Heparinase II from Flavobacterium heparinum

ROLE OF CYSTEINE IN ENZYMATIC ACTIVITY AS PROBED BY CHEMICAL MODIFICATION AND SITE–DIRECTED MUTAGENESIS*

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Heparinase II (no EC number) is one of three lyases isolated from Flavobacterium heparinum that degrade heparin-like complex polysaccharides. Heparinase II is unique among the heparinas in that it has broad substrate requirements and possesses the ability to degrade both heparin and heparan sulfate-like regions of glycosaminoglycans. This study set out to investigate the role of cysteines in heparinase II activity. Through a series of chemical modification experiments, it was found that one of the three cysteines in heparinase II is surface-accessible and possesses unusual chemical reactivity toward cysteine-specific chemical modifying reagents. Substrate protection experiments suggest that this surface-accessible cysteine is proximate to the active site, since addition of substrate shields the cysteine from modifying reagents. The cysteine, present in an ionic environment, was mapped by radiolabeling with N-[3H]ethylmaleimide and identified as cysteine 348. Site-directed mutagenesis of cysteine 348 to an alanine resulted in loss of activity toward heparin but not heparan sulfate, indicating that cysteine 348 is required for heparinase II activity toward heparin but is not essential for the breakdown of heparan sulfate. Furthermore, we show in this study that cysteine 164 and cysteine 189 are functionally unimportant for heparinase II.

Heparin-like glycosaminoglycans (HLGAGs) are key components of the extracellular matrix that serve to regulate an array of biological functions (1, 2). HLGAGs are linear, sulfated, acetylated, polysaccharides consisting of 1–4-linked derivatives of hexosamine and uronic acid (3). One of the major challenges in elucidating a specific role for HLGAGs in certain biological systems is that the considerable chemical heterogeneity of HLGAGs has thwarted attempts to determine sequence-function relationships (4, 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 (3, 6).

To elucidate the mechanism by which sequence or sequences within HLGAGs bind to and regulate components of the ECM, it is critical to develop biochemical methods of structure-function analysis (4). HLGAG-degrading enzymes, or heparinas, a family of polysaccharide lyases that catalyze the eliminative cleavage of HLGAGs, have shown promise as potential tools to determine specific HLGAG sequences involved in HLGAG-protein interactions (7). Toward development of the heparinas, we have cloned and recombinantly expressed heparinas I, II, and III from Flavobacterium heparinum (8–11). In addition, we have carried out extensive biochemical characterization of heparinase I to determine the molecular basis of its substrate specificity (11–13). Such an understanding will facilitate both a general appreciation of the chemistry of polysaccharide lyases and also broaden their use in the study of HLGAGs, particularly in diverse biological processes, for example, in development or in neovascularization (14).

The three heparinas, from F. heparinum, are distinguished on the basis of their size, charge properties, and substrate specificities (4). Heparinase I, a 42-kDa protein with a pI of 8.5–9.3, primarily cleaves HLGAGs at sites with an O-sulfated L-iduronic acid linkage (i.e. heparin-like regions). Heparinase III, a 73-kDa protein with a pI of about 10, requires primarily an unsulfated D-glucuronic acid moiety (heparan sulfate-like regions). Whereas there is evidence for a secondary substrate specificity for heparinas I and III (15, 16), these enzymes do show a predominant enzymatic preference for a C5 epimer of uronic acid, with heparinase III primarily acting at hexosamine-glucuronic acid linkages and heparinase I acting primarily at hexosamine-iduronic acid linkages. Heparinase II is the largest of the heparinas and has the broadest substrate specificity. The 84-kDa protein has a pI of around 9 and cleaves both heparin and heparan sulfate-like regions of HLGAGs (4, 17). 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 (4).

Through extensive biochemical and site-directed mutagenesis experiments, our studies with heparinase I have led to the identification of three residues: cysteine 135, histidine 203, and lysine 199, that are critical for enzymatic function (10, 11, 12). A mechanism was proposed wherein cysteine 135 was the active site base that abstracted the C5 hydrogen from iduronic acid, which, when coupled to the cleavage of the glycosidic bond, lead to the formation of the 4,5-unsaturated uronate product (12). Thus, a stereospecific role for cysteine 135 was...
posed that allowed heparinase II to distinguish between heparin and heparan sulfate-like regions. This proposed mechanism further motivates the studies for heparinase II in that this enzyme acts in a non-stereospecific fashion, abstracting a proton from either heparin or heparan sulfate.

As one step toward elucidating a catalytic mechanism of heparinase II, we recently identified several histidinyl residues that were critical for the catalytic activity of the enzyme (18). Taken together with our earlier finding that cysteine 135 is catalytically critical in heparinase I, this study sought to address the question of whether a cysteine(s) is required for catalysis in heparinase II. In this study, we show that cysteine 348 is essential for the enzymatic activity of heparinase II, whereas cysteine 164 and cysteine 189 are functionally unimportant.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Materials**

Urea, Tris, and trifluoroacetic acid were from J. T. Baker Inc. DTT was obtained from Sigma. Sodium phosphate monobasic and dibasic and acetonitrile were from Mallinkrodt (Chesterfield, MO). Hydroxyapatite and bovine serum albumin were purchased from Bio-Rad. The chemical modification reagents: IAA, IAM, 4-VP, NEM, pCMB, and DTNB were all purchased from Aldrich. 4-VP, NEM, and pCMB were used as received. IAA, IAM, and DTNB were recrystallized prior to use. All reagents were stored under nitrogen. Lys-C from *Escherichia coli* (DE3) host was from Novagen (Madison, WI).

**Heparinase II Activity Assay**

Native heparinase II from *F. heparinum*, was purified as described previously (19). The UV 232 nm assay to quantify native heparinase II enzymatic activity was similar to that reported for heparinase I (20). 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 sodium phosphate buffer, pH 7.3. With 2 mg/ml heparin sulfate, the reaction was measured in 50 mM sodium phosphate buffer, pH 6.9 (14). The temperature for all enzymatic activity measurements was kept constant at 35 °C.

**HPLC Analysis of Saccharide Products of Heparinase II Activity**

Heparin or heparan sulfate was degraded by heparinase II or one of the recombinant heparinases for 18 h at 30 °C. The reaction was stopped by boiling, and the samples were injected onto a POROS Q/M anion-exchange column connected to a BIOCAD system (PerSeptive Biosystems, Framingham, MA) (19). A salt gradient of 0–2 M NaCl in 10 mM Tris, pH 7.0, was run, and products were monitored at 232 nm.

**pCMB Modification Studies**

**Inactivation with pCMB—Heparinase II (50 μg/ml) was incubated with 2.5–10 μM pCMB in 50 mM sodium phosphate buffer, pH 7.0, at 4 °C. pCMB was prepared according to published procedures (21). A control reaction mixture containing vehicle alone was run in tandem. At fixed time intervals, aliquots were withdrawn to determine the fractional activity retained.**

**Reactivation of pCMB-modified Heparinase II with DTT—Heparinase II (50 μg/ml) was incubated with 5 μM pCMB for 4 min, and an aliquot was withdrawn to determine the fractional activity retained. DTT (10 mM) was then immediately added to the reaction mixture and to the control, which contained no pCMB. The mixtures were incubated at 4 °C. Heparinase II activity was measured every half hour.**

**Effect of Salt on the Inactivation of Heparinase II by pCMB—Heparinase II was incubated with 5 μM pCMB in 50 mM sodium phosphate buffer, pH 7.0, with different salt concentrations (30, 60, 110, 180, 300, and 500 mM NaCl). A control mixture, which contained no additional salt, was included.**

**Substrate Protection of Heparinase II against pCMB Modification—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 5 μM pCMB, and then the time course of inactivation was determined with the heparinase II activity assay.**

**Quantification of pCMB-modified Residues of Heparinase II—**

Quantification of pCMB-modified residues of heparinase II was determined by difference spectra. At time 0, 19 μM pCMB was added to the sample cuvette containing heparinase II (825 μg/ml) in sodium phosphate buffer, pH 7.0. The change in absorbance at 250 nm was monitored every 30 s for 10 min. The number of modified residues was determined using ε = 7,600 M⁻¹ cm⁻¹ (22). Heparinase II activity assays were completed under identical conditions with heparin as the substrate.

**DTNB Modification Studies**

**Inactivation with DTNB—Heparinase II (100 μg/ml) was incubated with DTNB (0.1–0.5 mM). A DTNB stock solution was made by dissolving DTNB in ethanol, and an aliquot was added to the reaction mixture (23). The control reaction mixture contained an equivalent amount of ethanol instead of DTNB; the amount of ethanol added was 3% of the total volume and did not have a measurable effect on enzymatic activity.**

**Quantification of DTNB-modified Residues of Heparinase II—**

Quantification of DTNB-modified heparinase II residues was determined by difference spectra. At time 0, 2 mM DTNB was added to the sample cuvette containing heparinase II (825 μg/ml) in sodium phosphate buffer, pH 7.0. The change in absorbance at 410 nm was monitored every 30 s for 10 min. To account for the decomposition of DTNB in the reaction mixture, a control was also done by monitoring the change in absorbance at 410 nm with only DTNB (2 mM) in 50 mM sodium phosphate buffer, pH 7.0. Heparinase II activity assays were also performed on the reaction mixture. The number of heparinase II cysteine residues modified was determined using ε = 13,600 M⁻¹ cm⁻¹ (23). A similar experiment was completed after preincubating the enzyme with heparin for 30 min.

**[3H]NEM Labeling and Lys-C Digest of Heparinase II**

To determine which cysteine residues were modified by NEM and pCMB, mapping studies using the protease Lys-C were completed. In one study, heparinase II (1 nmol) was incubated with [3H]NEM for 30 min. Unreacted [3H]NEM was separated from the modified heparinase II by reverse phase HPLC (RP-HPLC), the protein was concentrated by lyophilization and digested with Lys-C under denaturing, nonreducing conditions.

In another study, heparinase II (3 nmol) was reacted with a stoichiometric amount of pCMB for 15 min. Less than 2% of total heparinase II enzymatic activity remained after this time interval. The protein was then denatured under strictly nonreducing conditions and reacted with an excess of IAA (4 mM). Modified heparinase II was separated and concentrated. pCMB was removed via addition of DTT. [3H]NEM was then added to the reaction mixture. Unreacted tritium was separated by RP-HPLC, and heparinase II was digested with Lys-C.

Peptides derived from heparinase II digested by Lys-C were separated by RP-HPLC and monitored at 210 and 277 nm. Peptide peaks were collected and counted for tritium incorporation. The tritium-incorporated peptide sequences were sequenced using an Applied Biosystems sequencer model 477 with an on-line model 120 phenylthiohydantoin derivative analyzer (Biopolymers Laboratory, MIT).

**Mutagenesis and Cloning of Mutant Heparinase II**

The C164A, C189A, and C345A mutations were introduced by the overlap extension PCR methodology (24) via 15 cycle PCR. PCR products were concentrated by 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 three mutants
were cleaved from pCR 2.1 via restriction digest with NdeI/SacI and cloned into pSE02 for expression. pSE02 is a construct derived from pET28a, which contains a His tag, for purification purposes, and an OmpT 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 4 h at room temperature, at which time the cells were harvested as described previously (11).

The cell pellet was resuspended in 1/50th of the original volume of 50 mM sodium phosphate, 50 mM NaCl, pH 7.0. The reconstituted culture was placed on ice and sonicated as described previously. The soluble portion of the cell lysate was isolated by centrifugation at 14,000 rpm for 20 min at 4 °C and then purified by hydroxyapatite chromatography. Briefly, before addition of the supernatant, the hydroxyapatite column was equilibrated with 50 mM sodium 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 sodium phosphate, 100 mM NaCl, and 50 mM sodium phosphate, 200 mM NaCl. r-heparinase II and the mutants were eluted by addition of 3 ml of 50 mM NaCl, 500 mM NaCl. SDS-polyacrylamide gel electrophoresis was carried out using precast 10% gels and a Mini Protein 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 (19). Quantification of protein concentrations were determined using the Micro BCA reagent (Pierce) relative to a bovine serum albumin standard.

RESULTS

Cysteine-specific Modification Inactivates Heparinase II—To address several questions pertaining to a possible role for a cysteine in heparinase II activity, we investigated the reactivity of a panel of cysteine-specific reagents towards heparinase II. We not only sought to establish the importance, if any, of cysteine residues in heparinase II activity, but also sought to probe the chemical nature of the reactive cysteine(s). Two reagents in particular, pCMB and DTNB, were used extensively to chemically characterize the cysteines of heparinase II. Whereas considered cysteine-specific, both these reagents differ markedly in their chemical characteristics, thus together, they can provide a more complete picture of the environment and reactivity of heparinase II cysteines than could an investigation using either reagent alone.

pCMB showed the greatest reactivity toward heparinase II, completely inactivating heparinase II at 10 μM within 15 min. The inactivation rate was concentration-dependent (Fig. 1), yielding a second-order rate constant of 0.040 μM⁻¹ min⁻¹. The sulphydryl-specific nature of the interaction was confirmed by the fact that the inactivation was readily reversed by the addition of 10 mM DTT.

To provide a foundation upon which to interpret other modification experiments, the inactivation kinetics of DTNB modification to heparinase II was also thoroughly investigated (Fig. 2). DTNB inhibited heparinase II following pseudo first-order kinetics, with a second-order rate constant of 0.609 mM⁻¹ min⁻¹. A plot of the log k versus log [DTNB] (data not shown) confirmed that the reaction was first order in DTNB and suggested that one cysteine, modified by DTNB, was required for heparinase II activity. Identical rate constants of inactivation were obtained whether heparin or heparan sulfate was used as the substrate to monitor heparinase II activity. Like pCMB and DTNB, IAM, 4-VP, and NEM also inhibited heparinase II activity in a dose-dependent fashion (data not shown). IAA was the least effective cysteine-specific reagent at inactivating heparinase II.

Having characterized the specific, stoichiometric interaction of pCMB and DTNB with the cysteines of heparinase II, we sought to extend the chemical modification studies with pCMB and DTNB to quantify the number of modified cysteines and loss of activity. Upon incubation with 20 μM pCMB, loss of 98% activity was correlated with modification of 7,600 M⁻¹ min⁻¹.

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 4 h at room temperature, at which time the cells were harvested as described previously (11).

The cell pellet was resuspended in 1/50th of the original volume of 50 mM sodium phosphate, 50 mM NaCl, pH 7.0. The reconstituted culture was placed on ice and sonicated as described previously. The soluble portion of the cell lysate was isolated by centrifugation at 14,000 rpm for 20 min at 4 °C and then purified by hydroxyapatite chromatography. Briefly, before addition of the supernatant, the hydroxyapatite column was equilibrated with 50 mM sodium 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 sodium phosphate, 100 mM NaCl, and 50 mM sodium phosphate, 200 mM NaCl. r-heparinase II and the mutants were eluted by addition of 3 ml of 50 mM NaCl, 500 mM NaCl. SDS-polyacrylamide gel electrophoresis was carried out using precast 10% gels and a Mini Protein 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 (19).

Quantification of protein concentrations were determined using the Micro BCA reagent (Pierce) relative to a bovine serum albumin standard.

FIG. 1. Effect of the concentration of pCMB on the inactivation of heparinase II. Heparinase II (50 μg/ml) was incubated with various concentrations of pCMB, and the time course of inactivation was followed (n = 3). A plot of the ln (percent activity) versus time yielded a straight line, the slope of which is the pseudo first-order rate constant. The pseudo first-order rate constants are plotted as a function of pCMB concentration. A second-order rate constant of 0.040 min⁻¹ μM⁻¹ was calculated from the plot.

FIG. 2. DTNB inactivation of heparinase II. The inactivation rate was followed exactly as for pCMB and the pseudo first-order rate constants determined (n = 3). A plot of these constants against the concentration of DTNB used yielded a straight line with a second-order rate constant of 0.609 min⁻¹ mM⁻¹.
Chemical Environment in the Active Site Affects Thiol Reactivity—One possible mechanism by which a surface-exposed reactive cysteine exists in heparinase II at physiological pH is its high reactivity due to the presence of nearby basic clusters. Since the enzyme's active site is predominantly located proximally to the binding site for heparin and/or heparan sulfate, preincubation with one or both of the substrates should serve to shield such a cysteine from modification (see "Discussion"). With pCMB as the modification reagent, both heparin and heparan sulfate were able to protect the enzyme from inactivation, albeit heparan sulfate was more effective (Table I).

To confirm our analysis of the pCMB results and to extend them to determine the effect of heparin preincubation on the number of cysteines modified, we used DTNB to determine the number of cysteines modified with and without heparin preincubation. One attribute of DTNB is the fact that the 2-nitro-5-mercaptopbenzoate anion released upon reaction with a cysteiny residue is readily monitored (ε = 13,600 M\(^{-1}\) cm\(^{-1}\) at 410 nm) (23) without interference from product formation (λ\(_{\text{max}}\) = 232 nm). Thus, it was uniquely suited for these experiments (see "Discussion"). The above experiments with pCMB and DTNB taken together suggest that one cysteine is surface-exposed and can be protected from modification by heparin or heparan sulfate.

To examine the reactivity of the surface-exposed "unique" cysteine in heparinase II that is modified by chemical reagents, the pH profile of pCMB inactivation was investigated at pH values of 5–8. It is known that the mercaptide anion is more susceptible to chemical modification, especially by electrophilic compounds such as IAA and pCMB (25). Table II shows the pH profile of pCMB inactivation as a function of pH. There is very little pH dependence of the first-order rate constant of inactivation, less than a 2-fold difference, indicating that the surface-exposed cysteine is present in one ionic state from pH 5 to 8. Based on similar results with heparinase I and taking into account the high susceptibility of heparinase II to pCMB modification, it is probable that the surface-exposed cysteine exists in the active site of heparinase II as the mercaptide anion.

Together, the results of the chemical modification studies support the hypothesis that one cysteine in heparinase II is susceptible to chemical modification due to its high reactivity and that this cysteine is surface-exposed and located at or near the active site of heparinase II.

### Table I

| Substrate                  | None | Heparin | Heparan sulfate |
|----------------------------|------|---------|----------------|
| Heparin                    | 0.22 | 0.16    | 0.10           |
| Heparan sulfate            | 0.21 | 0.16    | 0.09           |

### Table II

| pH  | 5.0  | 6.0  | 7.0  | 8.0  |
|-----|------|------|------|------|
| k (min\(^{-1}\)) | 0.16 | 0.14 | 0.21 | 0.24 |
To determine whether cysteine 348 is the cysteine susceptible to modification by pCMB as well as NEM, the labeling and site-directed mutagenesis experiments that confirmed the role of cysteine 348 as the reactive cysteine required for heparinase II activity, cysteine to alanine mutants were created for each of the three cysteines (C164A, C189A, C348A). In each case protein production was induced by addition of isopropyl-β-D-thiogalactopyranoside, and the protein was purified by hydroxyapatite chromatography. The recombinant proteins were analyzed by silver stain gel and found to be pure. Recombinant heparinase II was found to have a similar degradation product profile as native heparinase II with both heparin and heparan sulfate as substrates.

Each of the cysteine mutants, and r-heparinase II as a control, were expressed in BL21. After purification, each of these four wild-type and mutant enzymes was characterized via exhaustive substrate digests to determine product profiles. Overnight digests with both heparin and heparan sulfate were completed, and the products were analyzed with a Perfusion Chromatography system (Fig. 7, a and b).

Both C164A and C189A had product profiles that closely matched that of both r-heparinase II and native heparinase II. Interestingly, the C348A mutant was completely inactive toward heparin but had a product profile very similar to wild-type heparinase when heparan sulfate was the substrate. Taken together with the mapping studies and the chemical modification studies, cysteine 348 is proposed to be an essential residue in heparinase II involved in the breakdown of heparin but not heparan sulfate.

**FIG. 5. pCMB inactivation of heparinase II with varying salt concentrations.** Heparinase II was incubated with 5 μM pCMB in 50 mM sodium phosphate, pH 7.0, at 4 °C with increasing concentrations of salt: 0, 30, 60, 110, 180, 300, and 500 mM NaCl. After 1, 4, 7, and 10 min of incubation with pCMB, aliquots were withdrawn for activity measurements. Controls that contained only salt were run at the same time to account for loss of heparinase II activity with increasing salt concentration.

**DISCUSSION**

**Physical Chemical State of Cysteines 164, 189, and 348**—We have shown in this study through a combination of chemical modification and site-directed mutagenesis experiments that cysteine 348 is an essential residue for catalysis in heparinase II.

The chemical modification data unambiguously point to the fact that there is one cysteine that is solvent accessible and chemically more reactive toward modifying reagents than the other two cysteines. Protection experiments suggest that this cysteine is proximate to the active site since addition of substrate, either heparin or heparan sulfate, shields the cysteine from modifying reagents, such as pCMB and DTNB. One possible interpretation of the above data is that the chemical modification of the reactive surface accessible cysteine 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 some how affect the chemical modification reaction and hence reduce the labeling kinetics. It is also possible that the reactive cysteine is not in the active site, but rather is 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 cysteine from modification. Whereas the above interpretations cannot be disproven, several points argue for a catalytic role of cysteine 348. First, Cys348 is unusually reactive toward pCMB (and the modification is readily reversed upon addition of a sulfhydryl reagent like DTT) and is ionized at physiological pH. Second, CD profiles of native heparinase II and pCMB-modified hepa-
rinase II are superimposable (data not shown), indicating there are no gross distortions in the secondary structure of heparinase II upon binding of pCMB. Finally, the C348A mutant displays activity toward heparan sulfate.

Of interest is the fact that, upon chemical modification, enzymatic activity toward both heparin and heparan sulfate is inhibited to the same extent, but the C348A mutant is able to catalyze the breakdown of heparan sulfate, but not heparin. One possible interpretation of these results is that there is one substrate binding domain in heparinase II that accommodates both heparin and heparan sulfate. Within this binding domain, specific amino acids are involved in the active site chemistry that affords the enzymatic breakdown of heparin; within this same binding domain separate amino acids are involved in the breakdown of heparan sulfate. Cys348 is one residue that is required for the enzymatic cleavage of heparin but is not required for breakdown of heparan sulfate. This interpretation is also consistent with the observation that preincubation with heparan sulfate affords greater protection from modifying reagents, since heparan sulfate is known to bind to heparinase II with greater affinity than heparin.

The results presented in this study indicate that cysteine 164 and cysteine 189 do not have a functional role in catalysis. The observation has been made that heparinase II is more stable than either heparinase I or III (17). Furthermore, unlike heparinase II, which contains three cysteines, heparinase I contains two cysteines that are not disulfide bonded, and heparinase III contains no cysteine residues (19). This raised the question as to the presence of a disulfide bond in heparinase II that stabilizes heparinase’s II tertiary structure. This study, however, shows that heparinase II contains no disulfide bonds in its native state. Interaction with DTNB results in the modification of 2 cysteine residues. Unlike the highly ionic pCMB, which is present in low concentrations, and partitions almost exclusively to the active site, DTNB reacts with all three cysteines in heparinase II to a varying extent indicating none are involved in a disulfide bond. Aromatic disulfides in general, and DTNB in particular, react exclusively with free sulfhydryl groups. Furthermore, the results from the site-directed mutagenesis experiments are also consistent with the above biochemical observations.

Comparison of Heparinase II with Heparinase I and Heparinase III—In heparinase I, the highly charged environment of the active site is thought to facilitate binding of the polyanionic heparin substrate through charge complementarity (12). Support for this hypothesis includes the fact that charged reagents

![Graph](https://example.com/graph.png)
FIG. 7. Perfusion chromatography profiles of the three cysteine mutants (C164A, C189A, and C348A) and r-heparinase II for heparin (a) and heparan sulfate (b) as substrates. Running buffer was 10 mM Tris, pH 7.0, and the salt gradient was from 0 to 2 M NaCl over the course of 10 min. Samples were run after exhaustive digest (18 h at 30 °C). The C164A (panel B) and C189A (panel C) mutants show a similar
are much more facile inhibitors of heparinase I action as compared with neutral reagents. Via a methodical regiment of site-directed mutagenesis this hypothesis was strongly supported when specific lysine residues, lysine 199 and lysine 132, were found to be critical for enzymatic activity in heparinase I (13).

The region of heparinase II around Cys$^{348}$ appears to be positively charged as well, but it is less charged than the active site region of heparinase I. Evidence for this assertion includes the fact that neutral modification reagents, such as IAM and NEM, inactivate heparinase II much more readily than the charged reagent IAA. Heparinase I was found to be very susceptible to IAA, but not IAM or NEM, an unusual situation in that IAM is generally considered to be more reactive than IAA toward cysteine residues. This unusual reactivity was attributed to the charge environment of heparinase’s I active site facilitating partitioning of the charged reagent into the active site (12). With heparinase II, this partitioning does not occur, since IAA inhibits heparinase II activity less readily than IAM.

Additional evidence that the active site of heparinase II is charged, but less than the heparinase I active site, includes the observation that the decrease in inhibition by pCMB upon increasing salt concentration was much less marked for heparinase II than for heparinase I. For heparinase I, addition of 200 mM salt resulted in complete nullification of pCMB labeling. For heparinase II, addition of even 500 mM salt still resulted in a first-order rate constant of inactivation of 0.06 min$^{-1}$.

Thus, the single substrate binding domain of heparinase II possesses some of the characteristics of the heparinase I binding pocket, i.e. the ability to bind the highly charged heparin polymer through ionic interactions, but is also required to interact with the less ionic substrate heparan sulfate, presumably placing a ceiling on the possible ionic nature of the substrate binding pocket.

Proposing a charged environment in the binding pocket of heparinase II, albeit a less charged one than is present in heparinase I, also serves to explain the relatively low $pK_a$ of Cys$^{348}$. A positive environment surrounding Cys$^{348}$ would serve to stabilize the mercaptide anion, thus lowering its $pK_a$ and allowing it to exist as an anion at neutral pH. By analogy to Cys$^{135}$ of heparinase I, one possible role for Cys$^{348}$ of heparinase II is that it acts as a general base catalyst to abstract the C5 proton of iduronic acid within the heparin polymer. Thus, in this context, Cys$^{348}$ would be one residue within the substrate binding pocket of heparinase II required for the breakdown of heparin but not heparan sulfate. Alternatively, Cys$^{348}$ could be an essential residue for heparin, but not heparan sulfate, binding. However, based on its reactivity as well as by reference to what is known about heparinase I, positing a role for Cys$^{348}$ in the catalytic mechanism of heparinase II seems plausible.

That a cysteine is not required for the enzymatic depolymerization of heparan sulfate is not surprising considering that heparinase III, which cleaves unsulfated heparan sulfate-like regions of HLGAGs, contains no cysteines (4). Thus, the results of our experiment suggest that within the single substrate binding pocket of heparinase II, there are two “active sites” present. One site contains residues positioned for the catalytic cleavage of heparin and the other site contains residues that are positioned for the cleavage of heparan sulfate. One essential amino acid contained in the former site is Cys$^{348}$ that acts as a base to abstract the C5 proton of heparin. The two active sites are proximate to one another, within the substrate binding domain, as is shown by the protection experiments.

**Active Site Chemistry of Heparinase II**—The results presented in this study also suggest a conserved enzymatic strategy among the heparinases for the breakdown of both heparin and heparan sulfate. Like heparinase I, heparinase II requires a cysteine to depolymerize heparin. In addition, like heparinase III, which does not contain any cysteines, heparinase II does not require a cysteine to depolymerize heparan sulfate.

The mapping studies also indicate that Cys$^{348}$ is probably proximate to His$^{451}$, another putative active site residue (18). Tritium labeling of the pCMB-reactive cysteine resulted in a peptide that did not contain a cysteine but did contain His$^{451}$. It is possible that $[^{3}H]$NEM either labels the reactive histidine proximate to Cys$^{348}$ or that labeling of Cys$^{348}$ protecst the histidine—containing peptide from proteolytic cleavage. In either case, this result suggests that Cys$^{348}$ and His$^{451}$ are both present in the active site of heparinase II.

Therefore, through this study, as well as a previous study (18), we have begun to identify specific residues in heparinase II that are essential for the breakdown of heparin and heparan sulfate. Like heparinase I, heparinase II requires a cysteine and histidine residues for the breakdown of heparin-like regions of HLGAGs. It remains to be seen which other residues, besides histidines, are required for the breakdown of heparan sulfate-like regions of HLGAGs by heparinase II.

In summary, in this study we have provided evidence that a surface-accessible cysteine is essential for the enzymatic activity of heparinase II. Furthermore, we have identified the specific cysteine, Cys$^{348}$, and identified some of its unusual chemical characteristics, including a low $pK_a$. Like cysteine 135 of heparinase I, it is very likely that the mercaptide anion of Cys$^{348}$ acts as a base for the abstraction of the C5 proton from the uronic acid. It remains to be seen how heparinase II accommodates both heparin and heparan sulfate as substrates. However, it seems that one common strategy employed by heparin-degrading enzymes is activation of a cysteine for use as a general base catalyst.

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**Heparinase II from F. heparinum**

Disaccharide profile (9) as compared with r-heparinase II (panel A) for both heparin and heparan sulfate, whereas the C348A (panel D) mutant is inactive when heparin is used as a substrate, while it is active when heparan sulfate is used as the substrate.
Heparinase II from F. heparinum

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