Histidine 295 and Histidine 510 Are Crucial for the Enzymatic Degradation of Heparan Sulfate by Heparinase III[†]

Kevin Pojasek,[‡] Zachary Shriver,[‡] Yini Hu,[§] and Ram Sasisekharan*[,‡]

Division of Bioengineering & Environmental Health and Department of Biology, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

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ABSTRACT: The heparinases from *Flavobacterium heparinum* are powerful tools in understanding how heparin-like glycosaminoglycans function biologically. Heparinase III is the unique member of the heparinase family of heparin-degrading lyases that recognizes the ubiquitous cell-surface heparan sulfate proteoglycans as its primary substrate. Given that both heparinase I and heparinase II contain catalytically critical histidines, we examined the role of histidine in heparinase III. Through a series of diethyl pyrocarbonate modification experiments, it was found that surface-exposed histidines are modified in a concentration-dependent fashion and that this modification results in inactivation of the enzyme (k_{inact} = 0.20 ± 0.04 min^{-1} mM^{-1}). The DEPC modification was pH dependent and reversible by hydroxylamine, indicating that histidines are the sole residue being modified. As previously observed for heparinases I and II, substrate protection experiments slowed the inactivation kinetics, suggesting that the modified residue(s) was (were) in or proximal to the active site of the enzyme. Proteolytic mapping experiments, taken together with site-directed mutagenesis studies, confirm the chemical modification experiments and point to two histidines, histidine 295 and histidine 510, as being essential for heparinase III enzymatic activity.

Heparin sulfate and heparin-like glycosaminoglycans (henceforth referred to as HLGAGs),[1] ubiquitous components of the extracellular matrix, are emerging as important mediators of a variety of biological processes (*1*, *2*). These complex linear polysaccharides are composed of a disaccharide repeat unit of a hexosamine residue 1−4-linked to a uronic acid residue. These polymers of 20−100 disaccharide units can be additionally modified through *N*- and *O*-sulfation, epimerization at the C5 position of the uronic acid moiety and adding an additional microheterogeneity to these information-dense molecules (*3*, *4*). Heparan sulfate is found as a proteoglycan on the surface of many cell types, *O*-linked to a variety of core proteins, and plays an active role in mediating growth factor signaling (*5*).

Although the structure and chemistry of HLGAGs are fairly well understood, information on how specific HLGAG sequences modulate different biological processes has proven harder to obtain. Our laboratory has recently developed a rapid sequencing methodology for polysaccharides akin to those developed previously for polypeptides and polynucleotides (*6*). Use of this approach to sequence HLGAGs relies on chemical and enzymatic tools to modify or degrade an unknown HLGAG polymer in a sequence-specific manner. The molecular characterization of the enzymes that act on HLGAGs is crucial for the further development of tools for this sequencing methodology. One family of enzymes that act by degrading HLGAGs in a sequence-specific manner is the heparinases.

Heparinases I, II, and III are three HLGAG degrading enzymes produced by *Flavobacterium heparinum*. Each of the heparinases has its own unique HLGAG sequence at which it cleaves, making these enzymes valuable tools in obtaining sequence-specific information. Heparinase I primarily cleaves HLGAGs at the $H_{NS,6X}-I_{2S}$[2] linkage found primarily in heparin-like regions (*4*, *7*, *8*). Heparinase III cleaves at the $H_{NY}-I$ and $H_{NY,6X}-G$[2] linkages which are the major disaccharides found in heparan sulfate (*4*, *6*, *9*). Heparinase II is capable of recognizing and cleaving both sets of substrate linkages (*4*).

Our laboratory has also been actively investigating the enzymology of the heparinases. In the case of heparinase I, cysteine 135 and histidine 203 along with lysines 198, 199, and 132 were identified to be critical for HLGAG degradation (*10*−*12*). Also, cysteine 348 and histidines 238, 451, and 579 were determined crucial for heparinase II activity (*13*, *14*).

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* To whom correspondence should be addressed at 16-561, MIT, Cambridge, MA 02139. Telephone: (617) 258-9494. Fax: (617) 258-9409.

[‡] Division of Bioengineering & Environmental Health.

[§] Department of Biology.

[1] Abbreviations: HLGAGs, heparin/heparan-like glycosaminoglycans; DEPC, diethyl pyrocarbonate; RPHPLC, reverse-phase high-pressure liquid chromatography; IPTG, isopropyl-β-D-thiogalactopyranoside; CD, circular dichroism; ΔU, uronic acid moiety with a 4,5 double bond.

[2] X represents sulfate or a hydroxyl group. Y represents sulfate or acetyl group.

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Heparinase III is unique in that it is the only member of the heparinase family that recognizes and preferentially cleaves heparan sulfate. Heparinase III is also unique among its heparin-degrading family members in that it contains no cysteines in its amino acid sequence (*15*, *16*). Heparinase III, however, does contain 13 histidines of which 1 or several might be involved in the activity of the enzyme. Therefore, through a combination of chemical modification, peptide mapping, and site-directed mutagenesis studies, we sought to examine the potential role of histidine in the catalytic activity of heparinase III.

MATERIALS AND METHODS

Chemicals and Materials. Hydroxylamine hydrochloride and urea were from EM Science (Gibbstown, NJ). The chemical modification reagent diethyl pyrocarbonate (DEPC) was purchased from Sigma and used as received (Milwaukee, WI). Heparan sulfate was purchased from Celsus Laboratories (Cincinnati, OH). Lys-C from *Lysobacter enzymogenes* (EC 3.4.21.50) was from Roche Molecular Biochemicals (Indianapolis, IN). Heparinase III from *Flavobacterium heparinum* (EC 4.2.2.8) was purified as described previously (*11*, *17*) and was a gift from IBEX Technologies (Montreal, Canada).

Heparinase III Activity Assay. The activity of heparinase III was measured using a UV 232 nm assay similar to those reported for heparinase I and heparinase II (*11*, *14*, *17*). Briefly, the increase in absorbance at 232 nm as a function of time was monitored under saturating substrate conditions. All assays were performed with heparan sulfate at a concentration of 2 mg/mL in 50 mM sodium phosphate, pH 7.6. The temperature for enzymatic activity measurements was kept constant at 35 °C.

Chemical Modification of Heparinase III with DEPC. (A) DEPC Inactivation of Heparinase III. At pH values ranging from 6.0 to 8.0, heparinase III (50 μg/mL) was incubated with DEPC in 50 mM sodium phosphate buffer at 25 °C. The DEPC stock solution (6.9 M) was diluted with ethanol. Control reactions contained an equivalent amount of ethanol without DEPC, which did not affect enzymatic activity over the experimental time range. At each pH, three reactions were run using different concentrations of DEPC, ranging from 50 μM to 2.5 mM. At fixed time intervals, 25 μL aliquots were withdrawn from the reaction mixtures for the UV 232 nm activity assay.

The kinetics of DEPC inactivation of heparinase III were determined by plotting the natural log of percent activity versus an adjusted time term (to account for the decomposition of DEPC). Briefly, this adjusted time term (t') was calculated according to the equation:

$$t' = \frac{1 - e^{k't}}{k'}$$

In this equation, k' is the first-order rate constant for DEPC hydrolysis and t is the measured time after addition of DEPC to the heparinase III solution. At each pH, the order of the reaction in DEPC was determined by plotting the log of the observed rate constants of inactivation at each pH vs log [DEPC]. The slope of this line is n, the order of the reaction with respect to DEPC (*18*).

(B) Reactivation of DEPC-Modified Enzyme with Hydroxylamine. Similar to what was completed with heparinases I and II (*11*, *14*), heparinase III (50 μg/mL) was incubated with 0.97 mM DEPC, pH 6.5, until its enzymatic activity was reduced to 50% of its initial value. Hydroxylamine was then immediately added to the reaction mixture to a final concentration of 0.5 M. The reaction was incubated at room temperature for 6 h. Aliquots were withdrawn every hour for the activity assay. The control mixture contained no DEPC but the same concentration of hydroxylamine to account for the nonspecific loss of activity. The ratio of the activity of the reaction mixture over the activity of the control was calculated to determine recovery of enzymatic activity.

(C) Substrate Protection of Heparinase III against DEPC Inactivation. Heparinase III (50 μg/mL) was preincubated with 2 mg/mL heparan sulfate in 50 mM sodium phosphate, pH 7.6, for 30 min at room temperature prior to the addition of 1.5 mM DEPC. A control reaction with no prior incubation of substrate was also completed. The time course of inactivation for both was determined using the heparinase III activity assay.

(D) Quantification of the Number of Histidines Modified by DEPC. The extent of modification of an enzyme by DEPC can be determined by monitoring the formation of the *N*-carbethoxylhistidyl adduct at 240 nm. At time zero, DEPC was added to a final concentration of 1.5 mM to the cuvette containing heparinase III in sodium phosphate buffer, pH 7.0. The change in absorbance at 240 nm was monitored every minute for 10 min. The number of modified residues was determined using a molar extinction coefficient of 3200 M^{-1} cm^{-1} (*18*).

Peptide Mapping Studies. To determine which histidine residues were modified by DEPC, mapping studies using the protease Lys-C were completed. Heparinase III (1 nmol) was incubated with 4 mM DEPC for 15 min, denatured with 6.5 M urea at 55 °C, and diluted with water. The enzyme was digested with 1.6 μg of Lys-C at 37 °C for 12 h.

Peptides derived from heparinase III digestion were separated by RPHPLC and monitored at 210, 277, and 240 nm. Peptide peaks not present in the control digest were collected and sequenced using an Applied Biosystems Sequencer model 477 with an on-line model 120 PTH amino acid analyzer (Biopolymers Laboratory, MIT).

Site-Directed Mutagenesis. Each of the 13 histidine residues of heparinase III was mutated to alanine using overlap extension PCR for 15 cycles (*19*). The PCR reactions were separated on a low-melt agarose gel, and the band corresponding to the proper length was excised. The DNA was extracted from the gel using a Gel Purification Kit (Qiagen, Valencia, CA), the insert was subcloned into pCR 2.1 (Invitrogen, Carlsbad, CA), and the plasmid was prepared using a Miniprep kit (Qiagen, Valencia, CA). The validity of all the point mutations and the integrity of the rest of the gene were verified by sequencing (data not shown). The 13 mutant heparinase III clones, along with recombinant heparinase III, were excised from pCR2.1 using *Nde*I/*Bam*HI (New England Biolabs, Beverly, MA) and subcloned into a pET-15b expression vector (Novagen, Madison, WI) that had been previously digested with these same enzymes. The pET-15b plasmid contains an NH_2 -terminal His-Tag for Ni^{2+}-column purification. Recombinant heparinase III was also

expressed and compared to the native heparinase III isolated directly from *Flavobacterium heparinum*.

Expression and Purification of r-Heparinase III and Mutants in E. coli. Overnight cultures of Luria−Bertani (LB) broth (5 mL) containing 0.02 mg/mL ampicillin (amp) were used to inoculate 500 mL LB/amp cultures at an initial OD_{600} of 0.1. The cultures were induced with 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) in mid-log phase (OD_{600} 0.7−0.9) and incubated for another hour at 37 °C. To harvest the cells, the cultures were spun at 5000 rpm, and the supernatant was discarded.

The cell pellet was resuspended in 20 mM Tris, 500 mM NaCl, 5 mM immidazole hydrochloride, pH 7.9 (1/50 of the initial culture volume). The resuspended cells were placed on ice and sonicated as described previously (*20*). The soluble protein of the cell lysate was isolated by centrifugation at 12 000 rpm for 20 min at 4 °C. The supernant was filtered through a 0.45 μm filter and loaded onto a nickel POROS column using a Biocad Perfusion Chromatography system (PerSeptive Biosystems, Framingham, MA). The column was washed, and the protein was subsequently eluted in 20 mM Tris, 500 mM NaCl, 500 mM imidazole hydrochloride, pH 7.9. SDS−polyacrylamide gel electrophoresis analysis using precast 12% gels, the Mini-Protean II apparatus, and the Silver Stain Plus kit (Bio-Rad, Hercules, CA) was performed to determine the concentration, by using known amounts of heparinase III as standards, and the purity of the individual proteins. The concentrations of the individual proteins were confirmed using the Micro BCA Assay (Bio-Rad, Hercules, CA).

HPLC Analysis of Saccharide Products of Heparinase III Activity. Exhaustive digests of 3 mg/mL heparan sulfate in 50 mM sodium phosphate buffer, pH 7.6, were performed overnight at 37 °C for each of the mutants (20 μg of protein). The reactions were loaded onto a Spherisorb S5 SAX column (Waters) and eluted using a linear gradient of 0.2−1.0 M NaCl, pH 3.5. The products were monitored at 232 nm, and each of the major peaks was collected. To identify the products, the collected fractions were analyzed by capillary electrophoresis and identified by comigration with known standards.

Circular Dichroism (CD). Recombinantly expressed heparinase III and the heparinase III mutants, H295A and H510A, were concentrated and buffer-exchanged into 50 mM sodium phosphate, pH 7.0, using a Centricon 30 Filter (Millipore, Watertown, MA). CD spectra were collected on an Aviv 62DS spectropolarimeter equipped with a thermostatic temperature controller and interfaced to an IBM microcomputer. Measurements were performed in a quartz cell with a 1 mm path length. Spectra were recorded at 25 °C, in an average of 10 scans between 205 and 260 nm, with a 1.0 nm bandwidth and a scan rate of 3 nm/min. CD band intensities are expressed as molar ellipticities, θ_M, in deg·cm^2·dmol^{-1}.

RESULTS

DEPC Inactivates Heparinase III. As a first step toward identifying histidines that are critical for the enzymatic activity of heparinase III, we determined the effect of the modification reagent DEPC on the enzymatic activity of heparinase III. DEPC is a common reagent used for the modification of solvent-accessible histidines in enzymes. As stated in early publications (*11*, *14*), DEPC is useful for the determination of catalytically critical histidines that are solvent-accessible; however, care needs to be taken to ensure that other nucleophilic residues, namely, tyrosines, lysines, and cysteines, are not modified.

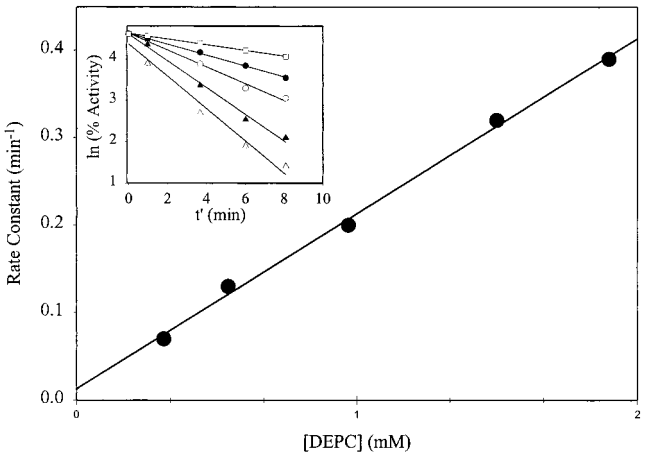

FIGURE 1: DEPC inactivation of heparinase III. (Inset) Heparinase III was incubated with 0.31 (□), 0.54 (●), 0.97 (○), 1.5 (▲), and 1.9 (△) mM DEPC at pH 6.5 and at 25 °C. The natural log of the percent activity remaining was plotted versus an adjusted time term (t') to account for the decomposition of DEPC. The slope of each of the lines at the various DEPC concentrations represents the pseudo-first-order rate constants of inactivation. Plotting these pseudo-first-order rate constants versus the respective DEPC concentrations yields a second-order rate constant of inactivation of 0.20 ± 0.04 mM^{-1} min^{-1}.

Table 1: Hydroxylamine Reversibility of DEPC Inactivation

time (min)	activity (%)
0	51
30	60
60	66
90	72
180	76
240	78
360	80

For heparinase III, we find that, similar to heparinases I and II, DEPC inhibits in a concentration-dependent fashion. A measured second-order rate constant of 0.20 ± 0.04 min^{-1} mM^{-1} (Figure 1) is obtained by varying the concentration of the inhibitor. Consistent with this reaction being first order in both heparinase III and DEPC, a plot of log k_{inact} vs log [DEPC] yielded a line with a slope of 1 (Figure 2).

To ensure that the interaction of DEPC with heparinase III is through histidine modification, we investigated whether other nucleophilic amino acids of heparinase III interact with DEPC. First, unlike with heparinase I or II, there is no possibility for cysteine modification since heparinase III contains no cysteines in its primary amino acid sequence. Furthermore, there was no loss of absorbance at 278 nm upon incubation of DEPC with heparinase III as would be expected if tyrosines were modified. Finally, addition of hydroxylamine to DEPC-modified heparinase III reversed most of the inactivation, indicating that strongly nucleophilic residues, such as lysine, were not modified by DEPC (Table 1).

In an attempt to further define the interaction of DEPC with the histidines of heparinase III, we examined the effect of the pH on the inactivation kinetics. Examination of the

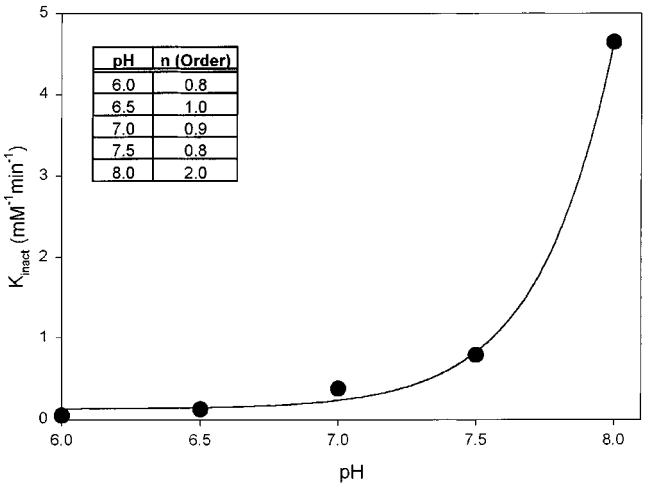

FIGURE 2: pH dependence of the second-order rate constant of inactivation. Heparinase III was incubated with 50 μM to 2.5 mM DEPC at pH's 6.0−8.0 at 25 °C. The second-order rate constant of inactivation was calculated for each pH.

rate of inactivation as a function of pH has been used to derive a pK_a for a modified residue, since, in the case of histidine, the unprotonated form is much more readily modified than is the protonated form. With heparinase III, increasing the pH of the reaction from 6 to 7.5 results in an increase in the inactivation kinetics without changing the order of the reaction (Figure 2). However, at pH 8.0 and higher, the reaction is no longer first order in DEPC, indicating other residues (possibly lysines) are interacting with DEPC at this pH. Consistent with this interpretation, hydroxylamine is no longer able to reverse inactivation at pH 8.0. Therefore, the mapping studies and substrate protection experiments discussed below were conducted at pH 7.0 which maximized the reactivity while ensuring that only histidines were the target of DEPC modification.

Consistent with the idea that DEPC is interacting with a histidine residue in heparinase III, there is an increase in absorbance at 240 nm as a function of time, resulting from *N*-carbethoxyhistdyl derivatives. The number of modified histidines was quantified (Figure 3). Over the course of 10 min, 1.8 histidines are modified per enzyme molecule, resulting in a loss of greater than 90% activity. Thus, it appears that one or possibly two histidines, modified by DEPC, result in loss of enzymatic activity for heparinase III.

Preincubation of heparinase III with heparan sulfate substrate before addition of DEPC resulted in lower inactivation kinetics (Figure 4), suggesting that the histidine(s) modified by DEPC is (are) proximate to the substrate binding and/or active site of heparinase III, similar to what was observed for heparinases I and II (*11, 14*).

Peptide Mapping of the Histidine Modified by DEPC. To identify the histidine(s) modified by DEPC that resulted in the loss of enzymatic activity, DEPC-modified heparinase III was digested with Lys-C. Peptides that had altered retention times and an increased in absorbance at 240 nm as compared to a control digest were collected and sequenced (Figure 5). Three peptides that had altered retention times and increased absorbance at 240 nm were isolated and sequenced. Two of the peptides contained histidine 295, and one contained no modified histidine residues (see Discussion).

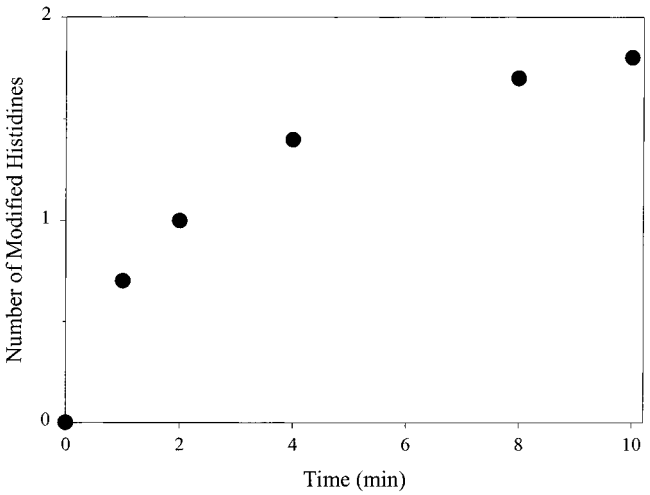

FIGURE 3: Quantification of DEPC-modified histidine residues in heparinase III. At time zero, 1.5 mM DEPC was added to a cuvette containing heparinase III (540 μg/mL) in sodium phosphate buffer, pH 7.0. The change in absorbance at 240 nm was monitored at time intervals for 10 min. The number of modified histidines were calculated using $\epsilon = 3200\ M^{-1}\ cm^{-1}$. At the beginning and end of the experiment, aliquots of heparinase III were withdrawn and tested for activity. Less than 5% of the initial activity remained after 10 min incubation with DEPC.

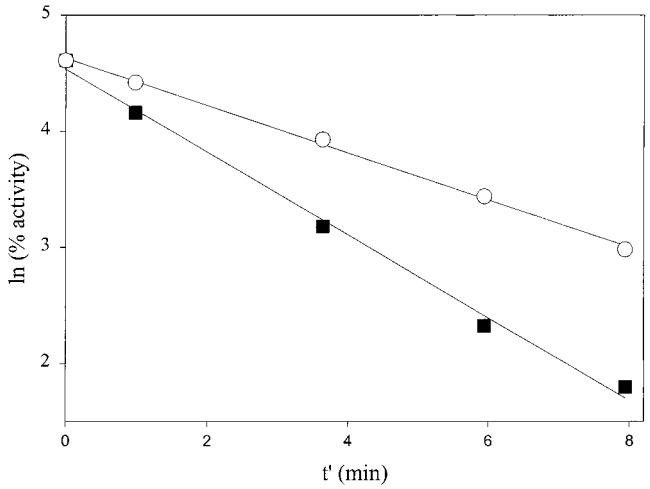

FIGURE 4: Substrate protection of heparinase III inactivation by DEPC. Heparinase III (50 μg/mL) was incubated with 2 mg/mL heparan sulfate for 30 min. 1.5 mM DEPC was added to the reaction, and the time course of inactivation was completed using the heparinase III activity assay (○). A control reaction without preincubation with heparan sulfate was also done (■).

Site-Directed Mutagenesis of Heparinase III. In parallel to the mapping studies and to confirm the results of the chemical modification experiments, each of the 13 histidine residues present in heparinase III was mutated to alanine. The recombinant heparinase III mutant proteins were expressed, purified, and assessed for enzymatic activity toward heparan sulfate (Table 2). As a control, the r-heparinase III construct without its putative signal sequence was expressed. The concentration and purity of all recombinant enzyme preparations were determined using SDS−PAGE and a Micro BCA assay (data not shown). All of the enzyme preparations were of sufficient purity for the subsequent experiments. The recombinant heparinase III was also compared to the heparinase III isolated from *F. heparinum* to ensure that they were the same molecular weight. Both enzymes displayed similar kinetic activity toward heparan

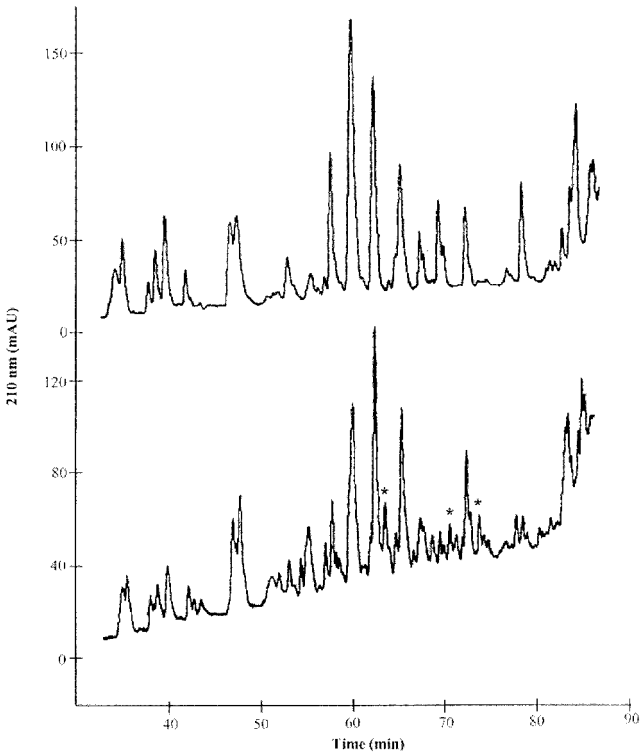

FIGURE 5: (A, top) C4 RPHPLC profile of the Lys-C digest of heparinase III which was not exposed to DEPC. (B, bottom) Peptide profile of heparinase III labeled with DEPC. Labeling of the DEPC-reactive histidines was completed by first reacting heparinase III with DEPC, then denaturing the protein in urea. Following an overnight digest with Lys-C, the resultant peptides were separated by using a 1.6−78.4% acetonitrile gradient over 120 min, which included a 5 min isocratic phase (1.6% acetonitrile, 0.1% trifluoroacetic acid) at the beginning of the run. Lys-C peptides were monitored at 210, 240, and 277 nm. New peptide peaks, not present in the control digest and with a marked absorbance at 277 nm were collected and sequenced. These peptides are marked with an asterisk in the chromatogram. The peptides migrating at 62 and 71 min contained the sequence QVYADGMQFELSPIY**H**VAAIDIFLK including histidine 295. The other consistently labeled peptide did not contain a histidine.

sulfate and yielded the same degradation profiles as determined by SAX-HPLC (Figure 6). The products of the exhaustive digests were then analyzed using capillary electrophoresis. The first major peak (5 min) observed in the SAX-HPLC chromatograms has a migration time that is identical to ΔU-H_{NAc}. The second peak (7.5 min) has a migration time that is identical to ΔU-H_{NS} (data not shown). Thus, the heparan sulfate degradation by recombinant heparinase III produces an identical product profile to that of wild-type heparinase III, indicating that, at least functionally, these enzymes are the same.

The replacement of histidine 295 and histidine 510 with alanine residues completely eliminated the activity of heparinase III toward heparan sulfate (Table 2). Kinetic data for the wild-type heparinase III are included to demonstrate its similarity to the recombinbant heparinase IIII. The H295A and H510A mutant enzymes showed no differences in terms of expression level or molecular weight. However, both the kinetic data and the exhaustive digest profile for H295A and H510A suggest that the enzymes are completely inactive (Figure 6). Nine of the histidine mutants (H36A, H152A, H225A, H234A, H241A, H469A, H424A, H510A, and H539A) showed no significant changes in recombinant

Table 2: Kinetic Constants for r-Heparinase III and the Histidine Mutants

enzyme	K_M (μM)[a]	k_{cat} (s^{-1})
wild-type heparinase III	143[b]	94[b]
wild-type r-heparinase III	80	78
H36A	98	86
H105A	ND[c]	ND[c]
H110A	9	37
H139A	191	68
H152A	58	83
H225A	80	22
H234A	75	23
H241A	16	5
H295A	ND	ND
H424A	59	24
H469A	71	100
H510A	ND	ND
H539A	92	132

[a] Calculated assuming a molecular mass for heparan sulfate of 15 kDa. [b] Kinetic values were calculated in phosphate buffer without imidazole. [c] Protein expression levels were too low for heparinase III kinetic assay.

protein yield, enzyme activity, or kinetic parameters when compared with r-heparinase III. Interestingly enough, 3 (H105A, H110A, and H139A) of the 13 histidine mutants yielded much less recombinant protein than either recombinant heparinase III or the other mutants. Despite lower protein levels, the H110A and H139A mutant proteins were amenable to kinetic analysis whereas the H105A mutant protein was not. However, SAX-HPLC analysis of overnight heparan sulfate digests confirmed that despite lower levels of recombinant expression, all three of these underexpressed enzymes retain their catalytic activity. The results for H105A are shown in Figure 6.

The wild-type heparinase III, the recombinantly expressed heparinase III, the H295A mutant, and the H510A mutant were compared using circular dichroism (CD). The possibility remained that the histidine 295 and/or histidine 510 were somehow responsible for the folding or the tertiary structure of the enzyme and not directly involved in catalysis. However, the CD spectra for H295A and H510A were nearly identical to those of wild-type and recombinant heparinase III (Figure 7). While the near-identity of the CD profiles does not preclude the possibility that there are perturbations in the local environment surrounding histidine 295 and histidine 510 that are not represented in the CD profile, it does suggest there are no gross conformational changes induced by mutating histidine 295 and histidine 510 to alanine.

DISCUSSION

We have shown through a combination of chemical modification, peptide mapping, and site-directed mutagenesis experiments that two histidines, histidine 295 and histidine 510, are critical for the enzymatic degradation of heparan sulfate by heparinase III. To the best of our knowledge, this represents the first biochemical characterization of an enzyme that exclusively degrades heparan sulfate.

Chemical Modification. We find that DEPC inactivates heparinase III in a pseudo-first-order, concentration-dependent manner (Figure 1). This suggests that DPEC is directly modifying a residue involved in the catalytic degradation of heparan sulfate. The second-order rate constant of inactiva-

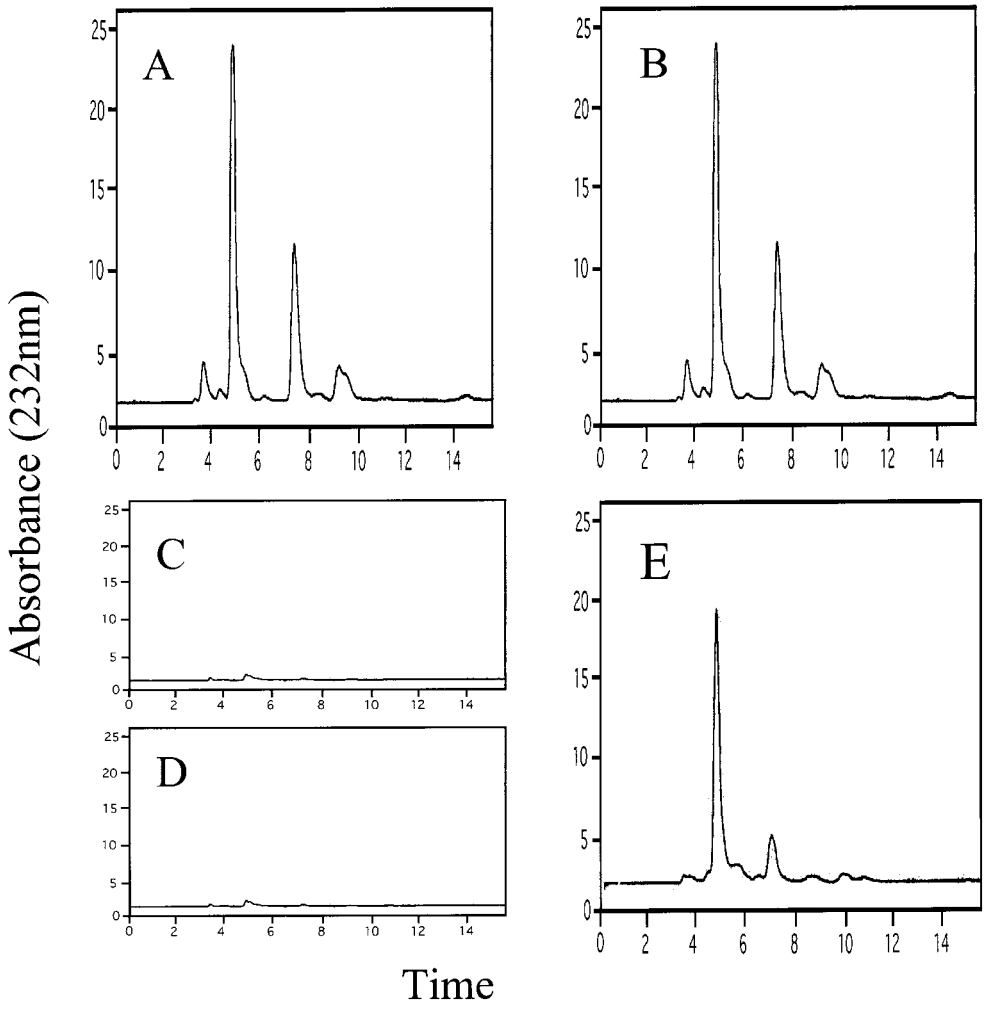

FIGURE 6: SAX analysis of exhaustive heparinase III digests of heparan sulfate. Heparinase III (20 μg/mL) was incubated with 4 mg/mL heparan sulfate overnight at 37 °C. The reaction was loaded onto a SAX column, and the saccharide products were eluted using a gradient of 0.2−1.0 M NaCl, pH 3.5, over 30 min and monitored at 232 nm. (A) Heparan sulfate digested with heparinase III from *F. heparinum*. (B) Heparan sulfate digested with recombinant heparinase III. (C) Heparan sulfate digested with the H295A mutant enzyme. (D) Heparan sulfate digested with the H510A mutant enzyme. (E) Heparan sulfate digested with the H105A mutant enzyme.

tion (0.20 ± 0.04 min^{-1} mM^{-1}) also suggests that DEPC is a potent inhibitor of heparinase III function.

DEPC is extremely useful in elucidating the role of histidines in enzymatic function. Care has to be taken, though, to ensure that DEPC does not modify other nucleophilic amino acids such as tyrosine, lysine, or cysteine (*11*, *14*). In the case of heparinase III, there are no cysteine residues in the amino acid sequence, eliminating this amino acid as a potential confounding factor in our chemical modification studies. Also, no decrease in the absorbance at 278 nm was observed after heparinase III was incubated with DEPC, suggesting that tyrosine residues were not modified (data not shown). An increase in the inactivation kinetics without a change in the order of the reaction was observed from pH 6.0 to pH 7.5 (Figure 2). Furthermore, the DEPC modification was 80% reversible upon incubation with 500 mM hydroxylamine (Table 1). Above pH 8.0, the inactivation kinetics were no longer first order for DEPC, and the modification could not be reversed by hydroxylamine, suggesting that residues other than histidines (i.e., lysines) were being modified. However, at neutral pH, the data suggest that DEPC specifically modifies the histidine residues of heparinase III.

Consistent with the observation that DEPC is modifying a histidine residue, there is an increase in the absorbance at 240 nm as a function of time. This is indicative of formation of an *N*-carbethoxyhistidyl derivative, the product of a reaction between DEPC and a histidine residue. Over the course of 10 min, 1.8 histidine residues are modified, and the enzymatic activity is decreased by 90% (Figure 3). Also, preincubation with heparan sulfate resulted in lower inactivation kinetics of heparinase III by DEPC (Figure 4). These data suggest that DEPC specifically modifies a critical histidine residue proximate to the substrate binding/active site of heparinase III, inactivating the enzyme.

An apparent discrepancy arises from these results in that the reaction of DEPC with heparinase III follows pseudo-first-order kinetics, yet two histidines appear to be independently modified. It is possible that two surface-accessible histidines react with DEPC at identical rates. It could be the case that either one or both of the modified residues is responsible for inactivating the enzyme. The limits of these chemical modification experiments prevent us from distinguishing between these two scenarios. However, the site-directed mutagenesis experiments discussed below point to

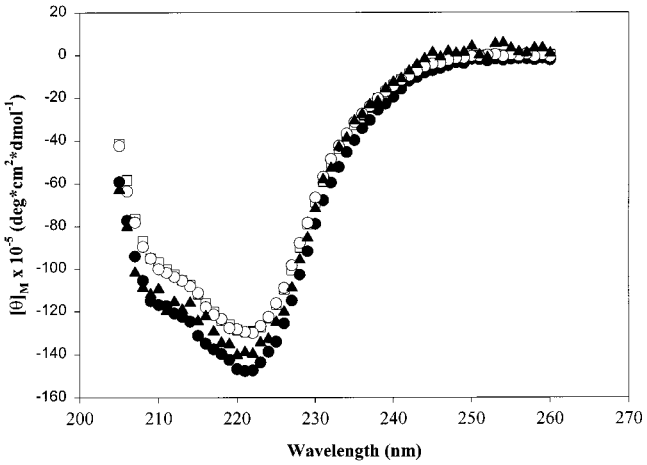

FIGURE 7: Circular dichroism analysis of recombinant heparinase III and the H295A mutant enzyme. The recombinant heparinase III (●), the H295A mutant enzyme (○), the H510A mutant enzyme (■), and the native heparinase III (□) were concentrated and buffer-exchanged into 50 mM sodium phosphate buffer, pH 7.0. Readings were taken using a quartz cell with a 1 mm path length at 25 °C. Spectra were recorded between 205 and 260 nm with an average of 10 scans; the bandwidth was 1.0 nm; and the scan rate was 3 nm/min. The CD band intensities are expressed as molar ellipticities, θ_M, in deg·cm^2·dmol^{-1}.

two histidines as being essential for heparinase III's catalytic activity.

Site-Directed Mutagenesis. The results from the site-directed mutagenesis experiments confirm and expand upon the chemical modification data in that surface-accessible histidines are critical for heparinase III activity. These results identify histidine 295 and histidine 510 as the primary histidines involved in the degradation of heparan sulfate by heparinase III. When these residues are replaced with alanines, the enzyme loses all activity toward its substrate (Table 2). None of the other histidine residues when mutated to alanine show a complete loss of activity (Figure 6 and Table 2). The results from the peptide mapping studies confirm the importance of the surface accessibility of histidine 295 (Figure 5). For some reason, histidine 510 does not appear in the chromatogram as modified under the present reaction conditions. Another possibility is that the histidine 510 is part of a peptide which is eluted in the void volume and not observed under the present experimental conditions (*11*).

The loss of activity with the H295A and H510A enzymes can be explained in several ways. It may be that these histidines are necessary for proper folding of heparinase III. However, the CD spectra of H295A, H510A, and recombinant heparinase III are nearly identical, suggesting that this is not the case (Figure 7). A second explanation is that histidine 295 and histidine 510 play a direct role in the binding of heparan sulfate to the enzyme. While this may be the case for heparinase III, prior studies on heparinase I suggest that other residues fulfill this role, namely, lysines (*21*). A final possibility is that histidine 295 and histidine 510 are critical active site residues directly involved in the catalytic degradation of heparan sulfate.

Histidine has been shown to be a critical residue in the function of the other heparinase family members, heparinases I and II (*14*, *22*). The results of this study are consistent with what was observed with heparinase II. In heparinase II, several histidines, specifically histidines 238, 451, and 579, were shown to be catalytically active in the depolymerization of heparan sulfate (*14*). It is also worth noting that when the catalytically critical cysteine of heparinase II, cysteine 348, is mutated to alanine, the enzyme loses the ability to depolymerize heparin but not heparan sulfate (*13*). This observation, along with the fact that there are no cysteines in the primary sequence of heparinase III, suggests that cysteine is not important in the degradation of heparan sulfate.

Although the role of histidine has not been clearly elucidated for heparinase II, a model exists for the role of histidine in heparinase I (*12*). It has been proposed that histidine 203 forms part of a catalytic triad also consisting of lysine 199 and cysteine 135 in heparinase I. In this model, histidine acts to stabilize the unprotonated form of the cysteine residue, allowing the thiolate anion to abstract the C5 hydrogen of the uronic acid moiety in the heparin chain. Lysine 199, along with calcium, is thought to stabilize the developing negative charge on the carboxylate of the uronic acid. Histidine may also directly protonate the anomeric oxygen in the leaving group. Histidine 295 or histidine 510 may be playing a similar acid−base catalysis role in the active site of heparinase III with another residue acting as the base directly involved in the abstraction of the C5 hydrogen. Future studies will hopefully identify other residues critical for heparinase III activity. This paper represents the first step in an effort to fully understand the mechanism by which heparinase III depolymerizes heparan sulfate.

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