with HA1. In the complex structure, 4 arginine residues (Table II) modify the orientation of the electronegative HA2 ΔDi-HA away from the position of alignment along the cleft to a new rotated orientation (Fig. 3B). There are four possible ways to fit the tetrasaccharide into the cleft. The tetrasaccharide position in the cleft can be end-flipped (the reducing versus the nonreducing end) or side-flipped (the inner versus the outer side of the cleft). Tetrasaccharide in Fig. 4 has no bad interactions with the enzyme residues in the cleft, and the positions of two ΔDi-HA molecules in the complex need the least movement to obtain this arrangement, whereas in any other three positions, tetrasaccharide has significant number

**Fig. 3.** Protein-product interfaces in the SagHL-ΔDi-HA complex structure. Residues from the α-domain were placed above the ΔDi-HA position and residues from the βII-domain below the ΔDi-HA. ΔDi-HA molecules are colored lavender and protein residues are colored green. H-bonds and/or salt bridges are shown in dotted lines. The figure was produced using Ribbons software (40). A, protein-HA1 ΔDi-HA interface; B, protein-HA2 ΔDi-HA interface.

**Table II**

Selected enzyme interactions with the disaccharide product of degradation

<table>
<thead>
<tr>
<th>Disaccharide</th>
<th>Contacting residues</th>
<th>No. of contacts</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA1</td>
<td>Asn(^{370}) (7), Trp(^{377}) (11), Arg(^{416}) (16), Asn(^{429}) (4), Phe(^{423}) (8), Asn(^{429}) (4), water (1)</td>
<td>48</td>
</tr>
<tr>
<td>HA2</td>
<td>Arg(^{321}) (8), Trp(^{372}) (5), Arg(^{390}) (2), Asp(^{432}) (3), Arg(^{546}) (7), water (1)</td>
<td>27</td>
</tr>
</tbody>
</table>

b. Selected distances\(^{a}\) between protein and ΔDi-HAs for the SagHL-ΔDi-HA complex

<table>
<thead>
<tr>
<th>Atom in disaccharides</th>
<th>Atoms in contacting residues</th>
<th>Distance Å</th>
</tr>
</thead>
<tbody>
<tr>
<td>UA1 of HA1(^{c})</td>
<td>Asn(^{429}) ND2</td>
<td>3.01</td>
</tr>
<tr>
<td>O3(^{d})</td>
<td>Arg(^{416}) NH1</td>
<td>3.06</td>
</tr>
<tr>
<td>O6</td>
<td>Trp(^{377}) NE1</td>
<td>3.07</td>
</tr>
<tr>
<td>O6</td>
<td>Asn(^{429}) ND2</td>
<td>3.81</td>
</tr>
<tr>
<td>NAc1 of HA1</td>
<td>C6</td>
<td>3.58</td>
</tr>
<tr>
<td>C6</td>
<td>Asn(^{429}) ND2</td>
<td>3.58</td>
</tr>
<tr>
<td>O6</td>
<td>Asn(^{429}) OD1</td>
<td>3.43</td>
</tr>
<tr>
<td>UA2 of HA2</td>
<td>O3</td>
<td>2.82</td>
</tr>
<tr>
<td>O3</td>
<td>Asp(^{432}) OD2</td>
<td>2.83</td>
</tr>
<tr>
<td>O6</td>
<td>Arg(^{390}) NH2</td>
<td>2.78</td>
</tr>
<tr>
<td>O6</td>
<td>Arg(^{546}) NH2</td>
<td>3.38</td>
</tr>
<tr>
<td>NAc2 of HA2</td>
<td>O7</td>
<td>3.40</td>
</tr>
<tr>
<td>O7</td>
<td>Arg(^{416}) NH1</td>
<td>3.08</td>
</tr>
</tbody>
</table>

\(^{a}\) The numbers in parentheses show the number of contacts that particular residue made with ΔDi-HA molecules. Atom pairs within 4.1 Å were considered to be in contact.

\(^{b}\) These selected distances were also marked using dotted lines on Fig. 3 (A and B).

\(^{c}\) UA1 and UA2 denote the unsaturated analog for β-D-glucuronic acid of HA1 and HA2, whereas NAc1 and NAc2 denote N-acetyl-β-D-glucosamine of HA1 and HA2, respectively.

\(^{d}\) The symbols assigned to atoms of the disaccharide product follow standard chemical nomenclature.
of bad interactions with the residues of the cleft. The interactions were considered too close (or bad) when any two-atom pairs between the modeled substrate and the enzyme were in a distance of 2.2 Å or closer. In addition in this orientation, there is enough space left in the cleft to accommodate one more disaccharide unit at the nonreducing end of the HA chain, which was named the HA3 position.

At this modeled and refined position, the tetrasaccharide substrate makes 16 interactions with 10 enzyme residues, which include Arg321, Asn370, Trp371, Trp372, Arg380, Arg416, Asn421, Phe423, Asn429, and Asp432. No bad contacts (distance between atom pairs within 2.4 Å) were present at the enzyme-substrate interface.

Although not in the above list, His479 was the initial residue identified earlier by site-directed mutagenesis studies as a possible catalytic residue in SagHL (16, 26). In the SagHL crystal structure, this residue is located at the bottom of the cleft. In the tetrasaccharide complex model, the NH2 side chain atom of His479 was pointing toward the C5 atom of the HA1 glucuronate sugar residue (Fig. 4). Site-directed mutagenesis studies of SagHL performed by Pritchard et al. (26) showed that single mutations of N429A, H479G, H479A, Y488F, Y488T, and Y488L inactivated the enzyme completely. It suggested their possible involvement in the enzyme catalysis. Based on the mutations and the native structure, the disaccharide complex structure, and the tetrasaccharide substrate model, the active site residues can be classified into two parts: Trp371, Trp372, and Phe423 forming a hydrophobic patch, and His479, Tyr488, and Asn429 forming a catalytic group. Their proposed function in the degradation of HA and CS will be discussed in detail in the following section. The relative positions of these residues with respect to the tetrasaccharide substrate are shown in Fig. 4.

Proposed Mechanism of Hyaluronan Degradation—Based on the SagHL native, the product complex structures, the modeled tetrasaccharide complex, the site-directed mutagenesis studies (26), and our previous studies on SpnHL (12), the enzymatic HA degradation by SagHL can be postulated as the following five successive steps (Fig. 5). First, the positively charged cleft of the enzyme attracts the anionic HA substrate chain and binds to it. In the second step, the aromatic patch of the enzyme composed of Trp371, Trp372, and Phe423 accurately anchors and positions the tape-shaped, ambidextrous substrate chain into the correct degradation position. At this stage the side chain of Trp372 is likely parallel to the hydrophobic patch on the N-acetylglucosamine residue of the HA2 disaccharide unit and the side chain of Phe423 is parallel to the hydrophobic patch on the glucuronic residue of the HA1 disaccharide unit, which stabilizes and positions these two disaccharide units in the precise position in the cleft. Such interactions between the polysaccharide chain and the aromatic residues are widely seen in protein-carbohydrate complexes (27). In this way, the β1,4 glycosidic linkage cleavage site on the bound in the cleft HA chain is properly positioned and selected for degradation. At this position, the carboxylate group of the HA1 glucuronate sugar residue is oriented toward the side chain of catalytic residue Asn429. The glycosidic oxygen O4 points toward the Oγ atom of Tyr488, and the C5 of the HA1 glucuronate points toward His479.

The side chain of Asn429 forms a couple of hydrogen bonds with the carboxylate group of the HA1 glucuronate moiety of HA, attracting the negative charges on the carboxylate group away from C5 and resulting in a relatively acidic C5 hydrogen. In the third step, this C5 atom loses a proton that is withdrawn by His479, resulting in the rehybridization of the C5 carbon from sp3 to sp2. In the fourth step, at the same time as His479 withdraws one proton from HA1 glucuronic C5, Tyr488, located at the bottom of the cleft and interacting with the O4 oxygen atom of the glycosidic linkage, donates a proton to this glycosidic oxygen (O4) and breaks the glycosidic linkage. HA1 glucuronic C4 is thus rehybridized from sp3 to sp2 similarly to the C5 carbon. Therefore, a double bond is formed between the two (C4 and C5) sp2 hybridized carbon atoms of the HA1 disaccharide, which accomplished the unsaturation of the product. We observed that, among the dominantly positively charged residues in the cleft, there are also three negatively charged residues, Glu468, Asp478, and Thr480, clustered together at the HA1 position, opposite to Phe423. These three residues form a negative patch (Fig. 6). After the covalent glycosidic linkage is broken, this negative patch is likely responsible for repelling the negatively charged products out of the cleft (product release). In the fifth and the final step, His479 loses its acquired proton and Tyr488 attracts a proton probably from the surrounding water molecules; thus, the enzyme returns to its original state and is ready for the next round of catalysis. We
Tyr246 was suggested to be both general acid and base. Such a sink for the alginate carboxyl group. Additionally, the role of the group I enzymes. An example of group II enzyme is chondroitinase AC lyase (28) and alginate by alginate lyase (29) suggest slightly different mechanisms of action of these two enzymes. For the chondroitinase AC lyase, in addition to the PAD mechanism, a catalytic Tyr residue (Tyr234) was suggested to act as both, general base and acid. For SagHL, the role of a homologous Arg residue is only to stabilize the environment (Arg288) was suggested as a general acid. For SagHL, the role of the catalytic Tyr residue (Tyr234) was suggested to act as both, general base and acid. In the mechanism proposed above, the aromatic patch is important in the selection of cleavage site on the substrate chain. Through hydrophobic interactions, the two tryptophan residues anchor the HA2 disaccharide unit and Phe423 anchors the HA1 disaccharide position through their hydrophobic interactions, whereas the glycosidic linkage between HA1 and HA2 is being degraded (Fig. 4). Single mutations of either of the two neighboring tryptophan residues did not significantly affect the enzyme activity, whereas double mutation W371F/W372V completely eliminated the enzyme activity (26). The two tryptophan residues together are likely predominantly responsible for the binding and positioning of the HA2 disaccharide unit. However, without Phe423, the substrate cannot be properly positioned at the HA1 position, and, therefore, the substrate most likely cannot be degraded. The binding of the substrate at the HA3 position in the cleft is different among group II enzymes. There are no aromatic residues around the HA3 position, and there are no hydrophobic interactions involved in the HA3 disaccharide binding. Ionic interactions and hydrogen bonds that are different from the HA1 and HA2 disaccharide binding mainly bind the HA3 disaccharide. In summary, the phenylalanine residue corresponding to Phe423 in SagHL in group I bacterial lyases stabilizes and positions the HA1 disaccharide in the active site.

**Action Pattern**—The difference in the aromatic patch composition between group I and group II bacterial hyaluronate lyases may also explain their different action patterns. The physiological molecular weight of HA is usually several million daltons (7). One HA chain may contain a few hundred to over 5,000 disaccharide units. Bacterial hyaluronate lyases may act on the HA chain in two different patterns of degradation, either in a processive or nonprocessive way (31, 32). In a nonprocessive degradation pattern, the enzyme leaves the substrate chain after the first degradation, then binds randomly to another cleavage site on the same or different substrate chain. In a processive degradation pattern, the enzyme binds to a cleavage site of the polymeric substrate chain, degrades it and

![Fig. 6. Electrostatic potential distribution in the catalytic cleft. The cleft is placed in the same orientation as in Figs. 1–4. The positive potential is shown in blue and negative potential in red. Majority of the cleft is highly positively charged, whereas at the product-releasing end of the cleft (HA1 position) a negative patch is clearly present as labeled. The position of the hydrophobic patch is also labeled. The figure was made using Grasp (41).](Image 256x520 to 554x729)

**Substrate Binding in the Catalytic Cleft**—Based on the products produced, bacterial hyaluronate lyases can be divided into two groups. Group I enzymes degrade HA into unsaturated disaccharide only, whereas group II enzymes degrade HA into unsaturated oligosaccharides, usually tetra- or hexasaccharides, or a mixture of the two (30). SagHL produces unsaturated disaccharide units only (16, 18) and is, therefore, a member of the group I enzymes. An example of group II enzyme is the *Streptomyces hyaloxyticus* hyaluronate lyase, which was shown to produce a mixture of tetra- or hexasaccharides (30).

The three catalytic residues, Asn191, His179, and Tyr468, in SagHL are conserved among all known bacterial hyaluronate lyases, but the aromatic patch residues (Trp371, Trp372, and Phe423) are only partly conserved (Fig. 7). The two neighboring tryptophan residues of the patch, Trp371 and Trp372 in SagHL, are uniformly conserved whereas Phe423 is only conserved in group I lyases such as SpnHL and SagHL, not in group II enzymes (Fig. 7). In the mechanism proposed above, the aromatic patch is important in the selection of cleavage site on the substrate chain. Through hydrophobic interactions, the two tryptophan residues anchor the HA2 disaccharide unit and Phe423 anchors the HA1 disaccharide position through their hydrophobic interactions, whereas the glycosidic linkage between HA1 and HA2 is being degraded (Fig. 4). Single mutations of either of the two neighboring tryptophan residues did not significantly affect the enzyme activity, whereas double mutation W371F/W372V completely eliminated the enzyme activity (26). The two tryptophan residues together are likely predominantly responsible for the binding and positioning of the HA2 disaccharide unit. However, without Phe423, the substrate cannot be properly positioned at the HA1 position, and, therefore, the substrate most likely cannot be degraded. The binding of the substrate at the HA3 position in the cleft is different among group II enzymes. There are no aromatic residues around the HA3 position, and there are no hydrophobic interactions involved in the HA3 disaccharide binding. Ionic interactions and hydrogen bonds that are different from the HA1 and HA2 disaccharide binding mainly bind the HA3 disaccharide. In summary, the phenylalanine residue corresponding to Phe423 in SagHL in group I bacterial lyases stabilizes and positions the HA1 disaccharide in the active site.

**Experimental Procedures**). Under physiological conditions, the concentration of the product to be released because of a very high concentration of the substrate chain after the first degradation, then binds randomly to another cleavage site on the same or different substrate chain.
moves along the substrate chain continually until the whole chain is degraded.

Biochemical studies of the hyaluronan degradation and product analysis showed that the action pattern of the group I enzyme SagHL is processive and produces disaccharide only (32). In contrast, the action pattern of the group II enzyme, for example, S. hyalurolyticus hyaluronate lyase, is nonprocessive and produces a mixture of tetra- and hexasaccharides (31). Among the active site residues, the difference between these two groups of bacterial hyaluronate lyases is in the phenylalanine residue of the aromatic patch. In group I bacterial hyaluronate lyases, after the initial degradation is accomplished and the product is released, HA1 position is emptied. Phe\(^{423}\) immediately attracts the next HA unit into the degradation position through hydrophobic interactions between the phenyl group of Phe\(^{423}\) and the next disaccharide unit of the HA substrate chain before the remaining substrate chain can leave the cleft. Through this hydrophobic matching, the next disaccharide unit is accurately anchored into the HA1 position for the next round of substrate degradation process. Because the HA substrate chain is moved by one disaccharide unit at each time, only \(\Delta\)Di-HA is produced. Additionally, the enzyme action pattern is processive. In group II bacterial hyaluronate lyases, without this phenylalanine, the hydrophobic stability force provided by the side chain of phenylalanine is missing, the remaining substrate chain leaves the cleft, and the enzyme randomly chooses and binds to another cleavage site on the substrate chain. In this case, the enzyme action pattern will be nonprocessive as in the group II bacterial hyaluronate lyases.

**Direction of Hyaluronan Chain Degradation—SagHL degrades HA in the processive pattern (32), which leads to the question of the direction of the enzyme movement during the degradation of the HA substrate chain. Several lines of structural evidence suggest that this processive degradation of the HA substrate chain is accomplished from the reducing end to the nonreducing end. First, the catalytic residues of the enzyme are located at the reducing end of the bound HA2 substrate-like molecules, a disaccharide product. The structural arrangement of the catalytic residues provides the enzyme with the ability to cleave the substrate chain at the reducing end of the \(\beta\)-1,4 glycosidic linkage and the product unsaturation happens at the nonreducing end glucurionate C4-C5 of the HA1 disaccharide unit. Second, the negative patch, composed of Glu\(^{468}\) Asp\(^{478}\) and Thr\(^{490}\) is located at the \(\beta\)I-domain side of the HA1 position, opposite to Phe\(^{423}\) in the cleft (Fig. 6). This negative patch repels the negatively charged product, HA1, out of the cleft while the HA2 end of the chain (which corresponds to the nonreducing end of the substrate chain) is still bound to the cleft. Third, the product release allows the HA1 position in the cleft to accept another disaccharide unit of the substrate chain. The Phe\(^{423}\) residue guides the substrate chain into the emptied HA1 position before the long HA chain leaves the cleft. In this way, the disaccharide unit at the HA2 position moves into the HA1 position and HA3 moves into HA2. The disaccharide unit next to HA3 at the nonreducing end of the HA chain moves into the HA3 position. Therefore, the HA chain is degraded in the way that the HA substrate chain moves from reducing end successively to the nonreducing end. As a comparison, the cellulose degradation by cellobiohydrolase I is also performed in a processive pattern and the polysaccharide chain is degraded from the reducing end to the nonreducing end (33).

At this time, the molecular directionality of polymerization of vertebrate and streptococcal HA is unclear. Early reports suggested that the streptococcal HA is elongated at the nonreducing end extrudes through the plasma membrane into the pericellular space (34).**

**Degradation of CS—CS is another substrate of SagHL (35). Both HA and CS are members of a large polysaccharide family known as glycosaminoglycans. Glycosaminoglycans are a group of heterogeneous polysaccharides built of repeating disaccharide units and contribute to the complex architecture of the extracellular matrix. The disaccharide unit employed to build CS is constructed by \(\beta\)-1,3-linked glucuronate and galactosamine. The glycosidic linkage between disaccharide units is \(\beta\)-1,4. CS is usually 4- and/or 6-sulfated on the N-acetylgalactosamine residue (35). The product unsaturation also occurs on the glucuronate residues of the CS chain in the lyase degradation process. When chondroitin molecules were modeled into the cleft of the SagHL structure (data not shown), the same group of catalytic residues targeted very well for the CS degradation. The unsulfated regions of CS can be degraded the same way through the degradation mechanism as shown earlier for HA. For the sulfated CS regions, the HA1 4-sulfated galactosamine is in conflict with Phe\(^{423}\). Therefore, SagHL likely can not degrade 4-sulfated CS. The 6-sulfated CS at the HA1 and other positions is not in conflict with Phe\(^{423}\) or any other enzyme residues. It can be expected from this structure analysis that, in the CS degradation by SagHL, only unsulfated or 6-sulfated galactosamine were detected (35).**

**Structural Comparison to S. pneumoniae Hyaluronate Lyase—The crystal structure of SpnHL has recently been de-
The field with the largest end of the barrel structure (the whole structure is 2.47 Å). In SpnHL, the catalytic residues are His399, Tyr408, and Asn349. Site-directed mutagenesis and enzymatic activity measurements on the SpnHL enzyme had shown that mutations Y408F, H399A, and N349A eliminate the enzyme function.

The catalytic residues are conserved between these two enzymes (Fig. 7). In SpnHL, the catalytic residues are His266, His266, and Tyr265. Site-directed mutagenesis and enzymatic activity measurements on the SpnHL enzyme had shown that mutations Y408F, H399A, and N349A eliminate the enzyme function.

Conclusions—HA plays an important role in the construction of the extracellular matrix of virtually all animal tissues. In humans, HA metabolism is a fast process as compared with the metabolism of other polysaccharides. One third of HA in a human body is overturned every day (37). The pathogenic strains of S. agalactiae that cause meningitis and septicaemia and other serious diseases in humans, especially neonates, usually secrete more hyaluronate lyase to degrade HA and CS, two major kinds of polysaccharide. This degradation process is linked to catalytic and aromatic patch residues identified for SpnHL (39). Sequence alignment revealed that all catalytic and aromatic patch residues identified for SpnHL are also present in the xanthan lyase sequence. Therefore, it can be anticipated that the catalytic center in xanthan lyase is likely to be composed by Asn349, His399, and Tyr408, and the aromatic patch is likely participated in the cleavage sites selection on the xanthan substrate chain. Xanthan lyase is likely to utilize similar PAD degradation mechanism for the xanthan degradation.

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