The Function of Hydrophobic Residues in the Catalytic Cleft of Streptococcus pneumoniae Hyaluronate Lyase

KINETIC CHARACTERIZATION OF MUTANT ENZYME FORMS*

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Streptococcus pneumoniae hyaluronate lyase is a surface antigen of this Gram-positive human bacterial pathogen. The primary function of this enzyme is the degradation of hyaluronan, which is a major component of the extracellular matrix of the tissues of vertebrates and of some bacteria. The enzyme degrades its substrate through a β-elimination process called proton acceptance and donation. The inherent part of this degradation is a processive mode of action of the enzyme degrading hyaluronan into unsaturated disaccharide hyaluronic acid blocks from the reducing to the nonreducing end of the polymer following the initial random endolytic binding to the substrate. The final degradation product is the unsaturated disaccharide hyaluronic acid. The residues of the enzyme that are involved in various aspects of such degradation were identified based on the three-dimensional structures of the native enzyme and its complexes with hyaluronan substrates of various lengths. The catalytic residues were identified to be Asn349, His399, and Tyr408. The residues responsible for the release of the product of the reaction were identified as Glu388, Asp386, and Thr409, and they were termed negative patch. The hydrophobic residues Trp291, Trp292, and Phe343 were found to be responsible for the precise positioning of the substrate for enzyme catalysis and named hydrophobic patch. The comparison of the specific activities and kinetic properties of the wild type and the mutant enzymes involving the hydrophobic patch residues W292A, F343V, W291A/W292A, W292A/F343V, and W291A/W292A/F343V allowed for the characterization of every mutant and for the correlation of the activity and kinetic properties of the enzyme with its structure as well as the mechanism of catalysis.

Hyaluronan (HA)† is a glycan that is abundantly present in nearly all vertebrate tissues, especially the extracellular matrix, and in some bacteria such as Streptococcus zooepidemicus. It is a polymeric substance built from a repeating disaccharide unit of hyaluronic acid with the chemical formula \([\beta 1\rightarrow 3]\text{GlcNAc(}\beta 1\rightarrow 4)\text{GlcUA}\) (Fig. 1). Hyaluronan isolated from natural sources has an enormous size, up to 25,000 disaccharide units or 10^7 Da. The polymer interacts with water to create a strikingly viscoelastic solution. These unique mechanical properties are utilized in, for example, joints as a shock absorber (2). In addition to the mechanical properties, hyaluronan synthesis and degradation is finely regulated to allow initiation of other biochemical processes. For example, hyaluronan is involved in multiple signal transduction processes (3, 4), often utilizing other macromolecules for interactions such as CD44 or RHAM (5, 6). Through these molecules or these transduction processes, HA influences many essential processes, for example, cell migration and development.

Exogenous elements, such as bacteria including members of the Streptococcus species, degrade the hyaluronan of its host organism, including humans, through the action of hyaluronate lyase enzymes by the process of β-elimination (7–9). In contrast, the endogenous degradation of hyaluronan is performed by the hyaluronidase enzymes of the host that utilize a mechanistically distinct hydrolysis mechanism (7). The exact molecular mechanism of the lyase action was largely unknown until relatively recently when the first structural information on bacterial hyaluronate lyase enzymes was obtained by means of x-ray crystallography (10). Similar structural information for the endogenous hyaluronidases is not available at present except for that of the homologous bee venom enzyme (11). Therefore, the details of this process are still unclear and are based on comparison with other hydrolytic polysaccharide degrading enzymes.

Hyaluronate lyase from Streptococcus pneumoniae, a Gram-positive human pathogen (12–14), has recently been cloned, overexpressed, and purified (15, 16). The availability of large quantities of the protein led to extensive biochemical and biophysical characterization of the enzyme together with its crystallization (17) and three-dimensional crystal structure determination (10). Structure determination of the native form of the enzyme was followed by molecular modeling and characterization of crystal-based structures of enzyme-substrate complexes using di-, tetra-, and hexasaccharide units of hyaluronan (18, 19) (Fig. 2). Finally, a crystal structure of hyaluronate lyase from another Streptococcus species, Streptococcus agalactiae, was elucidated in its native and complex forms with the disaccharide unit of HA degradation (20, 21). The three-dimensional x-ray crystal structures of hyaluronate lyases show the enzyme as a globular protein built from at least two
distinct domains: a helical α-domain and a β-sheet β-domain (Fig. 2). The α-domain is traversed by an elongated deep cleft where the HA substrate binds and where it is degraded to disaccharides.

The comparison of both structure groups, from *S. pneumoniae* and *S. agalactiae*, allowed for additional conclusions and generalization of the proposed mechanism of action of the lyase (22, 23). The catalytic residues (Asn\(^{549}\), His\(^{599}\), and Tyr\(^{608}\)) as well as the residues of the hydrophobic (Trp\(^{291}\), Trp\(^{292}\), and Phe\(^{343}\)) and the negative (Glu\(^{388}\), Asp\(^{398}\), and Thr\(^{408}\)) patches that were implicated in the activity of the lyase were clearly identified and analyzed based on approaches similar to those used in the structural studies described above (see Figs. 3) (10, 19, 23). These studies include the structures of the native *S. pneumoniae* and *S. agalactiae* hyaluronate lyase, the structures of complexes with the substrates or products (disaccharide product of degradation, tetra- and hexasaccharide hyaluronan units), and site-directed mutation studies used in conjunction with kinetic studies of the corresponding mutant enzyme forms (22, 24).

The enzymes with mutated catalytic residues Asn\(^{549}\), His\(^{599}\), and Tyr\(^{608}\) were generated and isolated previously, and their enzymatic properties, including specific activities along with kinetic parameters, were determined and analyzed as described (23). The correlation between the activity of the mutant enzymes, kinetic properties of the mutant enzymes, and their structures clearly confirmed the Asn\(^{549}\), His\(^{599}\), and Tyr\(^{608}\) residues as being directly involved in the catalysis. A detailed catalytic mechanism of β-elimination based degradation of HA, termed proton acceptance and donation (PAD), was proposed (see Figs. 3 and 4). The proposed mechanism involves five distinctive steps: (i) binding to the negatively charged hyaluronan substrate to the enzyme binding cleft with precise positioning of the substrate being directed by the hydrophobic patch consisting of residues Trp\(^{291}\), Trp\(^{295}\), and Phe\(^{343}\); (ii) catalysis involving primarily the catalytic residues Asn\(^{549}\), His\(^{599}\), and Tyr\(^{608}\) with the resulting cleavage of the glycosidic β,1,4 bond and generation of the disaccharide product; (iii) proton (hydrogen) exchange between the corresponding His\(^{599}\) and Tyr\(^{608}\) residues with the water microenvironment, a step that readies the enzyme for the next round of catalysis; (iv) release of the disaccharide product utilizing the negative patch composed of the Glu\(^{508}\), Asp\(^{596}\), and Thr\(^{408}\) residues; and finally (v) translocation of the remaining polymeric hyaluronan substrate by 1 disaccharide unit toward the reducing end of the substrate (see Figs. 3 and 4) (10, 19, 23).

Here we report further analysis of the properties of this enzyme by mutating the residues of the hydrophobic patch and providing the analysis of their role and importance in catalysis. Additional insights into this catalytic mechanism are supplied by the investigations of the specific activities and the kinetic properties of the wild type and mutant forms of this lyase.

**EXPERIMENTAL PROCEDURES**

**Materials**—Hyaluronan used in this study was from human umbilical cord (Sigma; sodium salt, Lot 1X1515). All other chemicals were purchased from either Fisher or Sigma.

**Cloning of the Mutant Form of the Enzyme**—Plasmid DNA preparations were obtained using the QiAprep kit from Qiagen. Mutagenesis was performed using the QuikChange kit and procedure of Stratagene. The template for the single mutations, W292A and F343V, was plasmid pET-SpiHyal-His\(_{6}\)_pMMJ004 (15), containing a truncated but active *S. pneumoniae* hyaluronate lyase (Ala\(^{168}\)-Glu\(^{596}\) of native enzyme). Multiple mutations were made progressively by performing mutagenesis using templates and primers with the desired combination of mutations. Mutants were identified by automated DNA sequence analysis. The following primers, with the desired changes indicated in bold italic type, were used in the mutagenesis procedures (+—denote the upstream and the downstream primers, respectively): W291A+; GAGCATGGTGGAGGACCTGCTCAAGTTATGAAAATCGG; W291A--; CCGATTTCAATATCTGCAGCGTTCACCAATAATGTCT; W292A+; CATTGTGGTGAACCTGCGAGATTATGAAATCGGT; W292A--; GTGTCAGTTCATATCTGCGCCAGTTCCAACAAATGG; F343V+; GAGCACTGATACCCATATGACGAGCAGCGTTCATCGTGTTAATCGTCAGG.

**Production of Enzyme Forms**—The recombinant wild type *S. pneumoniae* hyaluronate lyase enzyme from *E. coli* was overexpressed and purified as previously described (10, 15, 17). The mutant forms of the enzyme: (i) single mutants W292A and F343V; (ii) double mutants the triple mutants were generated and purified in the same fashion as the wild type enzyme with activity. The wild type and the mutant enzymes were stored in 10 mM Tris-HCl (pH 7.4), 2 mM EDTA, 150 mM NaCl, and 1 mM dithiothreitol buffer at 5 mg/ml protein concentration for further use. The activity unit for the enzyme was defined as the molar amount of the enzyme that produces 1 μmol product/min (15). The presence of the mutations in the produced proteins was additionally identified (in addition to DNA sequence-based analysis) by mass spectrometry experiments as described under “Other Methods.”

**Initial Velocity and Specific Activity Measurement and Data Analysis**—During the process of enzyme-catalyzed degradation of polymeric hyaluronan, a double bond in the glucronic moiety is introduced between carbon atoms C-4 and C-5. This bond formation in the product of HA degradation induces a spectral shift with the absorption maximum at ~232 nm. This absorption was followed experimentally to detect changes in the progress of the reaction for the wild type and the mutant enzymes. The HA degradation reaction was carried out in quartz cuvets containing 300 μl of a reaction buffer with the HA substrate. The reaction buffer was 50 mM sodium acetate, 10 mM NaCl, and 5 mM dithiothreitol buffer at 5 mg/ml protein concentration for further use. The activity unit for the enzyme was defined as the molar amount of the enzyme that produces 1 μmol product/min (15). The reaction was initiated by the addition of 10 μl of enzyme in the same buffer to the mixture of buffer and the specified amounts of the substrate. The amounts of the enzyme in the reaction mixture were modified as needed because of different abilities to degrade HA, and they were as follows: wild type, 10 ng; F343V, 33 ng; W292A, 500 ng; and F343V/W292A, 4000 ng. The measurement was initiated exactly 5 s after the enzyme was added to the reaction mixture. The progress of the reaction of HA degradation was followed by detecting the absorbance at 232 nm. The spectral measurements were performed using the BioSpec-1601PC UV-visible spectrophotometer (Shimadzu) equipped with a thermoelectrically temperature controlled cell holder to perform the reaction when temperatures were raised above room temperature. The limits for the detection of the specific activity were directly related to the sensitivity of the spectrophotometer used, which was the absorbance at 232 nm of 0.001 or specific activity of 0.01 unit/min. Enzymes with smaller activity were considered inactive. All of the materials used in this study were preincubated at 37 °C using a water bath, and all of the subsequent procedures were also performed at this temperature. Concentration determinations used a molar absorption coefficient for the product of HA degradation of 5.5 × 10\(^{3}\) liter/mole \(^{1}\) cm \(^{-1}\) as described (15, 26).

For the specific activity measurements, the enzyme was added to a cuvette containing 300 μl of a 0.2 mg/ml of polymeric HA in the reaction buffer. To investigate the influence of the hydrophobic effect versus hydrogen bonding on activity, the specific activity measurements were also performed with the addition of 150 mM NaCl in the reaction buffer. The product absorbance was measured every 5 s for 6 min. The specific activity was calculated using UV-1601 PC Kinetics software (Shimadzu) and previously reported methodologies (10, 15).
For the initial velocity measurements the polymeric hyaluronan substrate concentration ranged from 0.012 to 2 mM. The moles of HA were expressed as moles of its disaccharide unit based on its molecular weight. The measurements were performed similarly to the initial velocity measurements with the exception that the data points were recorded in 6-s intervals for 1.5 min. The initial velocity for the degradation reaction was calculated based on the increase of absorbance during the first 90 s of the reaction.

The measured data for initial velocity, $v$, and the varied concentrations of substrate, $S$, were fit to the Michaelis-Menten equation, $v = \frac{V_{\text{max}} S}{K_{\text{m}} + S}$, with a nonlinear regression program (Scientist Micromath). In this equation, $K_{\text{m}}$ is the Michaelis constant, and $V_{\text{max}}$ is the maximum velocity. For all of the experiments, goodness-of-fit statistics showed that $R$-squared and correlation values were greater than 0.957 and 0.982, respectively. As the results of the curve fitting show, the maximum velocity measurements with the exception that the data points were weighted. The measurements were performed similarly to the initial velocity measurements with the exception that the data points were weighted. The measurements were performed similarly to the initial velocity measurements with the exception that the data points were weighted.

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**Determination of the Size of the Degradation Product—**Hyaluronan was degraded as previously described (10). Briefly, for 1000 µl of 2.0 mg/ml of hyaluronan solution in 50 mM sodium acetate, 10 mM CaCl$_2$ at pH 6.0, the following amounts of the enzyme forms were used: wild type, 35 ng; F343V, 2.5 µg; W292A/F343V, 50 µg; W291A/W292A, 50 µg; and W291A/W292A/F343V, 50 µg. The mixture was incubated at 37 °C and collected at 1, 5, and 10 min and 3, 7, 20, and 24 h (100 µl each). The degradation product mixtures were immediately separated on a Superdex peptide HR 10/30 column (Amersham Biosciences) using 10 mM ammonium acetate buffer at pH 7.4. The eluting peak fraction identities were confirmed by electrospray mass spectrometry on a Micromass Quattro LCZ tandem mass spectrometer using atmospheric pressure ionization and the conditions previously reported (10, 27).

**Crystallization of Mutants and Data Collection—**The crystallization of the mutant forms of the enzyme was performed similarly to the crystallization of the wild type hyaluronan lyase and as described (17). A hanging drop vapor diffusion with VDX culture plates and siliconized glass cover slides were used. Briefly, equal volumes (~1 µl each) were mixed of reservoir solution and mutant proteins W292A, F343V, W292A/F343V, W291A/W292A, and W291A/W292A/F343V in buffer described earlier and at protein concentrations of 6.2, 5.5, 5.1, 5.6, and 5.10 mg/ml, respectively. The reservoir solution contained 60–65% saturated ammonium sulfate, 0.2 M sodium chloride, 2% dioxane, and 50 mM sodium citrate buffer at pH 6.0.

The crystals of the inactive mutant proteins W291A/W292A and W291A/W292A/F343V were in addition soaked in a hexasaccharide hyaluronan substrate (HA$_6$) for 10 h prior to cryo-freezing. The soaking solution contained 75% saturated ammonium sulfate, 10 mM sodium citrate buffer at pH 6.0 and 10 mM HA$_6$. All of the mutant crystals were cryo-protected in 30% xylitol (w/v), 80% saturated ammonium sulfate, and 10 mM sodium citrate buffer, pH 6.0, and flash frozen in liquid nitrogen before diffraction data collection.

The diffraction data collection was performed at a synchrotron source utilizing Berkeley Center for Structural Biology, Advanced Light Source, Lawrence Berkeley National Laboratory beamline 5.0.1. The x-ray wavelength was 1.0 Å, and the crystal diffraction was recorded on a Quantum 4u CCD detector using the oscillation method. The data were integrated and scaled using the HKL2000 package (28). The unit cells of crystals of mutants and their complexes were isomorphous to the native ones. The final data processing parameters are reported in Table I.

**Determination of Structures of Mutants and Their Complexes—**The native SnpHL crystal structure without water molecules (10) was used as the model for the mutant and complex structure solution. The $R_{	ext{free}}$ flag was assigned to 1% of reflection for W292A, F343V, and W292A/F343V mutant diffraction data sets and 2% for W291A/W292A and W291A/W292A/F343V mutant complexes with HA$_6$ data sets to validate the refinement progress (29, 30). Initially, a round of rigid body refinements using only the model structure was performed using the refmac5 maximum likelihood protocol (31, 32). The mutated residues were modified manually using the program O (33). The refinements were continued using the restrained and unrestrained protocols of refmac5 (31, 32) and were traced using inspection on graphics with the program O (33). The structures were refined against all reflections from 20.0 Å to the highest resolution available without any $\sigma(F)$ cut-off (see Table I). The electron density for the HA$_6$ substrate for the W291A/W292A and W291A/W292A/F343V complex structures was clearly identified and was followed by the incorporation of the HA$_6$ substrates into this density as previously described (19, 34, 35). The topologies/parameter files for the substrate were manually created following our earlier studies to reflect ideal stereochemical values. Additional refinements including individual anisotropic B-factor refinements for all structures, inspection, and manipulation of structures on graphics using O together with incorporation of water molecules placed into 3σ peaks in sigma-A weighted $F_o - F_c$ maps following standard criteria were performed. After further refinements, water molecules whose positions were not supported by electron density, at 1σ contouring, in sigma-A weighted $2F_o - F_c$ maps were deleted. A variety of stereochemical (36) and other analyses (33, 37) were periodically performed to locate possible model errors (38). The number of water molecules incorporated and the final refinement parameters are reported in Table I.

**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 (15, 39). The calculated molar extinction coefficients were 127,090 for the native enzyme and the F343V mutant, 121,590 for the W292A and W292A/F343V mutants, and 116,090 for the W291A/W292A and W291A/W292A/F343V mutants.

Mass spectrometry experiments to confirm the presence of mutations were collected on an LCQ quadrupole ion trap (ThermoFinnigan) mass spectrometry.
Crystallographic statistics of diffraction data and structure refinement of *S. pneumoniae* hyaluronate lyase mutant enzymes and their complexes with the hexasaccharide hyaluronan substrate

| Complex                  | Space group | a (Å) | b (Å) | c (Å) | \(\alpha\) (deg) | \(\beta\) (deg) | \(\gamma\) (deg) | Rmerge (% | Rfree (% | Res. range (Å) |
|--------------------------|-------------|------|------|------|----------------|----------------|----------------|--------|-------|-------------|
| W292A                   | P2₁ 2₁ 2₁ ¹ | 50.00 | 50.00 | 50.00 | 84.0           | 103.7          | 84.3           | 18.0   | 18.3  | 1.50–1.55   |
| W292A–Hyal–HA complex   | P2₁ 2₁ 2₁ ¹ | 50.00 | 50.00 | 50.00 | 84.0           | 103.7          | 84.3           | 1.50   | 1.50  | 1.50–1.55   |
| W292A/F343V             | P2₁ 2₁ 2₁ ¹ | 50.00 | 50.00 | 50.00 | 84.0           | 103.7          | 84.3           | 1.50   | 1.50  | 1.50–1.55   |
| W291A/W292A–Hyal–HA 6 complex | P2₁ 2₁ 2₁ ¹ | 50.00 | 50.00 | 50.00 | 84.0           | 103.7          | 84.3           | 1.50   | 1.50  | 1.50–1.55   |
| W291A/W292A/F343V–Hyal 6 complex | P2₁ 2₁ 2₁ ¹ | 50.00 | 50.00 | 50.00 | 84.0           | 103.7          | 84.3           | 1.50   | 1.50  | 1.50–1.55   |

**RESULTS**

**Construction of the Mutant Forms of the Enzyme**—A plasmid containing a partial (Ala₁⁶₈–Glu₈₉¹) but active *S. pneumoniae* hyaluronate lyase, pET-Sphyal-His₆ (pMJ0004 (15)), was used as the template to create the single mutants SpHyal–W292A (pMJ020) and SpHyal–F343V (pMJ021) by site-directed mutagenesis. These two mutants then served as templates with the primers encoding W291A and W292A, respectively, to create the double mutants SpHyal–W291A/W292A (pMJ022) and SpHyal–W292A/F343V (pMJ023). To make the triple mutant, SpHyal–W291A/W292A/F343V (pMJ024), the SpHyal–W292A/F343V clone served as the template with the mutagenesis primer encoding W292A. The generated clones were used to transform the *E. coli* overexpressing cells BL21 (DE3) (25).

**Overexpression and Purification of the Wild Type and Mutant Enzymes**—The recombinant wild type *S. pneumoniae* hyaluronate lyase protein from *E. coli* was obtained as previously described (10, 15). The mutant forms of the enzyme, W292A, F343V, W291A/W292A, W292A/F343V, and W291A/W292A/F343V, were overexpressed and purified following the same procedure as that for the wild type enzyme (15) or other mutant forms reported previously (10). Briefly, the overexpression was performed by growing *E. coli* BL21 (DE3), harboring the appropriate clone, in LB medium with ampicillin selection and isopropyl-thio-β-D-galactopyranoside (1 mM) induction. The purification procedure consisted of enzyme isolation from a cell lysate using a chelating Sepharose fast flow nickel affinity chromatographic step (Novagen) followed by a size exclusion column on Superdex 75 (Amersham Biosciences) and a high resolution anion exchanger using a MonoQ column (Amersham Biosciences).

The presence of mutations was confirmed by mass spectrometry experiments using electrospray ionization of the protein samples. The results yielded molecular masses for W292A, F343V, W291A/W292A, W292A/F343V, and W291A/W292A/F343V of 83,240, 83,058, 83,125, and 83,010 Da, respectively. These masses correspond to the calculated molecular masses: 83,173, 83,240, 83,058, 83,125, and 83,010 Da, respectively. The mutations were also confirmed by obtaining their three-dimensional structures (see below).

To confirm the maintenance of the overall fold of the produced mutant enzymes, they were crystallized. The crystallization experiments yielded crystals in essentially the same conditions and of the same habit as the native enzyme crystals previously reported by us (10, 17). To confirm this information and to confirm the structural fold in the active site of the enzyme together with the presence of the expected mutated residues, for all mutants three-dimensional crystal structures were obtained (Table I). The structures showed no significant differences with the native enzyme in their overall fold as well as in the fold in the catalytic cleft (Figs. 3 and 4). The mutated residues were identified in the electron densities as expected. Therefore, the observed changes in activity are due to the changes of amino acid residues introduced by site-directed mutagenesis.

**Initial Velocity Experiments with a Hexasaccharide Hyaluronan Substrate**—The kinetic parameters of the wild type and mutant enzymes are interpreted in the context of the PAD mechanism (Fig. 5) including: (i) substrate binding, (ii) catalysis, (iii) proton exchange with microenvironment, (iv) product release, and (v) translocation of remaining HA. The fifth step, the translocation of remaining HA, accounts for the processive
character of the enzyme as shown previously (41). Also, the product release step may account for the nearly irreversible nature of this catalysis.

In our earlier mutation and kinetic studies that were designed to investigate the involvement of the PAD mechanism in pneumococcal lyase catalysis, we derived an equation using the method of net rate constants (42) to describe the initial velocity of the enzyme, $V_{\text{max}}$ (23). The effects described by this equation show that for the enzyme catalyzed reaction, a relationship between measured kinetic parameters and functional interpretation can be defined: (i) changes only in $V_{\text{max}}/K_m$ for various mutants indicate that the enzyme binds the substrate with a different affinity, (ii) changes only in $V_{\text{max}}$ indicate that the nascent product/substrate translocation proceed at a different rate, and (iii) changes in both $V_{\text{max}}/K_m$ and $V_{\text{max}}$ show that the mutation in the enzyme affects some combination of the binding, the catalysis, the release of product, and the translocation steps. Although the $K_m$ may approximate the substrate binding affinity, this parameter can also be affected by the other rate and equilibrium constants during the catalysis, and therefore in addition to the $K_m$ parameter, $V_{\text{max}}/K_m$ is used as a descriptive parameter for hyaluronate lyase catalyzed reaction (23).

Kinetic Properties of the Mutant Forms of the Enzyme—The wild type enzyme obeyed Michaelis-Menten kinetics. The values of $V_{\text{max}}$ and $K_m$ for degradation of the hyaluronate substrate were $0.23 \pm 0.01 \text{ mmol/(min} \times \text{mg)}$ and $0.09 \pm 0.03 \text{ mM}$, respectively (Table II). The availability of the three-dimensional crystal structures for the wild type hyaluronate lyases from the *Streptococcus* species and the complexes with substrates/products provided a unique opportunity to correlate the kinetic and structural properties of this enzyme. The mutations of the catalytic residues H399A, N349A, and Y408F have already been investigated, and all have significantly reduced values of $V_{\text{max}}$ and $V_{\text{max}}/K_m$ as compared with the wild type enzyme (23). In the current study we report the analysis of the residues of the hydrophobic patch residues Trp291, Trp292, and Phe343. Based on the three-dimensional structure analysis, the region of hydrophobic patch occupies ~5% of the total cleft area, and the hydrophobic character in this region of the enzyme is significantly higher than that of the surrounding areas (10, 18, 19). As implied in the proposed PAD mechanism, these amino acid residues should be responsible for the precise positioning of the polymeric substrate for catalysis. The analysis of the structural environment in the catalytic cleft also suggested that these residues might be responsible for the size of the generated product of HA degradation (10). Five mutants were produced, W291A/W292A/F343V, of these mutants, the W291A/W292A and W291A/W292A/F343V forms were totally inactive; therefore, no specific activities or kinetic parameters were determined. The remaining three mutant forms, W292A, F343V, and W292A/F343V, were fully analyzed, and the specific activities or kinetic parameters were determined as described under “Experimental Procedures” (Table II). The specific activities for all three partially active mutants were significantly compromised when compared with the wild type enzyme. Similarly the $V_{\text{max}}$ values for each of the characterized mutants were much
lower. The $K_m$ values for the W292A and W292A/F343V mutants were significantly lower than that of the wild type. The $K_m$ value for the F343V mutant was comparable with that of the wild type (Table II). The changes observed in $K_m$ for W292A and W292A/F343V mutants, but not for the F343V mutant, are likely caused by the small alterations in the positioning of the substrate in the cleft and the resultant change in the binding of substrate. The analysis of the structure of the native enzyme (10), its complexes with the substrate (19, 34), and the mutant enzymes reported here suggests that the F343V mutation alone likely results in the misalignment of the substrate along the cleft and therefore likely decreased binding. Similar changes in the alignment along the cleft axis and the resultant change in $K_m$ were observed in our earlier work for the R243V mutant (10, 34).

Based on the structural information available, the longest HA unit that entirely fits in the catalytic cleft of the enzyme is the hexasaccharide. The hexasaccharide is built from three disaccharide units, HA1, HA2, and HA3, which correspond to the units originating from the reducing end toward the nonreducing end of this polymer. The side chain of Trp292 interacts through a hydrophobic interaction with the sugar rings of the HA2 disaccharide of the substrate, primarily with the \(N\)-acetyl-D-glucosamine group (Fig. 3). Similarly, the Phe343 side chain interacts with the hydrophobic ring moieties of HA1, primarily with the \(N\)-acetyl-D-glucosamine group. Although the interactions of Phe343 with the substrate are significantly weaker than that of Trp292, they are still pronounced (longer distances and smaller number of them). Trp291 interacts primarily through a hydrogen bonding interaction network with the \(N\)-acetyl-D-glucosamine group of HA1. The $V_{\max}$ parameter is compromised for all three partially active mutant enzymes, suggesting a direct influence on the catalytic process, product release, and/or translocation and involvement of each of the affected residues in such process. The role of

![Fig. 4. Stereo diagram of the wild type and five mutant structures of hyaluronate lyase.](image)

**Fig. 4.** Stereo diagram of the wild type and five mutant structures of hyaluronate lyase. The cleft with the region of the hydrophobic patch built from Trp291, Trp292, and Phe343 residues (labeled) is shown. The orientation of structure in all the panels is the same. The figure was prepared with O (33). A, wild type-like enzyme complex with HA6 (based on Protein Data Bank code 1loh) (19). B, F343V mutant. C, W292A mutant. D, W292A/F343V double mutant. E, W291A/W292A double mutant complex with HA6. F, W291A/W292A/F343V triple mutant complex with HA6.

![Fig. 5. Proposed chemical formulation of the mechanism of S. pneumoniae hyaluronate.](image)

**Fig. 5.** Proposed chemical formulation of the mechanism of *S. pneumoniae* hyaluronate. Only the catalytic residues, Asn349, His399, and Tyr408, and two disaccharide units, HA1 and HA2, are shown. The movement of hydrogens relevant to the catalytic process is indicated by arrows.
Phe\textsuperscript{243} seems to be primarily related to catalysis and less in the binding of substrate. Based on the kinetic data, the Trp\textsuperscript{291} and Trp\textsuperscript{292} residues seem to be involved in both the binding as well as the precise positioning of the substrate for the catalytic process (34).

The mutations W291A/W292A and W291A/W292A/F343V have completely inactivated the enzyme, preventing the determination of kinetic parameters that are used to characterize these mutants (Table II). According to the structural data of the enzyme complexes with tetra- and hexasaccharides (19), the Trp\textsuperscript{292} residue interactions are more significant that those of the remaining residues of the hydrophobic patch, and therefore mutating this residue has a more detrimental influence on the activity, especially when combined with changes to other residues of the hydrophobic patch. All of the information presented in the analysis of the wild type and the mutant enzymes, their specific activities, and kinetic properties is consistent with and confirms our previous structural and mechanistic studies of the enzyme (10, 18, 19, 23).

**Size of the Degradation Product**—The wild type and all mutant enzymes were tested with respect to the size of the product of degradation. All of the active enzyme forms showed similar profiles of hyaluronan degradation with the final product of degradation identified as the unsaturated hyaluronic acid disaccharide, 2-acetamido-2-deoxy-3-degradation identified as the unsaturated hyaluronic acid disaccharide. With short degradation times (~1 min), a small population of tetra- and hexasaccharide products was present for the three characterized mutants and the wild type enzyme (Fig. 6). The inactive mutants (specific activity below the instrumental detection limit of 0.01 unit/min) were not tested, because they did not degrade the substrate.

**Crystal Structures of the Mutants and Their Complexes**—The protein components of all three crystal structures of the mutants (W292A, F343V, and W292A/F343V) and the two complexes of the two additional mutants with HA\textsubscript{6} (W291A/W292A and W291A/W292A/F343V) are nearly identical to one another as well as to the structure of the native enzyme (10). The detailed description of the native structure and its complexes with hyaluronan have been reported elsewhere (10, 18, 19). The small structural changes present in the catalytic cleft and in the area of the hydrophobic residues are limited to the differences specifically related to the missing hydrophobic side chains of the mutated residues only. For the inactive mutants W291A/W292A and W291A/W292A/F343V a hexasaccharide hyaluronan substrate is present in the catalytic cleft. The position and the orientation of the HA\textsubscript{6} substrate are similar to those in the structure reported earlier (19, 34). The substrate conformation, including the carbohydrate ring structures, is also similar to the structures reported earlier (19, 34, 35). The lack of hydrophobic residues in mutants did not significantly change the position of the substrate but caused, as expected, its small distortion out of the ideal position in the binding cleft with respect to the catalytic residues and therefore displacement from ideal position for catalysis (Figs. 3b and 4).

**DISCUSSION**

**Structural Properties of the Enzyme**—The structure of 4-hyaluronate lyase enzyme from *S. pneumoniae* shows a two-domain enzyme with a catalytic \(\alpha\)-domain at the N terminus with a structure of \(\alpha_d/\alpha_g\) barrel with two layers of \(\alpha\)-helices, five on the inside of the barrel and five on the outside (Fig. 2) (10). At one end of such a barrel, the helices and loops between them form an elongated, deep cleft characteristic of glycan-binding enzymes. Structural and site-directed mutagenesis studies proved that hyaluronan substrate binds in this cleft and is also degraded at this location. In addition to the \(\alpha\)-domain, there is an additional \(\beta\)-sheet domain located toward the C terminus of the enzyme. This domain extends further to facilitate the enzyme binding to the cross-bridges of the peptidoglycan structures of pneumococci. The \(\beta\)-sheets, in general, are arranged into a \(\beta\)-sandwich structure. The \(\beta\)-domain likely facilitates the access of the substrate to the catalytic cleft by covering up or opening the space over the cleft (10, 19).

The cleft region spanning the \(\alpha\)-domain is of appropriate size to accommodate binding of the polymeric hyaluronan, is positively charged, and has only a small number of interactions with the \(\beta\)-sheet domain. These interactions are limited only to the selected residues, mainly Asn\textsuperscript{230}, at the edge of the \(\beta\)-domain that is facing the cleft and the \(\alpha\)-domain (Fig. 2). The negatively charged hyaluronan substrate was found to bind to...
the cleft. The positive charges in the cleft that complement the negative charge of the substrate are due to the accumulation of lysines and arginines that appear to be highly conserved among different bacterial species expressing this enzyme (10). The active site residues are grouped together at one side of the cleft and are composed of three distinct groups of residues: (i) the catalytic group composed of His399, Tyr408, and Asn349, (ii) a hydrophobic (aromatic) patch composed of Trp291, Trp292, and Phe243, and (iii) a negative patch composed of Glu388, Asp348, and Thr405 (10, 19, 23) (Fig. 3). The hydrophobic patch function appears to involve the selection of cleavage sites on the substrate chain for catalysis and the precise positioning of the substrate for cleavage by the catalytic group of residues. The catalytic group of residues is responsible for the cleavage of the glycosidic β1,4 linkage between HA1 and HA2 chains. The substrate through a five-step PAD catalytic mechanism as described below (10). The structural studies showed that the side chain of catalytic Asn348 forms hydrogen bonds with the carboxyl group of glucuronate moiety of the HA1 disaccharide and acts as an electron sink. This in turn causes acidification of the C-5 hydrogen of the C-5 carbon of the same glucuronate. A base, the His399 residue, then withdraws and accepts this more acidic hydrogen, resulting in the rehybridization of the C-5 carbon of the glucuronate. Simultaneously, the third catalytic residue, Tyr408, acts as an acid and donates its phenolic proton (hydrogen) to the glycosidic oxygen of the β1,4 bond to be cleaved. Protonation of this oxygen induces bond cleavage, subsequent formation of a C-4-C-5 double bond of the same glucuronate, and release of the unsaturated disaccharide product from the cleft. The remaining hyaluronan substrate is translocated toward the reducing end direction of the chain so that the penultimate disaccharide may then interact with the three catalytic residues (Fig. 5). During the process the enzyme donates the acquired proton on His399 to the surrounding water molecules and attracts one proton to Tyr408. In this manner the enzyme is prepared for the next round of catalysis.

Selection of the Residues for Mutations—In our previous study we have studied the catalytic mutants of the enzyme N349A, H399A, and Y408F as well as two mutants modifying the binding of the enzyme to the substrates R243V and N580A (10, 23). For the current study, we selected residues of the hydrophobic patch Trp291, Trp292, and Phe243 for the site-directed mutations. Based on the structural studies, their suggested role in the substrate degradation was to select the β1,4 glycosidic bond for degradation and position it precisely with respect to the catalytic group residues. Also, a suggestion has been raised that the hydrophobic patch residues are essential for the determination of the size of the product of degradation, i.e. disaccharide instead of tetra- or hexasaccharide, and for the positioning of the hyaluronan substrate in the enzyme cleft for catalysis. The precise positioning of the hyaluronan substrate is primarily accomplished by the hydrophobic interactions of carbohydrate rings of the substrate and the hydrophobic/ aromatic side chains of the residues selected for the mutation studies, primarily Trp291 and Phe243. To eliminate this interaction to study its effect on the enzyme activity and the size of the degradation product, the substitution of these residues to smallest amino acids like Ala or Val was preferred. The Trp291 residue interacts with the substrate mostly through the hydrogen bonding of its NE1 nitrogen (Fig. 3a) (34) unlike the ring-hydrophobic interaction of the substrate with Trp292 and Phe243 (Table I). Therefore, this residue was not selected for single mutation. The double and triple mutants were generated to study the additive effects of such changes on the enzymatic activity and the size of the product of degradation.

Kinetic Properties of the Mutant Forms of the Enzyme—All five of the mutants involving the hydrophobic residues in the cleft of hyaluronate lyase were expected to reduce the values for both Vmax and Km. For example, the W291A mutation removes Trp291, and therefore the indole nitrogen of Trp291, which otherwise would participate in several hydrogen bonds with the N-acetyl-β-D-glucosamine moiety of HA1 of the reducing end of the disaccharide to be removed, is not available to participate in substrate binding or positioning (19). Thus Trp291 would appear to serve an important role in the catalytic mechanism of this enzyme because it acts to finely position the substrate for catalysis and therefore directly assists in the catalytic process. The W292A mutation voids the enzyme from establishing the key hydrophobic interaction of Trp292 with the N-acetyl-β-D-glucosamine moiety of the HA2 disaccharide. The kinetic analysis indicating disruption to enzymatic function is also supported by the structural studies that indicate the importance of this hydrophobic interaction between the substrate and the enzyme in substrate positioning for catalysis. The two Trp amino acid residues cooperatively interact with the substrate on both sides of the β1,4 glycosidic linkage; this interaction and the corresponding enzyme-catalyzed reaction are disrupted by mutating either or both residues. Site-directed mutagenesis of Trp292 in the W292A mutant causes the enzyme to lose 98% of its activity and compromises the binding of the substrate as reflected in the Ken and Vmax/Km parameters (Table II). Even though the single mutant of Trp291 was not produced, its influence on enzyme activity is expected to be even more detrimental than that of the Trp292 mutant alone. The double mutant produced, W291A/W292A, abolished the enzymatic activity totally, clearly illustrating the importance of both Trp residues in enzyme catalysis. Finally, the mutation of Phe243 in the F343V mutant has a significant role in activity, because the mutant enzyme preserves only 70% of its original activity. Even though the substrate binding characteristics of this mutant do not appear to be significantly affected, as approximated by a Km value of 0.08 ± 0.01 mM (comparable with that of the wild type enzyme (0.09 ± 0.03 mM)), the Vmax and Vmax/Km values are significantly smaller (Table II). The Km parameter might also be influenced by other rate or equilibrium constants. The availability of the structural information, especially that of a complex of HA with the enzyme (19), suggests that perhaps the binding changes are not too significant for this mutant as compared with the wild type enzyme. In principle, the F343V mutation, however, may influence both the binding and the translocation of the substrate. Alternatively, the mutant enzyme may perform catalysis and product release or both at different rates. In this specific case the changes in the catalysis and product release or both are more likely.

The combined mutants of any two of these residues or all three of them render the enzyme essentially inactive. For the W291A/W292A double mutant and the W291A/W292A/F343V triple mutant, no activity could be detected even while utilizing the excess of the enzyme and increasing the time course for the reaction to several days. The only double mutant with some residual activity was W292A/F343V, which retained only 0.08% activity of the wild type enzyme. The values of Vmax and Vmax/Km were drastically smaller, whereas the Km was determined to be 3.82 ± 0.36 mM. All three parameters showed an enzyme drastically compromised in its catalytic abilities. It is evident that the function of the hydrophobic patch residues, Trp291, Trp292, and Phe243, is very important for the catalytic process of the enzyme. The individual mutations of these residues impact the activity to a very significant degree, and the double mutants obtained render the enzyme either inactive or essentially inactive.
The mutations of the noncatalytic, hydrophobic patch residues of the hyaluronate lyase were designed to test the different roles of these residues, particularly those involving positioning of the substrate for catalysis and in substrate binding. None of the residues analyzed participate in the catalytic function of this enzyme. These conclusions are based on the intrinsic chemical properties of these residues as well as their positioning relative to the substrate molecule observed in the three dimensional structures of each of the corresponding substrate-enzyme complexes (19). All of these residues are part of the catalytic c-domain, and they are well separated from the β-sheet domain of the enzyme, which controls the access to the cleft. Therefore, these mutations provide insight into role of the catalytic domain and about the reaction catalyzed by the enzyme.

Selection Process of the Size of the Degradation Product—The size of the final degradation product for the wild type and the active mutant enzymes is the C-4—C-5 unsaturated disaccharide of hyaluronic acid. None of the mutants produced products different from that of the wild type (data not shown). At very short degradation times, all of the enzymes were shown to produce tetra- and hexasaccharides; however, with time, they were all eventually degraded to disaccharide units (data not shown). These data do not support earlier suggestions that the hydrophobic patch residues play a direct role in the determination of the size of the degradation product (10). However, the location of these three hydrophobic residues in the cleft appear to be crucial in defining the precise positioning of the substrate on both sides of the glycosidic bond to be degraded as well as the processive and efficient nature of the enzymatic degradation of all consecutive β,1,4 linkages (to be degraded). In this manner primarily disaccharides are generated by the enzyme because the β,1,4 linkages separate distinct disaccharide units of HA (Fig. 1). Production of small amounts of tetra- and hexasaccharide units of HA instead of only disaccharides might be a result of random errors in the selection process of the linkage to be degraded. The presence of either Trp292 or Phe343 or even the lack of these two residues (W292A/F343V mutant) seems to protect the selection process for substrate degradation. However, the specific activity of the mutants causes the process to slow considerably, because the mutant residues are not expected to optimally position the substrate for catalytic degradation. The catalysis proceeds even with some of these residues not being available to position the substrate perfectly for catalysis, and every β,1,4 linkage is still degraded, albeit less efficiently.

The determination of specific activities for the mutant enzymes was also performed at higher salt concentration (ionic strength) to test the relation of hydrophobic versus, for example, hydrogen bonding effects (Table I). At higher ionic strength one might expect that the hydrophobic effect would be the major component as compared with the hydrogen bonding network. The activities clearly show a slight decrease as the ionic strength rises, but the changes are not very significant. These results are, however, consistent with the assumption that hydrophobic forces are very important and are essential for catalysis.

Structures of the Mutants and Their Complexes with Substrate—The structures of three mutants W292A, F343V, and W292A/F343V and two mutants complexes with the HA substrate, W291A/W292A and W291A/W292A/F343V, fully support the conclusions made above. The mutagenesis of hydrophobic residues did not modify significantly, if at all, the positions of the mutated residues but deprived the substrate of this enzyme of hydrophobic interactions made possible by the selected residues. The kinetic measurements performed of the mutants reflect primarily the changes caused by the mutation changes in the side chains and their interactions. These changes were shown to be very important for the enzyme—catalyzed reaction (Figs. 3b and 4 and Table II). The lack of hydrophobic interaction with the substrate did not drastically misplace the substrate; the substrate, as shown in the two complex structures reported here, is only slightly misplaced as compared with the earlier structural information (19, 34). However, the small modifications to the position of the substrate, especially with respect to the catalytic residues, Asn349, His349, and Trp308, are detrimental for catalysis. The precise positioning of the substrate by the hydrophobic residues present in the cleft of the enzyme, Trp308, Trp308, and Phe343 through the hydrophobic interaction with the substrate, including interactions of hydrophobic sugar rings of hyaluronan with hydrophobic planar moieties of Trp308 and Phe343, are absolutely essential for full activity of the enzyme.

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