Biochemical Investigations and Mapping of the Calcium-binding Sites of Heparinase I from *Flavobacterium heparinum*

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Zachary Shriver‡, Dongfang Liu‡, Yini Hu§, and Ram Sasisekharan‡¶

From the ‡Division of Bioengineering and Environmental Health, the ¶Department of Biology, and the §Center for Biomedical Engineering, Massachusetts Institute of Technology, Cambridge, Massachusetts 02139

The heparinas from *Flavobacterium heparinum* are lyases that specifically cleave heparin-like glycosaminoglycans. Previously, amino acids located in the active site of heparinase I have been identified and mapped. In an effort to further understand the mechanism by which heparinase I cleaves its polymer substrate, we sought to understand the role of calcium, as a necessary cofactor, in the enzymatic activity of heparinase I. Specifically, we undertook a series of biochemical and biophysical experiments to answer the question of whether heparinase I binds to calcium and, if so, which regions of the protein are involved in calcium binding. Using the fluorescent calcium analog terbium, we found that heparinase I tightly bound divalent and trivalent cations. Furthermore, we established that this interaction was specific for ions that closely approximate the ionic radius of calcium. Through the use of the modification reagents N-ethyl-5-phenylisoxazolium-3-sulfonate (Woodward’s reagent K) and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride, we showed that the interaction between heparinase I and calcium was essential for proper functioning of the enzyme. Pre-incubation with either calcium alone or calcium in the presence of heparin was able to protect the enzyme from inactivation by these modifying reagents. In addition, through mapping studies of Woodward’s reagent K-modified heparinase I, we identified two putative calcium-binding sites, CB-1 (Glu207–Ala219) and CB-2 (Thr373–Arg384), in heparinase I that not only are specifically modified by Woodward’s reagent K, leading to loss of enzymatic activity, but also conform to the calcium-coordinating consensus motif.

Heparin-like glycosaminoglycans, such as heparin and heparan sulfate, are acidic polysaccharides that play a role in many central biological processes, such as cell proliferation and signaling (1, 2). However, attempts to determine whether heparin-like glycosaminoglycans are involved in a particular biological process have been hampered by the lack of tools available to study these substrates. One such tool, under development in our laboratory, is the heparinas, bacterial-derived lyases. Heparinase I from *Fla-

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‡To whom correspondence should be addressed: Div. of Bioengineering and Environmental Health, MIT, 16-561, 77 Massachusetts Ave., Cambridge, MA 02139. Tel.: 617-258-9494; Fax: 617-258-9409; E-mail: ramnat@mit.edu.

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EXPERIMENTAL PROCEDURES

Chemicals and Materials—Urea, Tris, and trifluoroacetic acid were from J. T. Baker Inc. Dithiobiotetra, EDTA, and MOPS were obtained from Sigma. Bovine serum albumin and Chelex resin were purchased from Bio-Rad. TbCl₃, LuCl₃, and CaCl₂ as well as the chemical modification reagents N-ethyl-5-phenylisoxazol-3'-sulfone (Woodward's reagent K (WRK)), 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (EDAC), and glycine methyl ester were all purchased from Aldrich. EDAC was used as received. WRK was recrystallized prior to use. Trypsin was obtained from Boehringer Mannheim. Heparin (from porcine intestinal mucosa with an average molecular mass of 13 kDa) was obtained from Celsus Laboratories (Cincinnati, OH).

Heparinase I Activity Assay—Native heparinase I from F. heparinum was crystallized as described previously (15, 16). The UV 232 nm assay to quantify heparinase I enzymatic activity was similar to that reported elsewhere (17). Briefly, the course of the reaction was monitored by measuring the increase in absorbance at 232 nm from the Δ4,5 bond of the product of heparinase cleavage 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 100 mM MOPS and 5 mM calcium acetate, pH 7.0. The temperature for all enzymatic activity measurements was kept constant at 30 °C. For the inactivation kinetic profiles, activity was measured as outlined above at precise time points after addition of the modifying reagent.

Terbium Titrations of Heparinase I—The titrations of heparinase I with terbium were completed by adding aliquots of a terbium stock solution (in 10 mM MOPS and 0.1 M KCl, pH 6.5) to a solution containing heparinase I (4.6 μM). To maintain a constant protein concentration, the same amount of heparinase I (4.6 μM) was present in the terbium stock solution as was present in the cuvette. The concentration of the terbium solution was determined by EDTA titration in the presence of a xylene orange indicator (18). To ensure accurate readings, all solutions, except the terbium stock solution, were run through a chelating column (Chelex resin) to remove trace contaminants. After addition of a terbium aliquot, the sample was mixed and allowed to come to equilibrium for 15 min. Fluorescence measurements were recorded on a FluoroMax fluorescence spectrometer (Spx Industries, Edison, NJ). The geometry of fluorescence detection was 90°. All measurements were recorded using a quartz cell (Starna Cells) with a 1.0-cm path length, and the sample temperature was maintained at 25 °C using a circulating water bath. The excitation wavelength was either 488 nm (direct excitation of terbium) or 280 nm (excitation of nearby tyrosine residues); the emission wavelength was 545 nm.

For the calcium competition titrations, to a solution of heparinase I (4.6 μM) plus 8 molar eq of terbium were added aliquots of a 50 mM calcium stock solution that also contained 4.6 μM heparinase I. After each addition, the solution was thoroughly mixed and allowed to stand for 15 min before a measurement was taken. In none of the experiments was protein precipitation evident.

Effect of Lanthanides on Heparinase I Activity—To determine the effect of Tb³⁺ and Lu³⁺ on heparinase I activity, heparinase I was preincubated for 15 min with increasing amounts of a solution of 10 mM MOPS and 0.1 mM KCl, pH 6.5, with either Tb³⁺ and Lu³⁺. At this point, the activity of the heparinase I solution was measured using the 232 nm assay. The substrate solution was 4 mg/ml heparin and 5 mM calcium in 10 mM MOPS and 0.1 mM KCl, pH 6.5. The concentrations of the lanthanide stock solutions were determined as outlined above. Control reactions were run in the absence of lanthanide.

Formation and Degradation of the Ketoketenimine Intermediate from WRK—Upon addition of WRK to an enzyme solution, the actual agent that modifies nucleophilic amino acids is not WRK itself; rather WRK is converted into a reactive intermediate that binds to selective amino acids in a protein (19). Therefore, to accurately model the kinetics of WRK modification of heparinase I, it is necessary to know the concentration of this intermediate, the ketoketenimine, as a function of time. The formation of the ketoketenimine can be determined by monitoring an aqueous solution of WRK at 340 nm, where the ketoketenimine is the only species of WRK that absorbs appreciably (τ = 4730 cm⁻¹ M⁻¹) (19, 20). At pH 7.0, the conversion of 50 μM WRK to WRK—was quenched with water by bringing the total reaction volume to 200 μl.

Activity (mAU)

\[ [\text{Ca}^{2+}] \] (μM)

10
8
6
4
2
0
0
2000
4000
6000
8000
10000

\[ \text{FIG. 1. Heparinase I activity as a function of calcium concentration.} \]

Heparinase I (2 μg) was incubated with 4 mg/ml heparin and various concentrations of calcium in 100 mM MOPS, pH 7.0, at 30 °C. The formation of the Δ4,5 product was monitored at 232 nm. mAU, milli-absorbance units.

for 10 min. Stock solutions of WRK were made fresh with 0.1 M HCl at 4 °C. A cuvette containing an equivalent amount of 0.1 M HCl instead of WRK was used as a blank. To determine the rate constants of formation (k⁺) and degradation (k⁻) of the ketoketenimine, Equation 1 was used.

\[ \frac{d[W]}{dt} = k_+ [W]_0 e^{-k_- t} - k_0 [W]_0 e^{-k_+ t} \]

In this equation, \( t \) is the measured time, \([W]\) is the concentration of the ketoketenimine intermediate, and \([W]_0\) is the initial concentration of WRK (50 μM) (19).

Inactivation of Heparinase I with WRK—Heparinase I (30 mg/ml) was inactivated with 0.1–0.4 mM WRK at room temperature. The control mixture contained no WRK, but an equivalent amount of 0.1 M HCl. Reactions were carried out in 100 mM MOPS, pH 7.0. At fixed time intervals, aliquots were withdrawn for the UV 232 nm activity assay. The kinetics of WRK inactivation of heparinase I were determined by plotting the natural log of percent activity versus an adjusted time term (to account for the formation and decomposition of the ketoketenimine intermediate). This adjusted time term was calculated according to Equation 2.

\[ t' = \frac{k^+}{k^-} \left( 1 - e^{-k_+ t} \right) - \frac{k^-}{k^+} \left( 1 - e^{-k_0 t} \right) \]

Ca²⁺ Protection of WRK Inactivation of Heparinase I—To investigate the ability of Ca²⁺ to protect the enzyme against modification by WRK, heparinase I (30 mg/ml) was first incubated with different concentrations of Ca²⁺ ranging from 100 μM to 20 mM) for 30 min at pH 7 before 50 μM WRK was added to the reaction mixtures. The time course of inactivation was then determined. An activity assay was also performed with a control mixture with no prior addition of Ca²⁺.

Tryptic Digest and Protein Sequence Analysis—Tryptic digests of heparinase I were performed essentially as described previously (7). To 16 μg of heparinase I was added 4 mM WRK; the sample was allowed to incubate for 30 min at room temperature. A 10-fold excess of glycine methyl ester was added to quench the reaction. The enzyme was then denatured in 50 μl of 8 M urea and 0.4 M ammonium carbonate, reduced with 5 mM dithiothreitol at 65 °C, cooled to room temperature, and alkylated with 10 mM iodoacetamide for 15 min. The reaction was quenched with water by bringing the total reaction volume to 200 μl. The above reaction was added 4% (w/v) trypsin, and the digestion was carried out at 67 °C for 24 h. The proteolytic reaction was terminated by freezing at −20 °C. The digest was separated using gradient reverse-phase HPLC (2–80% acetonitrile in 0.1% trifluoroacetic acid for 120 min). Tryptic peptides were monitored at 210, 277, and 320 nm and collected. Based on the peptide peaks monitored at 320 nm, five peaks were collected and sequenced using an on-line Model 120 phenylthiohydantoin-derivative analyzer (Biopolymers Laboratory, Center for
To determine whether preincubation with calcium protected the enzyme from WRK modification, heparinase I was first incubated with 100 mM CaCl₂ at room temperature. Heparinase I digests in the absence of WRK modification were included as controls.

RESULTS

Interaction of Terbium with Heparinase I—Fig. 1 shows that, in the presence of millimolar concentrations of calcium, heparinase I activity was increased 5-fold, consistent with earlier observations (11). To address whether heparinase I itself binds calcium, we studied the interaction of Tb³⁺ with heparinase I in the absence of heparin. In this way, interactions of heparinase I with terbium could be studied independently of confounding factors associated with terbium-heparin interactions. Tb³⁺ is a lanthanide analog of calcium often used to probe the nature of protein interactions with calcium (21, 22). Tb³⁺ possesses an ionic radius that is very similar to that of calcium in aqueous solution and has the advantage that, unlike calcium, the protein-Tb³⁺ complex is fluorescent. In addition, because of the increase in charge properties of terbium compared with calcium (i.e. 3+ as opposed to 2+), terbium very often has a higher affinity for calcium-binding sites than does calcium itself.

Upon titration of heparinase I with terbium, an increase in fluorescence was observed whether excitation was performed at 488 nm (direct excitation of the terbium adduct) or 280 nm (excitation of nearby tyrosine side chains, followed by energy transfer to the terbium adduct). Since, as has been observed with other protein systems, the fluorescence signal was enhanced upon indirect excitation at 280 nm, the most extensive studies were completed in this way (Fig. 2). Fluorescence intensity increased upon titration of terbium to heparinase I until 10 eq had been added. Beyond this point, the fluorescence
intensity did not increase further.

To ensure that the terbium-heparinase I interaction was specific, the ability of calcium to compete with terbium for binding to heparinase I was investigated. As noted above, terbium very often can have a 1000-fold higher affinity than calcium for calcium-binding sites; therefore, a large excess of calcium is required to reduce the binding of terbium to a protein. In the case of heparinase I, after addition of 8 eq of terbium to heparinase I, calcium was added to the terbium/enzyme solution, and the fluorescence was measured. As shown in Fig. 3, addition of calcium concentrations up to 2 mM was able to compete terbium off of heparinase I. These results indicate that the interaction of terbium with heparinase I is specific and that this interaction substitutes for calcium binding to heparinase I.

**Inactivation of Heparinase I with Tb³⁺ or Lu³⁺**—In an effort to confirm and extend the conclusions of the fluorescence study, the effect of terbium on heparinase I activity was determined. We found that heparinase I activity was inhibited in a dose-dependent fashion by terbium with a measured IC₅₀ of 39 μM (Fig. 4). This type of inhibition has been seen for other enzyme systems known to interact specifically with calcium (23). The effect of another lanthanide, lutetium, on heparinase I activity was also investigated. The ionic radius of Lu³⁺ is smaller than that of Tb³⁺; therefore, we expected that Lu³⁺, a less suitable replacement for calcium in heparinase I, would be a less potent...
inhibitor of heparinase I activity (24, 25). In fact, Lu\textsuperscript{3+} was also able to inhibit heparinase I activity; however, the IC\textsubscript{50} was increased to 212 μM. Together with the fluorescence experiments, these results indicate that heparinase I interacts in a highly specific manner with terbium and, by extension, with calcium.

Inactivation of Heparinase I by Chemical Modification—Having determined that there is a specific calcium-heparinase I interaction, we set out to determine which sites in heparinase I could potentially bind calcium. To investigate the roles of calcium in the enzymatic activity of heparinase I, we used a combination of chemical modification coupled with protection experiments (to ensure the specificity of the reaction) and proteolytic mapping studies (to identify particular amino acids that are modified). To complete these studies and to identify the calcium-binding site(s) in heparinase I, we used two modification reagents (WRK and EDAC) that are specific for carboxylate groups.

Formation and Degradation of the Ketoketenimine Intermediate from WRK—In the case of WRK, the active modification reagent is not WRK per se, but rather an unstable intermediate, the corresponding ketoketenimine (19). In an effort to understand the kinetics of heparinase I modification by WRK, the rates of formation and degradation of the ketoketenimine were followed by monitoring an aqueous solution of WRK at 340 nm. At this wavelength, the only species of WRK that possesses appreciable absorbance is the ketoketenimine ($\varepsilon = 4730$ cm\textsuperscript{-1} mM\textsuperscript{-1}) (19). The results were fit to a nonlinear equation as outlined under "Experimental Procedures" (Fig. 5). The derived rate constants for the formation (0.061 s\textsuperscript{-1}) and degradation (0.019 s\textsuperscript{-1}) of the ketoketenimine were used to accurately determine the kinetics of heparinase I inactivation by WRK.

WRK and EDAC Inactivate Heparinase I in a Dose-dependent Way—Having determined the kinetics of ketoketenimine formation, the kinetics of WRK inactivation of heparinase I were studied. WRK was found to inhibit heparinase I in a dose-dependent fashion (Fig. 6A). Plotting the pseudo first-order rate constants as a function of the WRK concentration yielded a second-order rate constant of 7.9 mM\textsuperscript{-1} min\textsuperscript{-1} (Fig. 6B).

To ensure that the reaction was specific for carboxylate-containing residues, the effect of another carboxylate-specific reagent (EDAC) on heparinase I activity was determined. Similarly to what was seen for WRK, EDAC (in the millimolar range) was found to inhibit heparinase I in a dose-dependent fashion (data not shown).

Protection of Heparinase I from WRK-mediated Inactivation by Calcium or Heparin—If WRK modifies the calcium-binding domain(s) of heparinase I, leading to inactivation because of disrupting interactions critical for proper enzymatic functioning, then preincubation with calcium, heparin, or both should
Biochemical Studies of Calcium Binding to Heparinase I

Primary amino acid sequence of heparinase I (minus the leader sequence) and identification of WRK-modified peptides

| aa sequence | E | K | D | G | K | I | T | Y | V | A |
|-------------|---|---|---|---|---|---|---|---|---|---|
| CB-1        |   |   |   |   |   |   |   |   |   |   |
| Consensus   |   |   |   |   |   |   |   |   |   |   |

Calcium-coordinating motifs in heparinase I

| EF-hand homology | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|------------------|---|---|---|---|---|---|---|---|---|----|----|----|
| AA               | X | Y | Z | G | -Y | -I | -X | -Z |    |    |    |    |

Identification of CB-1 and CB-2—Many studies have aimed at identifying consensus sequences for calcium-coordinating motifs, and most of these have focused on a particular calcium-coordinating motif, the EF-hand, present in many calcium-binding proteins (32, 33). To provide a framework for this study as well as for the accompanying site-directed mutagenesis study (14), we set out to determine whether any of the WRK-labeled peptides conformed to an EF-hand motif.

Table II lists the consensus sequence of the EF-hand calcium-coordinating motif. The canonical EF-hand consists of two α-helices interposed by a loop region that contains the calcium-chelating amino acids. These amino acids, identified as X, Y, Z, -Y, -X, and -Z in Table II (six ligands, typically in an octahedral geometry), chelate calcium either through an oxygen atom of a side chain or through a carbonyl atom of the peptide backbone. Examination of the modified tryptic peptides and comparison of their amino acid sequence with the EF-hand calcium-chelating consensus sequence (Table II) indicated that two of these modified peptides (viz. the C-terminal region of heparinase I and the region proximate to the heparin-binding site of heparinase I) share similarities with a EF-hand consensus sequence (30) and could potentially bind calcium.

The first site, hereafter referred to as CB-1, extends from Glu207 to Ala219 (Table II) and is proximate to the heparin-binding site that has also been shown to be critical for enzymatic functioning (11) and that contains His203, a putative active-site residue (8). Within CB-1, the potential calcium-chelating amino acids include Glu207, Asp215, Asp213, and Thr216. The second site, hereafter referred to as CB-2, is at the C terminus of heparinase I, extending from Thr273 to Arg384. Like CB-1, CB-2 contains amino acids that could potentially bind calcium. These include Thr273, Asn293, Ser297, Ser296, and Glu313. More importantly, both CB-1 and CB-2 are modified by WRK/glycine methyl ester.

To determine whether these peptides that were modified by WRK in the above experiment could be protected upon preincubation with calcium, 100 mM Ca2+ was added to heparinase I before addition of WRK and subsequent digestion. Under these conditions, td 54 and td 59 (corresponding to modification of CB-1) and td 95 (corresponding to modification of CB-2) were all protected from modification by preincubation with calcium, consistent with CB-1, CB-2, or both being involved in calcium binding by heparinase I.
tioning of the enzyme, and we have mapped the calcium-binding regions of heparinase I.

Fluorescence titration experiments have often been used to establish a specific interaction between calcium and a protein, including enzymes. We found that, in the presence of heparinase I, there is a fluorescence enhancement of terbium. This enhancement plateaus at a terbium/enzyme ratio of 10:1. To confirm that this interaction is specific for calcium, we found that terbium binding can be competed off by addition of an excess of calcium. Furthermore, terbium has a more pronounced effect on heparinase I activity than lutetium, a lanthanide with an ionic radius that does not as closely mimic that of calcium. Once again, by analogy to other enzyme systems, most notably trypsin (26), this finding supports the notion of a specific calcium-heparinase interaction being important for enzymatic activity.

To corroborate the findings of the terbium study, we studied the interaction of WRK with heparinase I. We found that heparinase I is inhibited by WRK in a dose-dependent manner, suggesting that WRK modifies carboxylate residues important for proper enzymatic functioning. WRK is well established in terms of its ability to modify glutamate and carboxylate amino acids (27–29), which are especially prevalent in calcium-coordinating motifs. However, recent reports have highlighted the fact WRK can be nonspecific (30, 31). In fact, WRK can possibly react with other exposed nucleophiles, including surface-accessible histidines or cysteines. This was especially of concern in our case since it is known, from a previous study, that heparinase I contains an unusually nucleophilic cysteine residue, cysteine 135 (7).

Several lines of evidence support the supposition that WRK modifies carboxylate residues in heparinase I. First, preincubation with calcium was found to protect heparinase I from inactivation by WRK in a dose-dependent fashion. Second, EDAC, like WRK, was found to modify heparinase I. Finally, we mapped the residues that were modified by WRK. These mapping studies revealed that all the peptides contained carboxylate-containing amino acids. In addition, no peptides contained cysteine 135, indicating that, under these reaction conditions, WRK is specific for carboxylate-containing amino acids. Together, these findings support the notion that WRK modifies carboxylate residues important for proper enzymatic functioning of calcium. Once again, by analogy to other enzyme systems, most notably trypsin (26), this finding supports the notion of a specific calcium-heparinase interaction being important for enzymatic activity.

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