Heparinase I from Flavobacterium heparinum

MAPPING AND CHARACTERIZATION OF THE HEPARIN BINDING DOMAIN*

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In this study we have identified the primary heparin binding site of heparinase I (EC 4.2.2.7). Chemical and proteolytic digests of heparinase I were used in direct binding and competition assays, to map the regions of heparinase I that interact specifically with heparin. We find the heparin binding site contains two Cardin-Weintraub heparin binding consensus sequences and a calcium co-ordination consensus motif. We show that heparin binding to heparinase I is independent of calcium (Kd of 60 nM) and that calcium is able to activate heparinase I catalytically. We find that sulfhydryl selective labeling of cysteine 135 of heparinase I protects the lysines of the heparin binding site from proteolytic cleavage, suggesting the close proximity of the heparin binding site to the active site. Site-directed mutagenesis of H203A (contained in the heparin binding site) inactivated heparinase I; however, a H203D mutant retained marginal activity, indicating a role for this residue in catalysis. The above results taken together suggest that histidine 203 (hence the heparin binding site) is immediately adjacent to the scissile bond. We propose that the heparin binding site and active site are in close proximity to each other and that the calcium coordination motif, contained in the heparin binding site, may bridge heparin to heparinase I through calcium in a ternary complex during catalysis.

Glycosaminoglycans, such as heparin, heparan sulfate and chondroitin sulfate, play a key role in the extracellular matrix (Jackson et al., 1991; Lindahl et al., 1994). Heparin is an acidic polysaccharide, characterized by a disaccharide repeating unit of hexosamine and uronic acid (l-iduronic or d-glucuronic acid) connected through 1–4 linkages. Heparin is heterogeneous due to the varying degree of modification of the functional groups in the disaccharide unit (Comper, 1981; Linhardt and Logathan, 1989). Heparinases are heparin-degrading enzymes that cleave certain sequences of heparin/heparan sulfate specifically (Lohse and Linhardt, 1992; Ernst et al., 1995). Heparinases have aided in the understanding of important physiological roles of heparin, which include anticoagulation, angiogenesis, etc. (Linhardt et al., 1993; Sasisekharan et al., 1994; Ernst et al., 1995). Additionally, heparinases themselves have therapeutic and diagnostic applications such as heparin detection and removal (Langer et al., 1988; Baugh et al., 1992; Tejidor et al., 1993; Linhardt et al., 1993; Sasisekharan et al., 1994; Ernst et al., 1995).

Flavobacterium heparinum produces three heparinases, which are specific for different sequences of heparin (Linhardt et al., 1990; Lohse and Linhardt, 1992; Desai et al., 1993). While there are several reports on the substrate specificity of heparinases (Rice et al., 1989; Linhardt et al., 1990; Nader et al., 1990; Desai et al., 1993; Ernst et al., 1995), there is limited information on the molecular features of the enzymes that confer this specificity (Linhardt et al., 1986; Lohse and Linhardt, 1992; Sasisekharan et al., 1995; Ernst et al., 1995). There have been numerous recent reports on the high degree of specificity in the binding interactions of heparin and heparan sulfate with cytokines, growth factors and other molecules of the extracellular matrix (J. Jackson et al., 1991; Varki, 1993; Ernst et al., 1995). In several heparin-binding proteins, the binding specificity is dependent on certain sequences of the polysaccharide as well as characteristic amino acid sequences of the protein (J. Jackson et al., 1991; Lindahl et al., 1994). On the protein side, Cardin and Weintraub identified two consensus sequences (XBBXXBX or XBBBXB (B = basic residues; X = hydrophobic or other residues)) found in many heparin-binding proteins (Cardin and Weintraub, 1989). Subsequently, site-directed mutagenesis and binding studies with synthetic or isolated peptides from several of these proteins have confirmed that this consensus region often is involved in binding specifically to heparin (Ba et al., 1994; Baird et al., 1988; Bober Barkalow and Schwarzauer, 1991; J. Jackson et al., 1991; Smith and Knauer, 1987; Thompson et al., 1994). On the polysaccharide side, oligosaccharides with certain sequences of modifications in the disaccharide repeat have been isolated and shown to confer binding specificity for antithrombin III, bFGF, and others (Lindahl et al., 1984; Maccarana et al., 1993; Maimone and Tolefsen, 1990; Parthasarathy et al., 1994). This is parallel to the substrate specificities of heparinases and suggests that the mechanism of substrate binding to heparinases may be involved in generating specificity.

Earlier studies suggested the importance of basic residues in heparinase I activity (Comfort et al., 1989; Leckband and Langer, 1991). Recently, we found that cysteine 135 of heparinase I is catalytically active (Sasisekharan et al., 1995). In addition, the observation of a decrease in the rate of heparinase I inactivation by chemical modification of cysteine 135 in the presence of heparin, along with other experiments, led to the hypothesis that a heparin binding domain is in close proximity

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1 The abbreviations used are: bFGF, basic fibroblast growth factor; IAA, iodoacetic acid; MOPS, 3-(N-morpholino)propanesulfonic acid; PCMB, p-chloromercuribenzoate; PAGE, polyacrylamide gel electrophoresis; HPLC, high pressure liquid chromatography; RPHPLC, reverse-phase HPLC; r-heparinase I, recombinant heparinase I; HBP, heparin-binding peptide; ACE, affinity co-electrophoresis.
The Heparin Binding Domain of Heparinase I from F. heparinum

Cyanogen Bromide Digest and Heparin Hybridization

On 1 nmol (40 µg) of heparinase I was digested with CNBr as described by Matsudaira (1993). The digest was diluted 10-fold with water and then lyophilized under vacuum. SDS-PAGE of the CNBr-digested heparinase I fragments was carried out on a 15% polyacrylamide gel using a Mini PROTEAN II electrophoresis apparatus (120 V for 90 min) (Bio-Rad). Proteins were visualized with a 0.1% Coomassie Brilliant Blue R-250 solution, followed by destaining with a 40% methanol and 10% acetic acid (v/v) solution.

Following gel electrophoresis CNBr digests of heparinase I were blotted onto nitrocellulose sheets (Schleicher & Schüll) according to the method of Matsudaira (1993). Transfer was carried out in the following ammonerage sequence: 30 min at 50 mA, 30 min at 100 mA, and 60 min at 275 mA. The nitrocellulose membrane was stained for 1 min with 0.1% Ponceau S solution (Fluka, Buchs, Switzerland) in 0.1% acetic acid and then washed with 1% acetic acid for 1 min (Tempst et al., 1990).

For heparin hybridization, the membrane was further processed by washing it with 10 mM Tris-HCl, 10 mM NaCl and 0.5 mM EDTA, pH 7.0 (TNE-10) twice, and once with the probing buffer. The heparin binding domain was probed using 125I-labeled heparin at a concentration 10^4 cpm/ml in the probing buffer. The membrane was hybridized at 37°C for 18 h following washing of the membrane to remove excess radio-labeled probe. The membrane was cut into bands corresponding to the stained peptide fragments, and each band was counted for 125I incorporation on a liquid scintillation counter (model LS 3801, Beckman, Fullerton, CA). The bands that were positive for 125I incorporation were washed with phosphate-buffered saline, pH 7.0, and sequenced as described below. After autoradiography, the stained membrane was cut into bands before hybridization and the bands were then washed in a microcentrifuge tube, prehybridized, and probed individually. CNBr peptides were extracted from the nitrocellulose membrane using essentially the procedure described by Matsudaira (1993) for sequencing (see below).

Tryptic Digest, Haparin Competition, Dot Blot Assay, and Peptide Sequencing

The tryptic digestion was carried out essentially as described by Sasisekharan et al., 1993. In some experiments, heparin was added to heparinase I before trypsin digestion. The reaction was terminated by heating the sample at 65°C for 2 min. In the PCMB protection experiment, heparinase I was first labeled with PCMB as described (Sasisekharan et al., 1995), under nonreduced conditions, and then digested with trypsin as described above.

A total of 2.5 nmol of heparinase I was digested and then divided into five batches (500 pmol each; ~20 µg) inclusive of one control digest. For the competition experiments, heparin was added in varying concentrations (5, 50, 100, and 200 µg) to the digests, which were subsequently separated using RP-HPLC, with a 20-min isocratic run, followed by a 20-min linear acetonitrile gradient. The assay was done essentially as described by Sasisekharan et al. (1993).

HPLC of Heparin Oligosaccharides—Heparinase (0.2 mg/ml) was incubated with heparinase I, r-heparinase I, or mutant enzymes in 5 mM calcium acetate, 100 mM MOPS buffer, pH 7.0, for 18 h. The reaction was then subjected to anion-exchange HPLC to separate the oligosaccharide products, as described by Sasisekharan et al. (1993).

Heparin Affinity Chromatography

Heparin affinity chromatography was carried out using a heparin POROS (4.6 mm × 100 mm) column (PerSeptive Biotechnologies, Cambridge, MA). A nanomole (~40 µg) of heparinase I in 100 mM MOPS buffer, pH 7.0 (with and without 5 mM calcium acetate), was loaded onto the heparin POROS column, connected to a BIOCAD workstation (PerSeptive Biotechnologies, Cambridge, MA). The protein was eluted in a salt gradient of 0–1 M NaCl (in 5 min.), in 10 mM Tris-HCl, pH 7.0, and monitored at 210 and 277 nm.

Affinity Co-electrophoresis

Affinity co-electrophoresis (ACE) on heparin binding to heparinase I was performed with 0 (buffer 1) or 5 mM (buffer 2) calcium acetate essentially as described (Lee and Lander, 1991). The heparinase I concentrations were from 0.05 to 25 µg/ml, either untreated, or pre-treated with IAA as reported previously (Sasisekharan et al., 1995). The binding constant was determined using Scatchard analysis, using weighted, nonlinear least-squares curve fitting (Lee and Lander, 1991).

For complete DNA and protein sequence of heparinase I, refer to Sasisekharan et al. (1993) or GenBank accession no. L12534.
The Heparin Binding Domain of Heparinase I from F. heparinum

Results

Heparin Blotting of CNBr Digests of Heparinase I—CNBr-digested heparinase I separated by SDS-PAGE, transferred onto nitrocellulose membranes, and hybridized with ¹²⁵I-heparin. The membranes were then washed and counted for ¹²⁵I incorporation. Lane 1, molecular size standards; lane 2, F. heparinum heparinase I; lane 3, CNBr-digested heparinase I. See text and Table for details.

Table shows peptides from CNBr digest of heparinase I. The amino acids in parentheses were determined with less accuracy, or several amino acids were possible. The residue number for the methionine is indicated. The % heparin bound values are averages of triplicate determinations.

| Peptides | Amino acid sequence | Region (residues) | Molecular mass kDa | % ¹²⁵I-heparin bound |
|----------|---------------------|-------------------|--------------------|---------------------|
| CNBr-1   | Blocked             |                  | 42                 | 45                  |
| CNBr-2   | Blocked             |                  | 35                 | 30                  |
| CNBr-3   | Blocked             |                  | 31                 | 62                  |
| CNBr-4   | (M)X(R)(D)...       | Contaminant       | 28                 | 40                  |
| CNBr-5   | Blocked             |                  | 21                 | 45                  |
| CNBr-6   | Blocked             | Contaminant       | 16                 | 20                  |
| CNBr-7   | (M)²⁷⁵(K/I/G)FKKN   | 272–384           | 13                 | 47                  |
| CNBr-8   | (M)³⁰⁶(K)/GFKKN     | 195–384           | 10                 | 100                 |
| CNBr-9   | (M)³⁵²MTYTK...      | 326–384           | 7                  | 56                  |
| CNBr-10  | (M)³⁸²MPFAQ...      | 288–325           | 5                  | 41                  |
| Control  |                     |                   |                   | 25                  |
| Heparinase I |                   |                   |                   | 80                  |

Heparinase I CNBr peptide sequences and the levels of bound ¹²⁵I-heparin

In a dot blot assay using ¹²⁵I-heparin and in the presence of a 100-fold excess of cold chondroitin sulfate, only td45 showed a ¹²⁵I signal (data not shown). It should be pointed out that td45 is a partial digest of td39, with the common region being residues 215–221. From the competition and dot blot experiments, it can be inferred that only td4, td39, and td45 bind heparin specifically.

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gone undetected in these experiments. To investigate the heparin-binding properties of these residues, a peptide corresponding to this region was synthesized and characterized as described below.

Synthetic Peptide Corresponding to the Heparin Binding Site of Heparinase I—A synthetic peptide (HBP-I) corresponding to the residues 196–213 has a 4 mM binding affinity for heparin dodecasaccharides. Interestingly, HBP-I affected the product profile of heparinase I degradation of heparin. As mentioned earlier, heparinase I depolymerization of heparin results in two disaccharides, three tetrasaccharides (1–3), and a hexasaccharide (Figs. 3A) (Rice and Linhardt, 1989). In a concentration-dependent manner, the addition of HBP-I to the reaction mixture caused the peak corresponding to tetrasaccharide 3 (DU25, H$_{NS,6S}$, 12S, H$_{NS,6S}$) to disappear (Fig. 3, B and C). When tetrasaccharide 3 was isolated and degraded with heparinase I in the presence of HBP-I, a marked increase in the amount of disaccharide was observed (data not shown). A control peptide (Maxadilan) with similar charge properties (and at the concentration ranges tested above) had no effect on the enzyme activity or on the oligosaccharide product profile (Fig. 3D). This demonstrates that HBP-I affects the selectivity of heparin degradation by heparinase I; tetrasaccharide 3, but not tetrasaccharides 1 or 2, is degraded in the presence of HBP-I. It must be pointed out that HBP-I does not degrade heparin or heparin oligosaccharides.

Role of Calcium—Region 206–220 was found to be homologous to the calcium binding loop of the EF-hand structural domain (Table II) (Kretsinger, 1975, 1980). Four of the five amino acids that are involved in coordinating calcium in the loop of the EF-hand structural domain are conserved in heparinase I. This led us to investigate the role of calcium in heparinase I activity. There are conflicting reports on the effect of calcium on heparinase I activity (Linker and Hovingh, 1965; Dietrich et al., 1973; Linhardt et al., 1986; Lohse and Linhardt, 1992). The relative heparinase I activity increases with calcium concentrations up to around 5 mM as shown in Fig. 4. In order to further investigate the calcium-based heparinase I activation, the role of calcium in heparin binding to heparinase I was studied using heparin affinity chromatography. These experiments indicate that heparin binding to heparinase I is independent of calcium (heparinase I elutes at a salt concentration of 200 mM) and the presence of calcium leads to a loss in binding to heparin column (as calcium activates heparinase I catalytically) (data not shown). The above results suggest a possible co-factor role for calcium in heparinase I activity.

Heparin Binding Constant of Heparinase I: Affinity Electrophoresis—The ability of heparinase I to bind heparin, in the absence of calcium, led us to investigate the heparin binding affinity of heparinase I. ACE was used to determine a heparin binding constant for heparinase I (Lee and Lander, 1991). ACE was carried out in the presence or absence of IAA to determine the importance of the active site cysteine 135 in the binding of heparin to heparinase I (Sasisekharan et al., 1995), and in the absence of calcium to prevent heparin degradation. The K$_d$ for heparinase-heparin binding was found to be 60 nM by this technique. Fig. 5A shows the ACE gel of native heparinase I (nondenatured), and Fig. 5B shows the ACE gel of heparinase I modified with IAA. There is no difference in the retardation of heparin for the IAA-modified heparinase I when compared to the unmodified heparinase I. This result indicates that blocking the active site cysteine does not alter heparin binding. An ACE gel of heparin-heparinase I carried out in the presence of calcium (data not shown) showed extensive smearing of the
heparin band.

Role of Heparin Binding in Catalysis—Heparinase I derivatization by sulfhydryl-specific reagent PCMB inactivated the enzyme due to selective modification of cysteine 135, and the inactivation kinetics were altered in the presence of heparin, suggesting a heparin binding site in close proximity to cysteine 135 (Sasisekharan et al., 1995). To test this hypothesis, tryptic digestion of PCMB-modified heparinase I was carried out to determine if PCMB (being a bulky negatively charged molecule) could impede trypsin access to the basic residues contained in the heparin binding domain. Such a protection of the basic residues from proteolytic digestion could argue for the close proximity of these residues to cysteine 135. The PCMB-heparinase I tryptic map (data not shown) has the appearance of a new peak (about 31 min) with a sequence representing residues 199–209. The results suggest that PCMB-labeled cysteine 135 protects the lysine-rich peptide (contained in the heparin binding sequence) from trypsin cleavage, when compared to a control digest where this peptide is not observed. This result supports the notion that a heparin binding site is in close proximity to the active site cysteine 135 (Sasisekharan et al., 1995).

To further investigate a functional role of the heparin binding domain, site-directed mutagenesis was carried out. Hist-
and mutant et al. It has been suggested that this tidine may act as a nucleo-
nase I in the absence of calcium.

In competition assays, we used peptides that interact specifically with heparin. CNBr digests of heparinase I repro-
ducedly gave eight fragments, six of which were partial digests from the blocked N terminus. Hybridization of the CNBr-gen-
erated fragments to radiolabeled heparin showed that only one fragment (CNBr-8) bound significant amounts of heparin (Ta-
ble I). CNBr-8 spanned residues 195–290 and is also a partial digest, as it contained a part of CNBr-7 (residues 273–290).

Since CNBr-7 did not bind heparin, the binding site is most likely located in the former half of CNBr-8 (residues 195–272).

It is noteworthy that undigested heparinase I and partial N-terminal CNBr-generated fragments (CNBr-1, -2, -3, and -5) that contained the heparin binding sequence bound relatively less heparin than the CNBr-8 fragment. It is possible that greater heparin binding to CNBr-8 was due to better accessi-

dibility of the heparin binding site in CNBr-8. Tryptic peptides td4, td39, and td45 were selectively affected by heparin, and not chondroitin sulfate, thus reinforcing the specificity of bind-
ing of these peptides to heparin. Peptides td39 and td45 are contained in CNBr-8, while td4 contains the active site cysteine 135 (see below). The results of heparin binding and competition experiments, taken together, point to the residues 195–221 as being directly involved in heparin binding. Furthermore, a synthetic 18-mer peptide (HBP-I; corresponding to residues 196–213 in the region) not only bound heparin specifically in a dot blot assay, but also altered enzyme activity. It can thus be concluded that residues 195–221 constitute the primary hepa-
rinase binding site in heparinase I. This site belongs to a very basic region of heparinase I, with two Cardin-Weintraub heparin

binding consensus sequences and a calcium coordination motif. However, heparin competition experiments indicate heparin binding to td4. It is possible that in the native heparinase I, protein folding brings the lysine and/or arginine residues con-
tained in td4 close to the heparin binding site, and all these residues together constitute a heparin binding domain in hepa-
rinase I. The presence of such a domain is supported by the PCMB labeling results, where PCMB-labeled heparinase I pro-

tects the lysine residues in the heparin binding site from tryptic cleavage.

Heparin binding to heparinase I (Kd 60 nM) was demon-

strated using ACE. This is about 1 order of magnitude higher than the dissociation constants determined by the same tech-
nique for binding of heparin to bFGF (2.2 nM) and AT III (11 nM) (Lee and Lander, 1991). This also is consistent with hepa-

rinase I eluting from a heparin affinity column at 200 mM NaCl, while bFGF and other heparin binding growth factors typically elute around 1–1.5 M NaCl (Folkman and Klagesbrun, 1987). When ACE was performed with modified heparinase I (where the cysteines were blocked with IAA), there was no alteration on the heparin retardation, indicating that cysteine 135 does not influence heparin binding, but affects only enzyme catalysis.

Role of Calcium—As a region of the heparin binding site of heparinase I (residues 206–220) is homologous to the calcium binding loop of EF-hand domains, we set out to investigate the role of calcium in heparinase I activity. We find that hepari-

nase I activity increases with calcium concentrations up to around 5 mM. It is possible that purification of heparinase I

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Role of Heparin Binding in Catalysis—Specific interactions between the heparin binding domain of heparinase I and unique heparin sequences might determine the catalytic specificity of heparinas. The three heparinas from F. heparinum are specific for different linkages in the heparin chain (Desai et al., 1993), and heparinase I is much less active in cleaving tetrasaccharides compared to full-length heparin (Rice and Linhardt, 1989). This argues that heparin binding to heparinase I involves at least a pentasaccharide or longer region of heparin, similar to the heparin binding regions of bFGF and antithrombin III, which have rather large (20 Å or more) binding regions to accommodate close contacts (Rapraeger, 1993; Margail, 1993; Schreuder et al., 1994). It is interesting to note that in the presence of HBP-I, tetrasaccharide 3 (L\textsubscript{18}E\textsubscript{5}N\textsubscript{5}O\textsubscript{5}) (Rice and Linhardt, 1989) was cleaved by heparinase I. It is possible that HBP-I "presents" this saccharide to heparinase I and facilitates cleavage. Alternately, tetrasaccharide 3 containing sequence in heparin perhaps is a "hot spot" region to which heparinase I binds during its random endolytic cleavage of heparin (Cohen and Linhardt, 1990).

Histidine residues are involved in several enzymatic reactions via different mechanisms, which include acid/base catalysis, electrophilic catalysis, and substrate binding through electrostatic interactions (Munier et al., 1992). To differentiate between a substrate binding and a catalytic role for histidine 203 in heparinase I, this residue was replaced with alanine (which can perform neither of the above functions) and with aspartic acid (which can play a role in acid/base catalysis but is not known to participate in heparin binding). The H203A mutation abolished enzyme activity, while the H203D mutant retained residual activity, strongly suggesting a catalytic role for this residue. 3 It is considered unlikely that a change in the binding site far away from the scissile bond could produce such a dramatic change in enzyme activity. It is possible that histidine acts as a nucleophilic residue in the elimination mecha-

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3 Godavarti, R., Ernst, S., Venkataraman, G., Cooney, C. L., Langer, R., and Sasisekharan, R., manuscript in preparation.
