Heparinase II from *Flavobacterium heparinum*

ROLE OF HISTIDINE RESIDUES IN ENZYMATIC ACTIVITY AS PROBED BY CHEMICAL MODIFICATION AND SITE-DIRECTED MUTAGENESIS*

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The three heparinases derived from *Flavobacterium heparinum* are powerful tools for studying heparin-like glycosaminoglycans in major biological processes, including angiogenesis and development. Heparinase II is unique among the three enzymes because it is able to catalytically cleave both heparin and heparan sulfate-like regions of heparin-like glycosaminoglycans. Toward understanding the catalytic mechanism of heparin-like glycosaminoglycan degradation by heparinase II, we set out to investigate the role of the histidines of heparinase II in catalysis. We observe concentration-dependent inactivation of heparinase II in the presence of the reversible histidine-modifying reagent diethylpyrocarbonate (DEPC). With heparin as the substrate, the rate constant of inactivation was found to be 0.16 min⁻¹ mM⁻¹; with heparan sulfate as the substrate, the rate constant was determined to be 0.24 min⁻¹ mM⁻¹. Heparinase II activity is restored following hydroxylamine treatment. This, along with other experiments, strongly suggests that the inactivation of heparinase II by DEPC is specific for histidine residues and that these histidines are modified by DEPC. Substrate protection experiments show that heparinase II preincubation with heparin followed by the addition of DEPC resulted in a loss of enzymatic activity toward heparan sulfate but not heparin. However, heparinase II preincubation with heparan sulfate was unable to protect heparinase II from DEPC inactivation for either of the substrates. Proteolytic mapping studies with Lys-C were consistent with the chemical modification experiments and identified histidines 238, 451, and 579 as being important for heparinase II activity. Further mapping studies identified histidine 451 as being essential for heparin degradation. Site-directed mutagenesis experiments on the 13 histidines of heparinase II corroborated the chemical modification and the peptide mapping studies, establishing the importance of histidines 238, 451, and 579 in heparinase II activity.

Heparin-like glycosaminoglycans (HLGAGs) are one of the major components of the extracellular matrix. Increasingly, evidence points to the fact that HLGAGs serve a critical regulatory role in numerous biological functions (1, 2). The chemistry and structure of HLGAGs are beginning to be well understood (3). HLGAGs are linear, sulfated, acetylated polysaccharides consisting of 1–4-linked derivatives of hexosamine and uronic acid (1, 4).

At present, however, there is little understanding, at the molecular level, of how specific sequences of HLGAGs modulate a given biological process (1, 2). The considerable chemical heterogeneity of HLGAGs provides the major challenge in determining these sequence-function relationships (3, 5). Heparin, one subset of HLGAGs, possesses predominantly L-iduronic acid with a high degree of sulfation (3, 4). Heparan sulfate, another subset of HLGAGs, is chemically similar to heparins but contains less 2-O-sulfate and N-sulfate groups than heparin and also possesses a higher percentage of d-glucuronic acid within the polymer (4, 6). Of importance, then, is the development of molecular tools that can serve to elucidate structure-function relationships between HLGAGs and biological molecules, such as growth factors and cytokines. One such tool has proved to be the three heparinases derived from *Flavobacterium heparinum* (7). Using heparinases, HLGAGs have been shown to be critical in major biological functions, including angiogenesis (8) and development (9, 10).

The three heparinases from *F. heparinum* are distinguished on the basis of their size, charge properties, and substrate specificities (3, 11). Heparinase I cleaves HLGAGs at sites with an O-sulfated L-iduronic acid linkage (i.e. heparin-like regions). Conversely, heparinase III requires primarily an unsulfated D-glucuronic acid moiety (heparan sulfate-like regions). Heparinase II, the largest of the heparinases, is unique in that it possesses the ability to cleave both heparin and heparan sulfate-like regions of HLGAGs (3, 11). Thus, unlike heparinase I and heparinase III, which distinguish between the C5 epimers L-iduronic acid and D-glucuronic acid, heparinase II is catalytically active toward both (3).

In an effort to develop heparinase II as a biological tool, it is important to understand the basis for heparinase’s II substrate specificity, viz. its ability to catalytically cleave both heparin and heparan sulfate-like regions of HLGAGs, and hence to elucidate its active site chemistry (3). With this understanding, the amino acid sequence of heparinase II potentially can be altered such that its substrate specificity or reaction rate is modified (12). To this end, we are attempting to identify residues in heparinase II that are important in the degradation of heparin and/or heparan sulfate.

The aim of this study is to determine whether a histidine(s) is important in the degradation of heparin and/or heparan sulfate by heparinase II. Of the four histidines present in heparinase I, histidine 203 was identified as being essential for catalysis (14). Heparinase II contains 13 histidines (15). Thus,

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§ The abbreviations used are: HLGAGs, heparin-like glycosaminoglycans; r-heparinase II, recombinant heparinase II; RPHPLC, reverse phase high pressure liquid chromatography; DEPC, diethylpyrocarbonate; pCMBl, p-(chloromercuri)benzoate.

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it is plausible that one or more histidines are critical for enzymatic activity. Combining chemical modification, mapping studies, and site-directed mutagenesis, this study set out to identify specific histidines that are important for the enzymatic depolymerization of heparin/heparan sulfate by heparinase II.

**MATERIALS AND METHODS**

**Chemicals and Materials**

Urea, Tris, and trifluoroacetic acid were from J. T. Baker (Phillipsburg, NJ). Diiodotheitol was obtained from Sigma. Sodium phosphate monobasic and dibasic and acetoneitrile were from Mallinckrodt (Chesterfield, MO). Hydroxyapatite and bovine serum albumin were purchased from Bio-Rad. The chemical modification reagent diethylpyrocarbonate (DEPC) was purchased from Aldrich and used as received. Lys-C from *Achromobacter lyticus* (EC 3.4.21.50) was obtained from Wako Bioproducts (Richmond, VA). Heparin, from porcine intestinal mucosa with an average molecular mass of 12 kDa, was obtained from Hepar (Franklin, OH). Heparan sulfate, also derived from porcine intestinal mucosa, was from Celsus Laboratories (Cincinnati, OH). *Escherichia coli* BL21 (DE3) host was from Novagen (Madison, WI).

**Heparinase II Activity Assay**

Native heparinase II from *F. heparinum* was purified as described previously (12). The UV 232 nm assay to quantify native heparinase II enzymatic activity was similar to that reported for heparinase I (16). Briefly, the course of the reaction is monitored by measuring the increase in absorbance at 232 nm as a function of time under saturating substrate concentrations. With heparin as the substrate, the reaction was carried out at a concentration of 4 mg/ml in 50 mM phosphate buffer, pH 7.3. With 2 mg/ml heparan sulfate, the reaction was measured in 50 mM phosphate buffer, pH 6.9 (11). The temperature for all enzymatic activity measurements was kept constant at 35 °C.

**Chemical Modification of Heparinase II with DEPC**

**Decomposition of DEPC in Sodium Phosphate Buffer**—At pH values ranging from 5.0 to 8.0, 9.9 mM DEPC was incubated with different concentrations of sodium phosphate buffer. At fixed time intervals, a 10-μl aliquot was withdrawn to react with 10 μl imidazole in 250 mM sodium phosphate buffer, pH 7.5. The concentration of intact DEPC remaining was measured from the increase in absorbance at 230 nm (ε = 3,000 cm⁻¹ mM⁻¹). A second-order rate constant for the decomposition of DEPC in sodium phosphate buffer was derived for each pH.

**Inactivation of Heparinase II with DEPC**—At pH values ranging from 5.5 to 8.0, heparinase II (100 μg/ml) was incubated with DEPC in 50 mM phosphate buffer at 35 °C. At fixed temperature, different concentrations of DEPC, ranging from 0.2 to 2.0 mM, were used to inactivate the enzyme. The 6.9 mM DEPC stock solution was diluted in ethanol. The control mixtures contained an equivalent amount of ethanol instead of DEPC; the amount of ethanol added was less than 3% of the total volume and was determined not to affect significantly the enzymatic activity of heparinase II. At fixed time intervals, aliquots were withdrawn from the reaction mixtures and enzymatic activity determined by the UV 232 nm activity assay. The time course of inactivation was determined by monitoring the enzymatic activity retained after each time interval.

The kinetics of DEPC inactivation of heparinase II 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 following equation.

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

(Eq. 1)

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 II solution.

**Reactivation of Modified Enzyme with Hydroxylamine**—Heparinase II (100 μg/ml) was incubated with 0.4 mM DEPC at pH 7.0 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 500 mM, and the reaction was incubated at room temperature for 8 h. Every hour aliquots were withdrawn for the activity assay. The control mixture contained no DEPC but contained the same concentration of hydroxylamine to account for nonspecific activity loss.

**Substrate Protection of Heparinase II against DEPC Inactivation**—Heparinase II (50 μg/ml) was preincubated with either 4 mg/ml heparin or 2 mg/ml heparan sulfate for 30 min prior to the addition of 0.8 mM DEPC. The time course of inactivation was determined with the heparinase II activity assay using both heparin and heparan sulfate as substrates.

**Quantification of the Number of Histidines Modified by DEPC**—Quantification of DEPC-modified residues of heparinase II was determined by difference spectra. At time 0, 2 mM DEPC was added to the sample cuvette containing heparinase II (825 μg/ml) 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 ε 3,200 M⁻¹ cm⁻¹ (17). Heparinase II activity assays were completed under identical conditions with heparin as the substrate.

**DEPC Labeling and Lys-C Digest of Heparinase II**

To determine which histidine residues were modified by DEPC, mapping studies using the protease Lys-C were completed. In one study, heparinase II (1 nmol) was incubated with 2 mM DEPC for 20 min. Unreacted DEPC was separated from the modified heparinase II by reverse phase HPLC (RPHPLC), the protein was concentrated by lyophilization and digested with Lys-C under denaturing, reducing conditions.

To differentiate between histidines responsible for the breakdown of heparin versus those that are responsible for the breakdown of heparan sulfate, the modification and mapping studies were completed again, but heparinase II was preincubated with 4 mg/ml of heparin or heparan sulfate. At 30 min, DEPC was added, the reaction was allowed to continue for 20 min, and heparinase II was digested with Lys-C under denaturing, reducing conditions.

Peptides derived from heparinase II digested by Lys-C were separated by RPHPLC and monitored at 210, 240, and 277 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 parathyroid hormone-derivative analyzer (Biopolymers Laboratory, Massachusetts Institute of Technology).

**Mutagenesis and Cloning of Recombinant Mutant Heparinases II**

The 13 histidines were individually mutated to alanine by overlap extension polymerase chain reaction (18) with 15 cycles. Polymerase chain reaction products were concentrated by a spin column (Invitrogen, Carlsbad, CA) and subcloned into pCR 2.1. The authenticity of all mutations was verified by sequencing (data not shown). Heparinase II and the 13 mutants were cleaved from pCR 2.1 via restriction digest with *Nde*I/SacI and cloned into pSE02 for expression. pSE02 is a construct derived from pET28a that contains a His-Tag, for purification purposes, and an Omp8 leader sequence for periplasmic export.²

**Expression, Isolation, and Purification of r-heparinase II and Mutants in E. coli**

The recombinant and mutant heparinases II were expressed with the putative *F. heparinum* leader sequence. Overnight cultures of BL21 were induced with isopropyl-β-D-thiogalactopyranoside in mid-log phase (OD 0.7–0.9) and allowed to grow for another four hours at room temperature, at which time the cells were harvested as described previously (19).

The cell pellet was re-suspended in 1/10 of the original volume of 50 mM phosphate, 50 mM NaCl, pH 7.0. The re-suspended culture was placed on ice and sonicated as described previously. The soluble portion of the extract was isolated by centrifugation at 5,000 ×g for 20 min at 4 °C. Hydroxyapatite chromatography was used to purify the heparinase II present in the supernatant after centrifugation. Briefly, before addition of the soluble cell extract, the column was equilibrated with 50 mM phosphate, 50 mM NaCl, pH 7.0, by washing with 5 column volumes. The -2 ml of supernatant was applied, followed by washes (× 2) of 50 mM phosphate, 100 mM NaCl, and 50 mM phosphate, 200 mM NaCl. r-Heparinase II and the mutants were eluted by addition of 3 ml of 50 mM phosphate, 500 mM NaCl. SDS-polyacrylamide gel electrophoresis was carried out using precast 7.5% gels and a Mini-PROTEAN II apparatus and stained with the Silver Stain Plus kit (Bio-Rad) to verify protein purity. Exhaustive digests of both 4 mg/ml heparin and 2 mg/ml heparan sulfate were completed and the resulting products analyzed with a Perfusion Chromatography system in a fashion similar to the analysis completed for heparinase I (20). Quantification of protein concentrations was determined using the Micro BCA reagent (Pierce) relative to

² Z. Shriver, Y. Hu, and R. Sassekhawan, manuscript in preparation.

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Heparin or heparan sulfate was degraded by heparinase II or one of the recombinant heparinasins for 18 h at 30 °C. The reaction was stopped by boiling, and the samples were injected onto a POROS QM (4.6 × 100 mm) anion-exchange column connected to a BIOCAD system (Perseptive Biosystems, Framigham, MA) (20). A salt gradient of 0–2 M NaCl in 10 mM Tris, pH 7.0, was run, and products were monitored at 232 nm.

RESULTS

For the specific modification of histidines, DEPC is the most suitable and widely used reagent. To determine whether histidine or histidine(s) are important for heparinase II activity, extensive characterization of the modification of heparinase II by DEPC was completed. In addition, by varying the reaction conditions (e.g., the pH), we were able to derive information pertaining to the chemical nature of the DEPC-reactive histidines in heparinase II.

DEPC Inactivates Heparinase II in a Dose-dependent Fashion—DEPC readily inactivates heparinase II in a concentration-dependent manner. One complicating factor is that DEPC is unstable in aqueous solution. Therefore, the decomposition of inhibitor was investigated at pHs from 5–8 (Fig. 1A). At each pH measured, the decomposition of DEPC followed first-order kinetics. To allow for the hydrolysis of DEPC, the inactivation data were plotted as the natural log of percentage activity versus an adjusted time factor (t′). For each concentration of DEPC used, this adjusted plot generated a straight line (Fig. 1B), indicating that the reaction was pseudo first-order. The rate of inactivation of heparinase II was determined when both heparin and heparan sulfate were used as substrate.

Second-order rate constants were determined by re-plotting the pseudo first-order rate constants as a function of DEPC concentration. As is shown in Fig. 2, such a plot yields a straight line from which the second-order rate constant can be derived. With heparin as the substrate, the rate constant of inactivation was found to be 0.16 min⁻¹ mM⁻¹; with heparan sulfate as the substrate, the rate constant was determined to be 0.24 min⁻¹ mM⁻¹.

Reactivation of DEPC-modified Enzyme with Hydroxylamine—DEPC is usually considered to be a histidine-specific reagent. However, besides the imidazole ring of histidine, DEPC can also react with the nucleophilic side chains of other amino acids, such as tyrosine, lysine, and cysteine. To ensure that DEPC was histidine-specific under the conditions of this experiment, the ability of hydroxylamine to reverse the inactivation of heparinase II was studied (Fig. 3). At pH 7.0, heparinase II was incubated with 0.4 mM DEPC until the enzymatic activity toward heparin as compared with heparan sulfate. To investigate the chemical characteristics of the different histidines, the inactivation of heparinase II was determined as a function of pH. For the pH range of 5.5–8.0, a graph of the second-order rate constants of inactivation versus pH yielded a hyperbolic curve (Fig. 4). A similar curve was generated with heparan sulfate as the substrate. To confirm that histidines, and not lysines or cysteines, were being modified at pH 8, the reversibility of the reaction was determined using hydroxylamine. At pH 8, but not above, over 70% of activity could be restored with hydroxylamine. Therefore, up to pH 8, the interaction of DEPC with heparinase II involves only histidine modification.

Determining Number of Histidine Residues Modified by DEPC—To quantify the number of histidines that reacted with DEPC, the absorbance at 240 nm was followed as a function of time. The DEPC-histidine adduct, in a stoichiometry of 1:1, absorbs strongly in the near UV region (λmax = 240 nm, ε = 3, 200 M⁻¹ cm⁻¹). Fig. 5 shows that after 10 min, roughly three
histidines are modified by DEPC. Interestingly, the absorbance increase is nearly linear, indicating that the three histidines that react with DEPC do so at nearly the same rate. This result is consistent with the DEPC inactivation data. Under the same conditions, an enzyme activity assay was completed to determine the effect on activity of the modification of the three histidine residues. After 10 min, there was loss of 90% of heparinase II enzymatic activity toward heparin and heparan sulfate substrates. Thus, there are three histidines that are more reactive toward DEPC than the rest of the histidines in heparinase II.

Location of DEPC-modified Residues—The chemical modification data points to three histidines being DEPC-reactive and essential for heparinase II activity. One possible role for either one, two, or all three of these histidines is that they are present in the active site of heparinase II. To attempt to understand whether any or all of the histidines are located at or near the active site of heparinase II, the enzyme was preincubated with either heparin or heparan sulfate before being subjected to chemical modification. Since the enzyme’s active site is presumably located in proximity to the binding site for heparin and/or heparan sulfate, preincubation with one or both of the substrates should serve to shield an active-site histidine from modification (see “Discussion”). Fig. 6A shows the results of the effect of heparin preincubation on heparinase II inactivation when heparin and heparan sulfate are substrates; Fig. 6B shows the results of the effect of heparan sulfate preincubation on heparinase II inactivation.

In the case of heparinase II preincubation with heparin, enzymatic activity toward heparan sulfate was lost but not enzymatic activity toward heparin upon addition of DEPC. Conversely, heparan sulfate was unable to protect heparinase II from DEPC inactivation regardless of the substrate used. These results indicate that at least one and presumably more histidines are proximate to the active site of heparinase II. Of these, one and probably more than one of the histidines are involved in the breakdown of heparin; a separate histidine or histidines are involved in the breakdown of heparan sulfate (see below).

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Identification of Active-site Residues—To identify the histidines that are susceptible to DEPC modification, DEPC-modified heparinase II was digested with Lys-C. Since a DEPC-modified residue should be more hydrophobic, thus eluting later on a RPHPLC C4 column, the proteolytic digest of DEPC-modified heparinase II was compared with a control digest (Fig. 7). Three peptides: ld4, ld5, and ld6 were found to migrate differently in the digest of DEPC-modified heparinase II as compared with the control digest. All three of these peptides also had significant absorbance at 240 nm, as compared with

FIG. 2. Second-order rate constant for the inactivation of heparinase II by DEPC with heparin (●) or heparan sulfate (○) as substrate. Graphs were generated by plotting the pseudo-first order rate constants as a function of DEPC concentration. The second-order rate constant was determined to be 0.24 min⁻¹mM⁻¹ for inactivation with heparan sulfate as the substrate and 0.16 min⁻¹mM⁻¹ for heparinase II inactivation with heparin as the substrate.

FIG. 3. Reversibility of DEPC modification of heparinase II. Heparinase II was incubated with 0.4 mM DEPC until 50% enzymatic activity remained. Hydroxylamine was added to a concentration of 500 mM at this point. Hydroxylamine was also added to a heparinase II mixture that was not exposed to DEPC. At various points, the enzymatic activity of both the control and the DEPC-treated heparinase II was measured. Heparin was used as substrate; similar results were obtained with heparan sulfate as substrate.

FIG. 4. pH dependence of DEPC inactivation of heparinase II with heparin (●) or heparan sulfate (○) as substrate. Second-order rate constants were determined in the pH range 5.5–8.

FIG. 5. Quantification of DEPC-modified residues of heparinase II. At time 0, 2 mM DEPC was added to the sample cuvette containing heparinase II (825 μg/ml) 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 ε = 3,200 M⁻¹cm⁻¹ (17). Heparinase II activity assays were completed under identical conditions with heparin as the substrate. After 10 min, roughly 80% of enzymatic activity toward heparin was lost.
non-adduct peptide peaks, indicative of a DEPC-histidine adduct. ld4, migrating at 45 min, contained the sequence KRTIAH451NSLLIYDPK, with a modified residue in the sixth cycle (histidine 451). ld5, migrating at 85 min, contained the sequence KEH238LVAR, with a modified residue in the second cycle (histidine 238). Finally, ld6, migrating at 87 min, contained the sequence KFWLLH579SIEQPEIK, with a modified residue in the fifth cycle (histidine 579).

To identify the histidine that was protected upon heparin preincubation of heparinase II, the mapping studies were completed in an identical fashion, except the enzyme was first preincubated with 4 mg/ml heparin for a period of 30 min. The Lys-C digest profile was identical to a profile of DEPC-modified heparinase II described above, except for the fact that ld4, containing histidine 451, was absent. This result identifies histidine 451 as the essential histidine for the breakdown of heparin (Fig. 7).

The chemical modification studies taken together with mapping studies point to histidines 238, 451, and 579 as being essential for heparinase II activity. These results also point to histidine 451 being an active site residue, responsible for the breakdown of heparin. Site-directed Mutagenesis of the Histidines in Heparinase II—Heparinase II contains 13 histidines in the mature protein. In an effort to corroborate the biochemical studies and determine which histidine residues were critical for heparinase II, each of the histidines was individually mutated into an alanine residue. The recombinant mutant heparinases II proteins were expressed, purified, and enzymatic activity of each mutant toward both heparin and heparan sulfate was assayed. The results are presented in Table I. Consistent with the biochemical experiments, histidines 238, 451, and 579, when changed to alanines, were inactive toward both heparin and heparan sulfate, such that no enzymatic products could be detected by HPLC analysis of the saccharide products of heparinase II even after an 18-h digest within the limits of this experimental procedure. While seven of the histidine mutants (H48A, H249A, H252A, H437A, H440A, H473A, and H682A) displayed detectable enzymatic activity, two other histidine mutants (H406A and H408A), in addition to the H238A, H451A, and H579A mutants, showed complete loss of enzymatic activity (see “Discussion”).

**DISCUSSION**

We have shown in this study through a combination of chemical modification and site-directed mutagenesis experiments that histidine residues play essential roles in heparinase II. Mature native heparinase II contains 13 histidine residues, and some of these residues may be essential in one of three ways: the histidine can be an important structural element, it can be involved in substrate binding, or it can be a catalytic residue.

Chemical Modification Studies—The chemical modification data points to three histidines that are solvent accessible, chemically more reactive toward modifying reagents, and essential for heparinase II activity. Furthermore, since the rate constant of inactivation with DEPC is different depending on whether heparin or heparan sulfate is the substrate, it argues that different histidines must be involved in the enzymatic breakdown of heparin versus heparan sulfate. In both cases, a plot of log k versus log [DEPC] yielded a straight line with a slope of one indicating either that one histidine is modified or more than one histidine, all with the same apparent rate constant, are modified and are essential for the degradation of heparin versus heparan sulfate. Protection experiments further supports this proposition. With heparin as substrate, only heparin, but not heparan sulfate, is able to protect heparinase II from inactivation. With heparan sulfate as substrate, neither heparin or heparan sulfate was able to protect heparinase II from DEPC inactivation. These results indicate that at least one histidine is proximate to the active site(s) since addition of substrate shields the histidine from modifying reagents, such as DEPC.

One possible interpretation of the above data is that the chemical modification of the reactive surface accessible histidines may alter the conformation of heparinase II or impede substrate access to the active site and thereby affect heparinase II activity. Also, the substrate protection could somehow affect the chemical modification reaction and hence reduce the labeling kinetics. It is also possible that the reactive histidines are not in the active site, but rather they might be necessary for stability. If this were the case, then the protection experiments would be interpreted as showing that heparin binding stabilizes the correct tertiary structure of heparinase II, protecting the critical histidines from modification. However, when heparin or heparan sulfate is used for protection experiments with either substrates, only heparin is able to protect heparinase II when heparin is used as a substrate and not heparan sulfate. This observation strongly points to the fact that the results of the protection experiments are not due to the artifacts caused or induced by heparin or heparan sulfate. In fact, when heparin...
Proteolytic Mapping Studies—Proteolytic digests of heparinase II to map the histidine residues are consistent with the chemical modification data. Mapping studies identified the three histidines that are modified by DEPC. Three peptides were found to shift retention times upon addition of DEPC to heparinase II. All three peptides, ld4, ld5, and ld6, contained histidine residues that were modified. ld4 contained a peptide that contained His451, ld5 contained a peptide that contained His238, and ld6 contained a peptide that contained His579.

Further evidence supports the supposition that His451 is present in the active site of heparinase II. The sequence surrounding His451, K446RTIAH451, shares high sequence homology to the sequence in heparinase I, K198KNIAH203, containing histidine 203, which has been shown to be critical for enzymatic
activity of heparinase I (14).

There is evidence suggesting that histidine 238 may also be present in the active site. With heparinase I, labeling with cold p-(chloromercuri)benzoate (pCMB) to modify the active site cysteine 135, followed by trypsin digest and RP-HPLC, caused a significant increase in migration time. This result strongly suggested that histidine 203, the putative active site histidine for heparinase I, was modified. With heparinase II, labeling with pCMB yielded a peptide that was the same as ld5, containing histidine 238 (data not shown). In either case, the shift in migration time could be indicative of one of two possibilities: either the histidine-containing peptide was “protected” from modification by the Lys-C digest by the cysteine/pCMB adduct, yielding an incomplete proteolysis product not present in the control digest, or the nucleophilic histidine reacted with the highly electrophilic pCMB to form an uncharacterized, irreversible adduct under the conditions utilized in these studies. Regardless, histidine 238 displays similar chemical reactivity as histidine 203 of heparinase I toward pCMB, suggesting that it too might be an active site residue. Future structural and mutagenesis studies will be necessary to confirm this proposition.

A specific role for histidine 579 in the catalytic breakdown of either heparin or heparan sulfate cannot be posited at this time. The above experiments interpreted together strongly suggest that histidines 238, 451, and 579 are essential for heparinase II activity.

Site-directed Mutagenesis—Site-directed mutagenesis experiments further corroborates the chemical modification and peptide mapping experiments in the identification of histidines 238, 451, and 579 as being essential for heparinase II activity. Site-directed mutagenesis experiments indicate that histidines 238, 451, and 579 are essential for the enzymatic activity of heparinase II, as these three histidine to alanine mutations rendered the mutant enzymes enzymatically inactive toward both heparin and heparan sulfate. These results were further supported with our finding that the histidine mutants H406A and H408A also showed loss of enzymatic activity. As the chemical modification and the peptide mapping experiments together did not indicate the involvement of histidine 406 and histidine 408 in heparinase II activity, it is possible that these residues can be involved in either proper folding of heparinase II and/or binding of the heparin/heparan sulfate substrates.

In Table I, the H252A, H347A, and H440A mutants were all scored as enzymatically active toward both heparin and heparan sulfate. These residues are all unmodified based on the chemical modification, and they are all contained in peptides that, if modified, would be detected in the Lys-C mapping studies, indicating that they are not reactive toward DEPC under the conditions of this study.

However, it is interesting to note that, unlike all the other enzymatically active histidine mutants, the H252A, H347A, and the H440A mutants displayed differential activity toward heparin or heparan sulfate. For instance, the H440A mutant displayed nearly the same enzymatic activity as recombinant heparinase II when heparan sulfate was used as the substrate. While enzymatically active toward heparin, its activity was less than recombinant heparinase II. These results suggest a plausible role of histidines 252, 347, and 440 in differential substrate binding viz., heparin versus heparan sulfate.

On the other hand, as histidine 347 appears not to undergo chemical modification, it is possible that histidine 347 is not sufficiently surface accessible for chemical modification, however, it may still play a role in catalysis. One potential explanation for the marked decrease in enzymatic activity for the H347A mutant is that histidine 347 is proximate to cysteine 348, which is potentially an active site residue. In the case of Yersinia protein tyrosine phosphatase, an enzyme that, like the heparinases, binds a highly anionic substrate, a cysteine was found to be important for activity (22). In addition, the neighboring histidine was found to also be essential for activity. In the case of Yersinia PTPase, the histidine served to lower the pK\textsubscript{a} of the active cysteine; mutagenesis of this histidine to alanine resulted in an increase of the cysteine pK\textsubscript{a} by 1.5 units (13). In a similar fashion, histidine 347 might be required to activate cysteine 348 by lowering its pK\textsubscript{a} through either ion interactions or hydrogen bonding.

In conclusion, we have shown through a combination of chemical modification, proteolytic mapping studies, and site-directed mutagenesis that histidines are essential amino acids in heparinase II. In specific, the biochemical and the site-directed mutagenesis experiments together point to three histidines (His\textsuperscript{238}, His\textsuperscript{451}, and His\textsuperscript{579}) presumably playing a key role in catalysis. Furthermore, it is possible that His\textsuperscript{451} plays an especially critical role in the breakdown of heparin as addition of heparin protects this residue from DEPC modification. It is possible that the other histidine residues are important structurally or may be critical for substrate binding. Future studies are necessary to delineate specific roles of the histidines in heparinase II for catalysis.

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