Mechanism of Hyaluronan Degradation by *Streptococcus pneumoniae* Hyaluronate Lyase

STRUCTURES OF COMPLEXES WITH THE SUBSTRATE*

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Hyaluronate lyase enzymes degrade hyaluronan, the main polysaccharide component of the host connective tissues, predominantly into unsaturated disaccharide units, thereby destroying the normal connective tissue structure and exposing the tissue cells to various endo- and exogenous factors, including bacterial toxins. The crystal structures of *Streptococcus pneumoniae* hyaluronate lyase with tetra- and hexasaccharide hyaluronan substrates bound in the active site were determined at 1.52- and 2.0-Å resolution, respectively. Hexasaccharide is the longest substrate segment that binds entirely within the active site of these enzymes. The enzyme residues responsible for substrate binding, positioning, catalysis, and product release were thereby identified and their specific roles characterized. The involvement of three residues in catalysis, Asn249, His209, and Tyr408, is confirmed, and the details of proton acceptance and donation within the catalytic machinery are described. The mechanism of processivity of the enzyme is analyzed. The flexibility (allosteric) behavior of the enzyme may be understood in terms of the results of flexibility analysis of this protein, which identified two modes of motion that are also proposed to be involved in the hyaluronan degradation process. The first motion describes an opening and closing of the catalytic cleft located between the α- and β-domains. The second motion demonstrates the mobility of a binding cleft, which may facilitate the binding of the negatively charged hyaluronan to the enzyme.

*Streptococcus pneumoniae* colonizes predominantly the upper respiratory tract of humans and is a major human pathogenic bacterium. It is one of the key causes of life-threatening disease such as pneumonia, bacteremia, and meningitis (1). It also causes less threatening diseases that are, however, very prevalent like otitis media and sinusitis (2). Pneumococci interact with the host and its tissues through the surface sugars (capsule) and a variety of usually surface-exposed protein molecules. These interactions are essential for the full pathogenicity of these bacteria and are likely involved in the disease-causing processes. The proteins known to be involved in this interaction include, among others, hyaluronate lyase (3, 4), pneumolysin (5), pneumococcal surface protein A (6), and pneumococcal surface antigen A (7, 8).

*S. pneumoniae* hyaluronate lyase (SpnHL)primarily degrades hyaluronan (HA), the predominant polysaccharide component of animal and human connective tissues and the nervous system, into unsaturated disaccharide units as the end products (9). Cells in the connective tissues are embedded in the strikingly viscoelastic HA matrix. Hyaluronan is a polymeric glycan composed of linear repeats of a few hundred to as many as 20,000 or more disaccharide units of glucuronic acid and N-acetyleglucosamine. The glycosidic linkage present within the disaccharide unit is β-1,3, whereas the disaccharide units are connected with the β-1,4-glycosidic linkage. The HA metabolism seems to be more significant than the metabolism of other polysaccharides in vivo due to the large turnover of this sugar. In human, one-third of the HA (about 5 g) is turned over daily (10). The rapid turnover rate facilitates the use of HA and its degradation products in many physiological processes, such as cell differentiation and development (11), cell proliferation, recognition, locomotion, and the immunological responses (12).

Enzymes of either mammalian (including host or human enzymes) or bacterial origin degrade HA at the β-1,4-linkage exerting the endo- and exogenous pressures on the host. Bacteria secrete hyaluronate lyases to degrade HA, and these enzymes usually produce unsaturated di-, tetra- (HA₄), or hexasaccharides (HA₆). The mechanism of this degradation by *Streptococcus* species hyaluronate lyase, which produces primarily unsaturated disaccharides of HA, 2-acetamido-2-deoxy-3-O-(β-D-glucopyranosyluronic acid)-D-glucose, as the end products, was recently proposed and involves the elimination reaction introducing an unsaturated bond to the product (9). Mammals, on the other hand, express hyaluronidasises to degrade HA and to produce relatively longer oligosaccharides (13). The detailed atomic mechanism of HA degradation by mammal hyaluronidasises is still largely unknown due, in part, to the lack of structural information. This process likely relies on the displacement mechanism based on hydrolysis, similar to

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‡ The atomic coordinates and structure factors (code 1Loh and 1Lxk) have been deposited in the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).

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1 The abbreviations used are: SpnHL, *S. pneumoniae* hyaluronate lyase; ED, essential dynamics; MD, molecular dynamics; HA, hyaluronan; HA₄, tetrasaccharide of HA; HA₆, hexasaccharide of HA; HA₁, and HA₂, HA₃, positions of hyaluronan building blocks of disaccharide for HA numbered from the reducing toward the non-reducing end; PAD, proton acceptance and donation.
that of celluases, to degrade the HA substrate (13). Mammalian hyaluronidases (mainly bovine hyaluronidase) are, however, widely used in clinics, for example as an additive to the local anesthesia for the fast spreading and penetration of medications through tissues (14, 15).

The structure of the native SpnHL (9) and its complex with the disaccharide products of HA degradation (16) were recently reported. This structural information together with further characterization of the enzyme (17, 18) allowed for the formulation of the proposed mechanism of HA degradation (9, 13). Briefly, the mechanism of catalysis, termed proton acceptance and donation, involves a five-step process involving neutralization of the carboxylate of a glucuronic moiety, extraction of a proton from C-5 followed by a double bond formation in the glucuronate and breaking of the β-1,4-glycosidic linkage. Additional comparison to other enzymes degrading glycans like Streptococcus agalactiae hyaluronate lyase (19), Flavobacterium heparinum chondroitin AC lyase (20), and Sphingomonas species alginate lyase A1-III (21) allowed for generalization of the pneumococcal enzyme mechanism and suggested a similar mechanism of action for these additional enzymes (13). Here we report the structures of the S. pneumoniae hyaluronate lyase complexes with its substrates, the tetra- and hexasaccharide units of hyaluronan, and we discuss their implications for the mechanism of action of the enzyme.

EXPERIMENTAL PROCEDURES

Enzyme and Substrate Preparation, Crystallization of Complexes, and Collection of Diffraction Data—The SpnHL Y408F mutant cloning, overexpression in Escherichia coli (pMMJ004), and purification were reported previously (9, 22). The mutant enzyme was concentrated to 5 mg/ml in 10 mM Tris-HCl, pH 7.4, 2 mM EDTA, and 1 mM Dl-thioglycolate and stored at −80 °C until their use. The tetra- and hexasaccharide substrates were also obtained as described previously (17, 18). To prevent degradation they were stored frozen at −80 °C in 10 mM Tris-HCl buffer, pH 8.0, until their use.

The crystals of Y408F mutant version of SpnHL were obtained using the same conditions as for the native enzyme crystals (100 mM sodium cacyclate buffer, pH 6.0, 2.9 M ammonium sulfate) but with the addition of 5 mM EDTA (23). Crystallization of Y408F mutant enzyme was accomplished using the vapor diffusion hanging drop method in 24-well Linbro culture plates using equal volumes of the protein sample and the reservoir solution (2 μl of each) at 22 °C (24, 25). The protein and reservoir solution were mixed and equilibrated against 1.0 ml of reservoir solution. Diffraction quality rectangular block shaped crystals of Y408F (0.5 × 0.3 × 0.3 mm) were grown within several days. The crystals of Y408F SpnHL were then soaked at room temperature with 20 mM tetra- and hexasaccharide substrates (both in 10 mM Tris-HCl buffer at pH 8.0) for 10 h directly before the x-ray diffraction data collection. The crystal soaking solution contained the crystallization conditions (100 mM sodium cacyclate buffer, pH 6.0) with increased concentration of the precipitation agent, ammonium sulfate, to 3.2 M.

The crystal was cryo-protected using the same conditions as for the native crystals (23) and flash freezing them at −170 °C in a nitrogen flow using a Cryostream Cooler (Cryosystems, Oxford, UK). The diffraction data sets were collected using synchrotron radiation at the wavelength of 1.0 Å at the 19-BM beamline at the Argonne National Laboratory, Advanced Photon Source, Structural Biology Center using an Oxford 3 × 3 charged-coupled device detector. The diffraction limits of the crystals were 1.52- and 2.0-Å resolution for the tetra- and hexasaccharide complexes, respectively. The data were processed and scaled using the HKL2000 package (26). The crystals of complexes were isomorphous to the native ones. The final data processing parameters are reported in Table I.

Determination of Structures of Complexes with Tetra- and Hexasaccharides—The native SpnHL crystal structure without water molecules (9) was used as the model for the complex structure solution. The Rmerge flag reflections (1.249 reflections, 2% (1,205 reflections), and 2% (1,205 reflections), respectively, to validate the refinement progress (27). Initially, a round of rigid body refinements using only the model was performed using X-PLOR (28). Refinements progress using program X-FLOR with the positional and simulated annealing protocols (28, 30) and was traced using inspection on graphics with program O (29). The structures were refined against 124,927 and 60,437 reflections for the tetra- and hexasaccharide complexes, respectively, and utilizing all data from 50 Å to the highest resolution available and a 2σ(F) cut-off (Table I). The electron density for the substrates was then clearly identified, and the incorporation of the substrates into this density was followed. The topologies and parameter files for both substrates were manually created following our earlier studies with disaccharide substrates and carefully corrected to reflect ideal stereochemical values (16). Additional refinements including individual restrained B-factor refinements, inspection, and manipulation of structure on graphics using O together with incorporation of the ligand molecules placed into the map were performed following standard criteria were performed. After refinement, water molecules whose positions were not supported by electron density, at 1σ contouring, in σ-A weighted Fσ − Fc maps were deleted. A variety of stereochemical (31) and other analyses (29, 32) was periodically performed in order to locate possible model errors. The tetra- and hexasaccharide substrate molecules and 685 and 590 waters were incorporated, respectively, into the final structures of these two complexes. Final refinement parameters are reported in Table I.

Flexibility Analysis and CONCOORD Simulations—The CONCOORD program (33, 34) generates uncorrelated protein structures fulfilling a set of upper and lower interatomic distance limits. These limits are derived from experimental structures through measurement of interatomic distances and eigenvalues of the interatomic length of the atoms involved. Thus, the separation of strongly interacting atoms was allowed to vary only slightly from the observed value, whereas weaker interactions have more relaxed limits. Special consideration was given to interacting atoms within the same secondary structure element in order to ensure the preservation of secondary structure. Although detailed aspects of dynamic variation may not be reproduced by this method, good qualitative agreement has been found between the results of the conventional molecular dynamics and CONCOORD simulations (33–35). Trajectories containing 500 conformations were calculated.

Essential Dynamics Calculations—The essential dynamics (ED) was performed by Gromacs 3.0 (36, 37). This method is based on the diagonalization of the covariance matrix of atomic fluctuations, which yields a set of eigenvalues and eigenvectors (38). The eigenvectors indicate directions in a 3N dimensional space (where N = number of atoms) and describe concerted fluctuations of the atoms, constructed such that the amount of fluctuation along a small number of eigenvectors is maximized. The eigenvalues reflect the magnitude of the fluctuation along with the respective eigenvectors. The central hypothesis of this method is that the motions along the eigenvectors with the largest eigenvalues are essential for describing the function. The motion along any desired eigenvector can be inspected by projecting the frames from the trajectory onto that eigenvector. The figures were prepared using Ribbons (39), Grasp (40), O (29), and Molscript (41).

RESULTS AND DISCUSSION

Choice of the Mutant Enzyme and Substrates—The native S. pneumoniae hyaluronate lyase enzyme used in our studies was active in the crystallized form. When crystal was combined with the hyaluronan substrates, the degradation to disaccharides was observed. This led to three-dimensional structures for the complexes identical to that of the complex with the disaccharide product reported earlier (16) (data not shown). The presence in crystals of EDTA, to chelate divalent cations such as calcium, which are required for full enzyme activity, was not sufficient to totally prevent substrate degradation by the enzyme (data not shown). The use of an inactive mutant of the enzyme was, therefore, necessary to obtain complexes of the hyaluronan-derived substrates larger than disaccharide HA with the enzyme. Our earlier studies (9) showed that the Y408F mutant of the enzyme is essentially inactive, and for this reason this mutant form of the enzyme was chosen for our work. Indeed, placed into 3σ peaks in σ-A weighted Fσ − Fc maps were not degraded by the enzyme during complex formation in the crystals. The Y408F mutant enzyme consisted of residues Ala168 to Gin892 of the full-length protein and a C-terminal His6 tag engineered for ease of purification (22).

The tetra- and hexasaccharide hyaluronan substrates were chosen because our earlier modeling studies (9, 16) suggested that...
that they should fit entirely within the catalytic cleft of the enzyme and not interfere with the crystallization process. These truncated hyaluronan substrates were produced in milligram quantities from a human umbilical cord hyaluronan by a controlled degradation with Streptomyces hyalurolyticus hyaluronate lyase as reported previously (9, 42, 43). This enzyme is an eliminase and produces tetra- and hexasaccharides with an unsaturated uronic acid at their non-reducing end as the final degradation products. These products were identified using ion spray mass spectrometry experiments.

Solution and Refinement of Structures of Complexes—The crystals of the inactive Y408F mutant form of the enzyme were obtained using the hanging drop vapor diffusion method and essentially the original crystallization condition of the native enzyme reported previously (23). The crystals of the complexes were obtained by soaking the Y408F mutant enzyme crystals with 20 mM tetra- and hexasaccharides of HA for a relatively short time directly before the freezing of the crystals and/or diffraction data collection. The best soaking time was determined to be 10 h. The diffraction data were collected and processed, and structures were solved and refined as described under “Experimental Procedures.” The data in Table 1 show well refined structures with excellent stereochemistry. In a Ramachandran plot (31), a majority of the residues for the tetrasaccharide complex and 2.0–2.07 Å for the hexasaccharide complex.

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The detailed description of the native structure has been reported elsewhere (9).

Structure and Conformation of the Tetra- and Hexasaccharide Hyaluronan Substrates—The structures of both tetra- and hexasaccharide HA substrates are similar in their orientation and placement in the cleft of the enzyme (Figs. 2 and 3). The tetrasaccharide position in the cleft essentially overlaps with a portion of the hexasaccharide substrate at its reducing end. In naming the disaccharide units of HA from the reducing to the non-reducing end, this portion consists of HA1 and HA2. HA3 is unique to the hexasaccharide complex. The sugar rings (N-acetyl-β-D-glucosamine and β-D-glucuronate rings) of both substrates are in chair conformations with the exception of the terminal sugar rings of the unsaturated β-D-glucuronates located at the nonreducing end of both substrates (which are unsaturated due to the presence of the double bond between C-4 and C-5 introduced during substrate production, HA3 for hexasaccharide and HA2 for tetrasaccharide) that are in distorted half-chair conformations. Both substrates, including the sugar ring substituents, fit well within clearly defined electron density. The majority of substituents of the sugar rings was clearly identified in such densities. The current structures closely resemble and agree with our earlier modeling studies (9, 16) of both tetra- and hexasaccharides in the cleft of the native enzyme. There are numerous interactions between the substrates and the enzyme residues including the catalytic, hydrophobic, positive, or negative patch residues. There are no predominant interactions responsible for substrate binding, rather the binding of the substrate is accomplished by numerous interactions of HA with multiple residues of the enzyme. Such binding is consistent with the processive character of the enzyme as described below.

The Catalytic Cleft and the Active Site—The substrate-binding cleft is located at the larger end of the α5β6β5 barrel, and its surface is built primarily from loop areas between the helices of the α-domain. The only residues from the β-domain contributing to the cleft are two loops between β-strands, encompassing residues 578–583 and 766–771. The cleft dimensions are 30 Å in length, 10 Å in width, and 14 Å in depth. Such an elongated and relatively wide cleft allows for easy access of the elongated...
HA substrate. The surface of the cleft is highly electropositive due to the abundance of basic residues at its surface. Hyaluronan is electronegative due to the negative charges of the carboxylate groups along its chain, and it also has significant hydrophobic character. The charge complementarity between the cleft and HA allows for electrostatic interactions between the enzyme and the substrate resulting in substrate binding. The predominant features of the cleft are as follows: (a) multiple basic residues lining the cleft surface making it highly positively charged and thereby facilitating substrate binding; (b) a cluster of three negatively charged residues, Glu388, Asp398, and Thr400, located at the terminal part of the cleft that form a negative patch implicated in product release (reducing end of the bound substrate, see below); (c) three closely placed aromatic/hydrophobic residues, Trp291, Trp292, and Phe343, located next to the catalytic residues of the cleft and forming a hydrophobic patch that is implicated in precise positioning of the substrate for the catalysis; and (d) three residues, Asn349, His399, and Tyr408, that are proposed to be directly involved in catalysis (Figs. 3 and 4).

Based on our earlier site-directed mutation studies, a group of 5 residues was considered to affect directly the catalytic process with 3 of them involved in the catalytic process, Asn349, His399, and Tyr408, and the remaining 2, Arg243 and Asn580, involved in substrate binding. These five mutant enzymes were produced (R243V, N349A, H399A, Y408F, and N580A) and characterized kinetically, and the correlations between the kinetic properties and the structure were made (Fig. 3) (9, 17). Both the structure and the kinetic analysis of the three catalytic residues Asn349, His399, and Tyr408 were shown to be involved directly in catalysis, and their mutations caused significant changes in the $K_{m}$, $V_{max}$, and $V_{max}/K_{m}$ kinetic parameters. Similarly, two additional residues, Arg243 and Asn580 were implicated in substrate binding and translocation but not catalysis (9, 17). The native enzyme obeyed Michaelis-Menten kinetics.

The degradation of HA is thought to be processive with the random initial endolytic enzyme binding to hyaluronan followed by exolytic degradation of the same substrate chain toward its non-reducing end until the whole substrate was degraded (Fig. 5) (17, 45). For the pneumococcal hyaluronate lyase, and for several other known bacterial hyaluronate lyases, the final degradation product is an unsaturated disaccharide derivative of hyaluronic acid (22). The analysis of the native structure, its modeled or x-ray crystal complex struc-
Degradation of Hyaluronan

Selected interactions of hyaluronan lyase residues with the substrate molecules

The symbols assigned to the substrate atoms follow standard chemical nomenclature for sugar molecules.

| Substrate/water atoms | Enzyme/water residues and atoms | Distance for HA<sub>A</sub> | Distance for HA<sub>B</sub> |
|-----------------------|---------------------------------|-----------------------------|-----------------------------|
| NAc<sup>a</sup> of HA1 |                                 |                             |                             |
| C-3                   | Trp<sup>291</sup> NE1           | 3.86                        | 3.95                        |
| C-6                   | Phe<sup>442</sup> CD1           | 3.80                        | 3.70                        |
| O-3                   | Trp<sup>291</sup> NE1           | 2.87                        | 2.89                        |
| O-4                   | Asn<sup>356</sup> NH2           | 3.94                        | 4.00                        |
| O-5                   | Asn<sup>356</sup> NH2           | 3.56                        | 3.66                        |
| O-6                   | Glu<sup>388</sup> OE2           | 3.83                        | 3.94                        |
| O-7                   | Trp<sup>291</sup> NE1           | 3.59                        | 3.61                        |
| UA1 of HA1            |                                 |                             |                             |
| O-4 (glycosidic oxygen between HA1 and HA2) | Phe<sup>408</sup> CZ (Tyr<sup>408</sup> OH)<sup>b</sup> | 4.63 (3.10) | 4.55 (3.18) |
|                       | Arg<sup>662</sup> NH2          | 3.02                        | 3.16                        |
|                       | Wat<sup>1</sup>                | 4.04                        | 4.07                        |
|                       | Wat<sup>2</sup>                | 4.61                        | 3.41                        |
| O-5                   | His<sup>399</sup> NE2           | 3.66                        | 3.73                        |
| O-6 (glucuronic CO<sub>3</sub>) | Asp<sup>449</sup> OD1            | 3.12                        | 2.98                        |
| O-7 (glucuronic CO<sub>3</sub>) | Asp<sup>449</sup> ND2            | 3.56                        | 3.34                        |
|                       | Wat<sup>3</sup>                | 6.99                        | 6.30                        |
|                       | Wat<sup>4</sup>                | 6.39                        | 6.73                        |
| NAc<sub>2</sub> of HA2 |                                 |                             |                             |
| O-3                   | Arg<sup>466</sup> NH1          | 3.34                        | 3.22                        |
|                       | Arg<sup>466</sup> NH2          | 3.67                        | 3.82                        |
| O-5                   | Trp<sup>292</sup> CD2          | 3.83                        | 3.89                        |
| O-7                   | Arg<sup>466</sup> NH2          | 2.94                        | 2.97                        |
| UA2 of HA2            |                                 |                             |                             |
| O-2                   | Arg<sup>466</sup> NH1          | 2.63                        | 3.74                        |
| O-6 (glucuronic CO<sub>3</sub>) | Arg<sup>443</sup> NH1            | 3.63                        | 2.24                        |
| O-7 (glucuronic CO<sub>3</sub>) | Arg<sup>443</sup> NH2            | 3.63                        | 2.90                        |
| NAc<sub>3</sub> of HA3 |                                 |                             |                             |
| O-3                   | Arg<sup>200</sup> NH2          | NA<sup>5</sup>             | 3.51                        |
| O-6                   | Asn<sup>318</sup> OE1          | NA                          | 3.59                        |
|                       | Asp<sup>314</sup> OD1          | NA                          | 3.52                        |
| UA3 of HA3            |                                 |                             |                             |
| O-2                   | Glu<sup>346</sup> OE2          | NA                          | 2.31                        |
| O-4                   | Lys<sup>230</sup> NZ           | NA                          | 2.93                        |
| O-6 (glucuronic CO<sub>3</sub>) | Asn<sup>318</sup> NE2            | NA                          | 3.39                        |
| O-7 (glucuronic CO<sub>3</sub>) | Asn<sup>318</sup> NE2            | NA                          | 3.34                        |

<sup>a</sup> UA1, UA2, and UA3 denote the β-ν-glucuronic acid of HA1, HA2, and HA3, respectively, whereas NAc1, NAc2, and NAc3 denote N-acetyl-β-ν-glucosamine of HA1, HA2, and HA3, respectively.

<sup>b</sup> The distance in parentheses corresponds to the distance between the modeled Tyr<sup>408</sup> OH atom position based on the native enzyme structure position of Tyr<sup>408</sup> residue and the glycosidic oxygen of the corresponding complex structure.

<sup>c</sup> NA, not applicable.

structures with substrates and products of degradation, mutation studies, and the kinetic analysis allowed for the formulation of a five-step catalytic process responsible for HA degradation. This process was proposed to consist from the following: (i) a hyaluronan substrate binding step, (ii) a catalytic step, (iii) hydrogen exchange with the water microenvironment, (iv) an irreversible product release step, and (v) a translocation of the remaining polymeric substrate step (Fig. 5a) (9). These steps are described in more detail below.

Hyaluronan Binding in the Substrate Binding Cleft of the Enzyme—In order for the hyaluronan degradation process to be feasible, the enzyme needs to bind to the substrate utilizing its elongated cleft. However, the immobilized structure of the enzyme in the crystals might not totally reflect the possible changes in and the dynamics of the structural properties of this enzyme. As shown below using a flexibility analysis based on dynamics calculations, the β-domain of the enzyme might have a significant degree of mobility with respect to the α-domain. Therefore, it is possible that the β-domain modulates the accessibility of substrate to the catalytic cleft by opening up or closing down the entrance to this cleft (see below). The Asn<sup>580</sup> residue of the β-domain contributes to the cleft formation in its narrowest part, and mutation of this residue to one with a smaller side chain, Gly, likely widens the cleft entrance. An N580G mutant showed significantly higher activity as the wild type enzyme (9, 17) suggesting that cleft opening had allowed for easier binding of the substrate. The Km and Vmax kinetic parameters of the N580G mutant enzyme are 0.99 ± 0.22 mM and 725 ± 61 mmol/min·mg, respectively. Corresponding values for the native enzyme were determined to be 0.08 ± 0.04 mM and 563 ± 18 mmol/min·mg (17). The comparison of these kinetic parameters clearly shows that the N580G binds the substrate with a lesser affinity (Vmax/Km = 732 for the mutant and 7038 for the wild type enzyme) but is a more efficient enzyme in translacating the nascent product/substrate (higher Vmax), a property that may be directly related to the width of the cleft opening.

After the initial binding or docking of the substrate in the cleft, hyaluronan is precisely positioned along the cleft inter-


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After the initial binding or docking of the substrate in the cleft, hyaluronan is precisely positioned along the cleft interacting predominantly with charged residues lining the cleft surface (9). The importance of this process is reflected by the loss of activity of the R243V mutant. As the current complex structures show, the Arg<sup>243</sup> residue interacts directly with the carboxylate of the glucuronic acid moiety of the penultimate disaccharide (Table II and Fig. 3a) and as such has the ability to align the substrate directly along the cleft. Mutation of Arg<sup>243</sup> to Val disturbs this precise alignment along the cleft axis, and the enzyme loses significant activity (17).

The substrate chain also is oriented such that the reducing end of the chain is located at the narrower end of the cleft where catalysis is performed (9, 16, 19). Such unidirectional binding of hyaluronan determines its direction of degradation by the enzyme from the reducing to the non-reducing end (see below). The positions of the three-disaccharide units of the HA<sub>A</sub> substrate bound in the cleft are termed HA1, HA2, and HA3.
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from the reducing to the non-reducing end of the substrate and similarly HA1 and HA2 disaccharide units for the HA4 substrate.

Finally, directly next to the catalytic residues determined by our earlier research (9), there are three hydrophobic residues, Trp292, Trp291, and Phe143, creating a hydrophobic area in the cleft termed aromatic/hydrophobic patch (Fig. 4). These residues interact with the substrate utilizing the hydrophobic in-character sugar rings of the first and the penultimate disaccharide units of the substrates. Trp292 interacts with the HA2 unit, whereas Trp291 and Phe143 interact with the HA1 disaccharide unit of both the hexa- and tetrasaccharides. In this way, these three residues precisely position the substrate and its chemical groups in positions most suitable for catalysis performed by three catalytic residues Asn349, His399, and Tyr408 (Figs. 4 and 5b). The binding of the substrate in the opposite direction is not favored because it creates significant steric clashes between the enzyme and the substrate, and the catalytic residues are not positioned favorably with respect to the substrate for catalysis.

Catalytic Cleavage of the Glycosidic Bond—We have proposed previously (9), based on the structure of the native enzyme and its modeled complex structure with the tetra- and hexasaccharide units of HA, its complex structure with the disaccharide product of degradation (16) and mutation studies (9, 17) that the catalytic residues are Asn349, His399, and Tyr408 (Fig 5b). The current complex structures with the tetra- and hexasaccharide HA units confirm our earlier conclusions. As shown in Table II, Asn349 directly interacts in a bidentate fashion with the carboxylate group of the glucuronate moiety of HA1. This interaction allows Asn349 to act as a partial electron sink by attracting the electronegative charge of the carboxyl group of HA1 away from the C-5 of glucuronate. This process leads to acidification of the C-5 hydrogen that normally has a high pK value, which is estimated to be in the range from 29 to 32 (46). As a consequence, His399 acting as a base can withdraw this hydrogen, and the C-5 is rehybridized to the sp2 hybridization. In the complex structures His399 is in direct contact with the C-5 of the glucuronate moiety (Table II), which supports our suggestion. At the same time Tyr408 acts as an acid and donates hydrogen to the glycosidic oxygen leading to cleavage of its β-1,4 covalent glycosidic bond between HA1 and HA2. The C-4 is then rehybridized to the sp2 hybridization, and a double bond is formed between C-4 and C-5 (Fig. 5b).

To avoid substrate degradation, we used a Y498F mutant enzyme to obtain the complex structures reported here. Therefore, the distance between the Tyr408 and the glycosidic oxygen could not be determined; however, the distances of 4.55 Å for the HA4 complex and 4.63 for the HA4 complex between Phe408 and the glycosidic oxygen, O4, are consistent with the proposed role of Tyr408. Furthermore, the corresponding distances based on the modeled position of Tyr408 (Table II) are 3.18 and 3.10 Å. There are two other residues, Arg262 and Arg426, that are in close proximity to the glycosidic oxygen and could in principle serve as acids to donate hydrogen to the glycosidic oxygen (Fig. 3). Their possible contribution to catalysis cannot be discarded although the pK of Arg is significantly higher than that of Tyr so that their participation seems unlikely.

Hydrogen Exchange with Microenvironment—In the catalytic process described above, the enzyme molecule loses one hydrogen from Tyr408 and gains another one at His399. As the final step, the hydrogen balance needs to be restored for the enzyme to return to its original state and be ready for the next round of catalysis. There is no evidence in the structure of hydrogen channeling using ordered water molecules or residues of the enzyme. The closest structured water molecules, Wat1 and Wat2, identified in the electron density in the proximity of His399 are more than 6 Å away (Table II and Fig. 3a). No structured waters were identified anywhere close to the Phe143 of Y498F enzyme complexes. Therefore, the hydrogens are likely equilibrated using the unstructured water microenvironment present in the cleft of the enzyme.

For the next round of catalysis to take place, the enzyme also needs to release the generated disaccharide product from the active site. This can happen by either translocation of the remaining polymeric HA substrate to the catalytic position by an advance by one disaccharide unit toward its reducing end (considered more likely) or by the release of the substrate from the cleft thereby allowing the binding of a new substrate molecule to the enzyme.

Disaccharide Product Release—At the HA1 position in the cleft, there are three negatively charged residues, Glu388, Asp398, and Thr400, grouped together at the bottom of the cleft surface, directly opposite the Phe143 amino acid residue of the hydrophobic patch (Fig. 4). These residues are responsible for the generation of a patch of negative potential (negative patch) in the area of the cleft where the HA1 disaccharide of the electronegative substrate binds (Fig. 6). The presence of this patch is likely not coincidental but is probably important for the function of the enzyme. Most likely, after catalysis when the disaccharide product is cleaved off from the polymeric HA substrate chain, this product is directly located in this potential. As a consequence, the product is electrostatically repelled from the cleft. Such behavior rationalizes the release of the product from the cleft. The electronegative patch simply facilitates this process and frees up this part of the cleft, making the enzyme ready for the next round of catalysis.

Translocation of the Substrate and the Processive Mode of Action of the Enzyme—After the release of the disaccharide product, the remaining polymeric substrate is translocated toward the non-reducing end of the HA molecule. The movement of the substrate molecule is hindered by the negative charge of the electronegative patch, and the flexibility of the HA substrate facilitates the threading of the substrate through the cleft. For the catalytic residues to be repositioned and the hydrogen balance to be restored, the distance between the HA4 substrate and the catalytic residues needs to increase (Fig. 6). The distance between Tyr408 and the glycosidic oxygen, O4, is consistent with the proposed role of Tyr408 (Fig 5).
product, there remains a truncated polymeric HA in the cleft that can become a substrate for further degradation. One possibility is that the substrate leaves the cleft and the enzyme binds again a new or the same hyaluronan molecule into the catalytic cleft (random endolytic or exolytic binding and then cleavage). Alternatively, the molecule already bound in the cleft could be translocated in the cleft by one disaccharide unit toward the reducing end for the next round of catalysis (subsequent processive exolytic binding and cleavage of unsaturated hyaluronan disaccharides by the enzyme). The latter seems to be the case as shown by the biochemical evidence collected for the similar *S. agalactiae* hyaluronate lyase enzyme (47). As the *S. pneumoniae* enzyme is very similar in its sequence and structural and functional properties to the *S. agalactiae* enzyme from the same genus, it is reasonable to assume that this enzyme behaves in the same way. The overall sequence identity between the two enzymes is 51%, and sequence homology is 66%. In addition, the structures of both enzymes show a high degree of similarity, especially in the active site area (19, 22) (Fig. 5).

**Direction of Hyaluronan Degradation.**—The processive nature of hyaluronan degradation leads to the question whether this degradative process is performed from the reducing end of the substrate to the nonreducing end or vice versa. Our current structural report including the complexes of tetra- and hexa- saccharide HA with the enzyme and our previous work (16, 19) on the complex structures with the disaccharide products suggests that the degradation takes place from the reducing to the nonreducing end of hyaluronan. The current structures clearly show that the cleavage of the β1,4-glycosidic bond between HA1 and HA2 disaccharide units of the tetra- and hexa-saccharide substrates will produce an unsaturated disaccharide unit of HA at the reducing end of the chain and a tetrasaccharide (for the hexasaccharide complex) or a disaccharide (for the tetrasaccharide complex). The tetrasaccharide product of the degradation of the hexasaccharide can be further degraded to disaccharides, whereas the disaccharide product of tetrasaccharide degradation cannot be further degraded (17). The translocation of the substrate in the reducing end direction advances HA polymer to the new degradation position. In this position the HA2 disaccharide took the place of the cleaved HA1 disaccharide, and HA3 took the place of HA2 (Figs. 4 and 5).

In addition, the presence of the negative patch at the reducing end of the substrate chain further identifies this reducing end as the releasing end of the substrate. The structures of the hyaluronate lyase enzyme with the bound product of degradation also support this analysis because no bound product was found at the HA3 non-reducing position in the cleft (16, 19). The possibility of the degradation of HA in the opposite direction from the non-reducing to the reducing end is clearly not supported by the current structures of the complexes or by our earlier studies.

**Comparison of Hyaluronan Degradation to the Degradation of Dermatan Sulfate, Chondroitin Sulfate, and Alginate.**—The degradation of polysaccharides by polycarboxylate lyases such as hyaluronate lyase has been investigated for some time by biochemical and structural methods (e.g., Refs. 49 and 50). Recent structural investigations of *F. heparinum* chondroitin AC lyase (20) and *Sphingomonas* species alginate lyase AI-III (21) provided some additional insight into this process. The overall fold of the *F. heparinum* chondroitin AC lyase (20) is similar to that of *S. pneumoniae* hyaluronate lyase consisting of two domains, a α-helical and a β-sheet domain. The α-helical domain contains a similar elongated cleft where the polymeric sugar molecules bind and are cleaved. Several scenarios were proposed for the chondroitin AC lyase mechanism of dermatan sulfate, chondroitin sulfate, and hyaluronan degradation, and these mechanisms involve His235 and Tyr234, residues corresponding to His399 and Tyr408, respectively, of the pneumococcal hyaluronate lyase. The homologous residue to Asn349 of the
pneumococcal hyaluronate lyase, Asn175, has not been directly implicated in the degradation mechanism for chondroitin AC lyase by Huang et al. (20), although modeling studies and the complex structures of the AC lyase showed that this residue could interact with the carboxylate group of the C-5 of all substrates (20). Also, the residue homologous to Arg462 of S. pneumoniae lyase, Arg488, was implicated in the chondroitin AC lyase mechanism as possibly a general acid acting as a donor of hydrogen to the glycosidic oxygen during the cleavage of this bond or as a charge-neutralizing residue for the enolate anion intermediate formed during catalysis. Our analysis of the active site of pneumococcal hyaluronate lyase does not support such role of Arg462 for the pneumococcal lyase. Even though Arg462 is in close proximity to the glycosidic oxygen of hyaluronan (Table II), its pKₐ for hydrogen donation is significantly higher than that of Tyr408, and therefore the latter residue is more likely to participate in this way. Mutation of the homologous residue to Arg462 for the hyaluronate lyase from S. agalactiae, Arg442, decreased but did not abolish the activity of this enzyme (51) supporting our conclusions. This Arg may have a structural role or be involved in substrate

![Fig. 7. Stereo view of the superposition of the minimum and the maximum projection structures of hyaluronate lyase.](image)

**Table III**

| Residues involved | Minimum projection distance (Å) | Maximum projection distance (Å) |
|-------------------|--------------------------------|---------------------------------|
| Thr234-Gly769     | 10.9                           | 14.0                            |
| Asn580-Asn290     | 6.6                            | 11.6                            |
| Ala220-Gly769     | 29.3                           | 36.8                            |
| Asn468-Lys515     | 10.0                           | 11.6                            |

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binding; either of these functions would reasonably explain the reduced enzyme activity on its mutation.

Another recently studied polysaccharide degrading enzyme is alginate lyase. The overall fold of the *Sphingomonas* species alginate lyase A1-III elucidated structurally only recently (21) is similar to that of the α-domain of *S. pneumoniae* hyaluronate lyase, but the β-sheet domain is not present in this enzyme. The α-helical domain also supports the formation of a similar elongated substrate-binding cleft to that of the pneumococcal lyase. For the alginate lyase A1-III a Tyr246, homologous to Tyr408 of the pneumococcal lyase, was suggested to act as both an acid donating hydrogen to the glycosidic oxygen (21). Although this may be possible for the alginate lyase, we do not see evidence for such behavior with hyaluronate lyase in our complex structures nor from earlier studies (9, 13, 17, 19).

**Protein Flexibility of Hyaluronate Lyase and Its Functional Implications—**Computational studies often can enable correlations between mobility of proteins or their segments and specific functions (52). Concerted motions involving protein segments may be deduced from ED analysis of molecular dynamics (MD) simulations (52–54). In the case of pneumococcal hyaluronate lyase, its size precludes the realistic calculation of MD trajectories at biologically relevant time scales. However, good qualitative agreement between the results of ED analyses of MD trajectories and the results of analysis of CONCOORD software generated structures has been obtained (see “Experimental Procedures”). We therefore applied the CONCOORD/ED methodology to seek independent support for the proposed roles of the dynamical features of the enzyme, in particular in the region of the binding cleft and its catalytic region.

The largest amplitude concerted fluctuation, mathematically described by the first eigenvector, is a rotation-twisting motion of the whole α-domain relative to the top half of the β-domain (Fig. 1). Concomitantly, the bottom half of the β-domain rotates in the opposite direction, relative to the top half of that domain. Fig. 7a shows the superimposition of the minimum and maximum projections for this first eigenvector. These projection structures correspond to the two extreme structures associated with this mode of fluctuation. The intra-β-domain motion seems to be of little functional consequence because the interface between the two dynamically independent portions of the β-domain lies distant to the active site. However, the residues from the positively charged majority of the binding cleft of the α-domain of the enzyme (a positive patch) are particularly mobile relative to residues from the active center and the negative patch. This is consistent with the previous hypothesis (19) that the mobility of the positive patch is related to drawing the ligand into the binding cleft.

Another major motion of the hyaluronate lyase is also related to an opening/closing of the substrate-binding cleft and is described by eigenvector 3. Fig. 7b shows the superimposition of the minimum and maximum projections for this mode of concerted fluctuation. The change of the distance between selected residues involved in the movement is shown in Table III. The residues from the active site and from the negative patch, whose relative orientation did not change significantly with the motion described by the first eigenvector, exhibit significant relative positional shifts along the motion described by eigenvector 3. This finding is interesting given the obvious need of the enzyme to release the electronegative disaccharide product of degradation from the active site in order to enable relocation and further processing of the remaining polysaccharide. In addition, there is evidence for the mobility, relative to the α-domain, of two loops from the β-domain, which are involved in the cleft formation (described earlier). For example, Asn580, which is located at the tip of one of the loops from the β-domain, shows a relatively large fluctuation (Table III). As described above and supported by the earlier kinetic analysis (17), the N508G mutant showed significantly higher activity when compared with the wild type enzyme, confirming the importance of this loop to catalysis. Therefore, the flexibility analysis suggests that this opening/closing motion of the enzyme is directly relevant not only to hyaluronan binding but also to the catalysis itself.

**Conclusions and Biological Implications—**The current structures of hyaluronan lyase complexes with substrates enable us to draw conclusions related to the functional properties of the enzyme. The mechanism of catalysis was well established as proton acceptance and donation. The comprehensive analysis of the entire process of hyaluronan degradation was proposed including the irreversible release of the disaccharide product and the nature of the processive mechanism of this important biological process. The residues involved in substrate binding, precise positioning for catalysis, catalysis itself, the release of the product, and the translocation of the remaining substrate were clearly identified, and their contribution to the entire degradation process was described. The nature of the processive behavior of hyaluronate lyase described here is likely relevant to other processive enzymes. Finally, we presented simulated molecular and essential dynamics analyses that shed light on the aspects of the function of the enzyme that would be inaccessible to evaluation of static crystal structures.

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