In the accompanying paper (Shriver, Z., Liu, D., Hu, Y., and Sasekharan, R. (1999) J. Biol. Chem. 274, 4082–4088), we have shown that calcium binds specifically to heparinase I and have identified two major calcium-binding sites (CB-1 and CB-2) that partly conform to the EF-hand calcium-binding motif. In this study, through systematic site-directed mutagenesis, we have confirmed the accompanying biochemical studies and have shown that both CB-1 and CB-2 are involved in calcium binding and enzymatic activity. More specifically, we identified critical residues (viz. Asp\textsuperscript{210}, Asp\textsuperscript{212}, Gly\textsuperscript{213}, and Thr\textsuperscript{216} in CB-1 and Asn\textsuperscript{375}, Tyr\textsuperscript{379}, and Glu\textsuperscript{381} in CB-2) that are important for calcium binding and heparinase I enzymatic activity. Mutations in CB-1 resulted in a lower k_{cat} but did not change the product profile of heparinase I action on heparin; conversely, mutations in CB-2 not only altered the k_{cat} for heparinase I, but also resulted in incomplete degradation, leading to longer saccharides. Fluorescence competition experiments along with heparin affinity chromatography suggested that mutations in CB-1 alter heparinase I activity primarily through decreasing the enzyme’s affinity for its calcium cofactor without altering heparin binding to heparinase I. Compared with CB-1 mutations, mutations in CB-2 affected calcium binding to a lesser extent, but they had a more pronounced effect on heparinase I activity, suggesting a different role for CB-2 in the enzymatic action of heparinase I. These results, together with our accompanying study, led us to propose a model for calcium binding to heparinase I that includes both CB-1 and CB-2 providing critical interactions, albeit via a different mechanism. Through binding to CB-1 and/or CB-2, we propose that calcium may play a role in the catalytic mechanism and/or in the exolytic processive mechanism of heparin-like glycosaminoglycan de-polymerization by heparinase I.

Heparin-like glycosaminoglycans (HLGAGs)\textsuperscript{1}, found both at the cell surface and in the extracellular matrix, bind to and regulate the biological activities of diverse molecules, including growth factors (1), thus influencing a range of physiological functions (2). HLGAGs are acidic polysaccharides, characterized by a disaccharide repeat unit of hexosamine and uronic acid (L-iduronic or D-glucuronic acid) connected through 1–4 linkages. Chemical heterogeneity in HLGAGs results from variation in the degree and distribution of sulfation. Heparinases I–III from Flavobacterium heparinum degrade HLGAGs with unique specificity and are thus useful tools in understanding the structure, composition, and physiological roles of HLGAGs (3–6). Moreover, heparinases have important clinical applications, such as in the neutralization of heparin in blood (approved by the Food and Drug Administration) (7), in the production of low molecular weight heparins for use in humans. In addition, heparinases I and III have been shown to be potent inhibitors of angiogenesis (8).

To determine structure-activity relationships for heparinase I, we are attempting to understand the mode of heparin degradation by heparinase I both at a mechanistic level and by identifying critical amino acids in the heparinase I active site through extensive biochemical and mutagenesis approaches (3–6, 9–11). We have shown that Cys\textsuperscript{135}, His\textsuperscript{203}, and Lys\textsuperscript{199} are important in the catalytic mechanism of heparinase I (9, 11, 12). Through extensive site-directed mutagenesis studies, we identified other positively charged residues (Lys\textsuperscript{112} and Lys\textsuperscript{196}) that provide the necessary microenvironment for heparinase I catalysis (12). Recent studies of heparin degradation by heparinase I revealed a processive catalytic mechanism in which heparin is predominantly degraded exolytically from the non-reducing end (13).

In the accompanying study (19), by using the fluorescent calcium analog terbium, we found that heparinase I binds calcium specifically. In addition, we have shown that Woodward’s reagent K and 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride can inactivate heparinase I, whereas preincubation with calcium and/or heparin affords protection from inactivation. Thus, it is clear that the interaction between heparinase I and calcium is essential for the proper functioning of the enzyme. Finally, tryptic mapping studies of Woodward’s reagent K-modified heparinase I revealed two putative calcium-binding sites in heparinase I, CB-1 and CB-2. As part of a primary heparin-binding site (residues 196–221), CB-1 contains residues 207–219 (19). CB-2 is located at the C terminus of heparinase I, spanning residues 373–384 (19). Preincubation with calcium also protected CB-1 and CB-2 from being modified by Woodward’s reagent K. We therefore suggest that either or both calcium-binding sites play an important role in calcium binding and/or enzymatic activity.

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1 The abbreviations used are: HLGAGs, heparin-like glycosaminoglycans; CB-1, calcium-binding site 1; CB-2, calcium-binding site 2; HPLC, high pressure liquid chromatography; MOPS, 4-morpholinepropanesulfonic acid.
In this paper, we confirm and extend the biochemical studies by identifying important calcium-coordinating residues in both CB-1 and CB-2. Furthermore, by analyzing the effects of mutations in CB-1 and CB-2 on enzymatic activity, calcium and heparin binding as well as the distribution and amount of different products formed from heparin degradation, we sought to outline possible roles for calcium in heparinase I activity.

**EXPERIMENTAL PROCEDURES**

**Chemicals and Materials**

Heparin (from porcine intestinal mucosa with an average molecular mass of 13 kDa and an activity of > 150 USP units/mg) was from Celsus Laboratories (Cincinnati, OH). Urea, dithiothreitol, and acetonitrile were from Allied Chemicals (Deerfield, IL). Molecular mass standards were obtained from Life Technologies, Inc. The fluorescence probe rhod-5N was obtained from Molecular Probes, Inc. (Eugene, OR). Chelex resin was purchased from Bio-Rad. All other chemicals were from Sigma. The Escherichia coli BL21(DE3) host was from Novagen (Madison, WI). Molecular biology reagents and their sources are listed in the appropriate sections below.

**Mutagenesis, Expression, and Purification of Recombinant Heparinase I**

**Mutagenesis**—The mutations were introduced via 12-cycle polymerase chain reaction by the method of Higuchi (14). All mutant genes were cloned into pET-15b and were sequenced to verify the mutations as described previously (9).

**Expression and Purification**—Mutant recombinant heparinases I were expressed without the putative F. heparinum leader sequence, i.e. as a construct (–L recombinant heparinase I) that reads Met-Gln22-Gln24-Gln25. (3). To facilitate purification, the heparinase I gene was expressed using the pET-15b system (Novagen). This construct has a polyhistidine tag and a thrombin cleavage site in a 21-amino acid N-terminal leader sequence (9).

All recombinant heparinases were constructed in the pET-15b expression system and expressed and purified in the BL21(DE3) host as described previously (9). SDS-polyacrylamide gel electrophoresis was carried out using 12% gels and a Mini Protean II apparatus and stained with the Silver Stain Plus kit (9). The level of protein expression was carried out using 12% gels and a Mini Protean II apparatus and stained with the Silver Stain Plus kit (9). The level of protein expression was determined by direct measurement from the increase in absorbance at 232 nm as a function of time. The UV 232 nm assay was performed essentially as described previously (10).

The heparin-POROS chromatography study of heparinase I was essentially carried out following the procedure described previously (12). Briefly, 30–40 µg of L recombinant heparinase I and the various mutant enzymes were injected into a heparin-POROS column (4.6 x 100 mm; PerSeptive BioSystems, Framingham, MA) connected to a BioCAD system (PerSeptive BioSystems). Proteins were eluted using a linear gradient of 0–1 M NaCl in 10 min (10 mM Tris and 1 mM EDTA, pH 7.0) and monitored at 210 nm. EDTA was added to chelate any calcium ions that may have been present in the buffers.

**Fluorescence Competition**—To determine the effect of mutations in CB-1 and CB-2 on the ability of heparinase I to bind calcium, mutants with a dramatic drop in activity were tested for their ability to bind calcium. To accomplish this, we examined the ability of heparinase I and mutants to compete for free calcium with the calcium-chelating fluorescence probe rhod-5N. The fluorescence probe rhod-5N was dissolved at a dilution in buffers (10 mM MOPS and 100 mM NaCl, pH 6.5). Buffer A and water used in the study were run through a Chelex resin column to remove trace amounts of calcium. In the absence of calcium, rhod-5N is not fluorescent; however, upon binding calcium, rhod-5N is fluorescent with an emission $\lambda_{em}$ of 576 nm. Before titration, rhod-5N and heparinase I were added to a quartz cuvette such that the final concentration of rhod-5N in the cuvette was 0.3 µM and that of heparinase I was 3 µM. To this solution was added aliquots of a calcium solution (20 mM) that had been previously equilibrated with 0.3 µM rhod-5N and 3 µM heparinase I. The stock was added such that the calcium concentration in the cuvette was 50, 100, 200, 500, 1000, or 5000 µM. After allowing the solution to come to equilibrium, the sample was scanned from 560 to 600 nm with the excitation wavelength fixed at 561 nm. The plot of $\Delta \psi_{max}$ versus $[Ca^{2+}]$ was fitted to a nonlinear equation to determine the apparent $K_{app}$ value. Thus, $K_{app}$ values represent the apparent dissociation constants for the fluorescence probe rhod-5N. Control samples without heparinase I were included in the study.

**Strategy for Site-directed Mutagenesis Studies**

In the accompanying study (19), we identified two putative calcium-binding sites, CB-1 and CB-2. CB-1 spans residues 207–219, and CB-2 includes residues 373–384. Also, as stated earlier, both CB-1 and CB-2 conform to the canonical calcium-binding consensus sequence, the EF-hand motif (16, 17) (Table I). This, in part, serves as the basis for the selection of amino acids for site-directed mutagenesis. Specifically, amino acids in CB-1 and CB-2 that contain oxygen side chains and/or that conform to the EF-hand consensus sequence were mutated.

**Heparinase I Activity Assays**

**UV 232 nm Assay**—The UV 232 nm assay was performed essentially as described previously (3, 15). Briefly, the enzymatic activity was directly measured from the increase in absorbance at 232 nm as a function of time. All assays were performed at 30 °C. When measuring the enzymatic activity as a function of heparin concentration, heparin concentrations varied from 0 to 4 mg/ml at a fixed calcium concentration of 5 mM (100 mM MOPS and 5 mM calcium acetate, pH 7.0). The data were then fit to a nonlinear equation to determine $K_{cat}$ and $K_{m}$ of heparinase I. The catalytic activity was also investigated as a function of calcium concentration ranging from 0 to 10 mM. These data were also fit to a nonlinear function to determine $K_{cat}$, i.e. the calcium concentration at which half of the maximum enzymatic activity was observed. Activity is expressed as international units/micrograms of product formed per minute using $E = 3800$ mU cm$^{-1}$.

**HPLC of Heparin Oligosaccharides**—Heparin (4 mg/ml) was incubated with L recombinant heparinase I or mutant enzymes in 100 mM MOPS and 5 mM calcium acetate buffer, pH 7.0, for 18 h. The reaction was terminated and subjected to anion-exchange HPLC to resolve the oligosaccharide products as described (3).

**Heparin-POROS Chromatography**—The heparin-POROS chromatography study of heparinase I was essentially carried out following the procedure described previously (12). Briefly, 30–40 µg of L recombinant heparinase I and the various mutant enzymes were injected into a heparin-POROS column (4.6 x 100 mm; PerSeptive BioSystems, Framingham, MA) connected to a BioCAD system (PerSeptive BioSystems). Proteins were eluted using a linear gradient of 0–1 M NaCl in 10 min (10 mM Tris and 1 mM EDTA, pH 7.0) and monitored at 210 nm. EDTA was added to chelate any calcium ions that may have been present in the buffers.

**Alignment of putative calcium-binding site amino acids in heparinase I with the EF-hand consensus sequence**

The canonical EF-hand consists of an α-helix, a loop wrapped around the calcium ion, and a second α-helix. The numbers given represent the positions of amino acids in the loop region, X, Y, Z, X, Y, and Z represent amino acid residues that coordinate the Ca$^{2+}$ ion (16, 17). CB-1 and CB-2 of heparinase I are aligned against the consensus sequence for calcium-binding EF-hands, which includes the helix-loop-helix motif (16, 17). Within the consensus sequence, boldface letters indicate the amino acids that have been observed among calcium-binding proteins in decreasing order of frequency.

| EF-hand homology | 1 | 3 | 5 | 6 | 7 | 8 | 9 | 12 |
|------------------|---|---|---|---|---|---|---|----|
| CB 1             | X | Y | Z | G | Y | I | X | -Z |
| CB-2             | V | T | Y | N | L | S | G | Y | S | E | T | A | R |
| Consensus        | D | X | K | D | G | K | I | T | Y | V | A |
|                 | N | N | L | T | S | V | G | D | N | E |

* Amino acid.
Mutagenesis Studies of the Heparinase I Calcium-binding Sites

CB-1

![Fig. 1. Schematic representation of the various heparinase I mutations completed for this study. Two putative calcium-binding consensus sequences (CB-1 and CB-2) were chosen as targets for mutagenesis study. Based on alignment with the classical EF-hand calcium-binding motif (Table I), Glu207, Asp210, Asp212, Gly213, and Thr216 in CB-1 were first individually changed to alanines. Then double and triple mutants (D210A/D212A, E207A/D210A, E207A/D212A, D212A/T216A, G213A/T216A, E207A/D210A/D212A, and D212A/G213A/T216A) were made to investigate the collective effect of mutations. In CB-2, the double mutants G378A/Y379A and E381A/T382A were first made. Since the double mutants affected enzymatic activity, Gly378, Tyr379, and Thr382 were individually changed to alanines to examine the possibility of one of these residues having a dominant effect on enzymatic activity. Based on homology to the EF-hand motif, the mutations N375A, S377A, and T373A were also made. Solid circles indicate amino acids that were chosen for the mutagenesis study.]

![Table II](https://www.jbc.org/figs/)

| Enzyme                  | \(k_{\text{cat}}\) | \(K_m\) | \(K_{0.5}\) | NaCl |
|-------------------------|-----------------|--------|------------|------|
| L                      | 92              | 3.9    | 270        | 482  |
| E207A                   | 92              | 3.1    | 520        | 483  |
| D210A                   | 74              | 2.6    | 310        | 500  |
| D212A                   | 65              | 1.9    | 370        | 498  |
| G213A                   | 28              | 1.0    | 740        | 476  |
| T216A                   | 50              | 1.5    | 430        | 479  |
| E207A/D210A             | 65              | 2.8    | 470        | 513  |
| D210A/D212A             | 21              | 0.3    | 700        | 515  |
| E207A/D212A             | 58              | 0.9    | 710        | 513  |
| D212A/T216A             | 52              | 1.5    | 320        | 497  |
| G213A/T216A             | 19              | 0.8    | 2200       | 498  |
| E207A/D210A/D212A       | 25              | 0.7    | 810        | 540  |
| D212A/G213A/T216A       | 20              | 3.2    | 730        | 495  |
| T373A                   | 85              | 1.0    | 50         | 480  |
| N375A                   | 10              | 0.9    | 60         | 490  |
| S377A                   | 71              | 2.7    | 50         | 486  |
| G378A                   | 41              | 0.7    | 80         | 489  |
| Y379A                   | 9                | 0.9    | 290        | 489  |
| E381A                   | 10              | 0.5    | 180        | 505  |
| T382A                   | 65              | 1.6    | 100        | 488  |
| G378A/Y379A             | 9                | 0.3    | 90         | 489  |
| E381A/T382A             | 10              | 1.8    | 180        | 505  |

\(k_{\text{cat}}\) refers to the number of product molecules catalyzed by heparinase I per second; \(K_m\) refers to the \(K_m\) value for the heparin substrate; \(K_{0.5}\) refers to the calcium concentration required for the half-maximal activity of heparinas I; NaCl refers to the concentration at which individual enzyme is eluted from a heparin affinity column.

\(^{a}\) The \(K_m\) value reported here is identical to the one reported by Ernst et al. (4) but differs from the \(K_m\) value of Godavarti and Sasisekharan (12). The discrepancy in \(K_m\) values for recombinant heparinase I are due to differences in the experimental determination of the initial velocity.

**Fig. 2. Plot of \(K_{0.5}\) versus \(k_{\text{cat}}\) for CB-1 mutants.** The data on the single, double, and triple mutants in CB-1, including both the turnover rate \(k_{\text{cat}}\) and the calcium concentration at which half-maximal activity was observed \(K_{0.5}\), were derived from Table II. A linear regression was run on these data and yielded an \(r^2\) value of 0.69.

Significantly, with \(3\) and 2-fold decreases in \(k_{\text{cat}}\) values, respectively, compared with that of wild-type recombinant heparinase I (Table II). Furthermore, the double mutants E207A/D210A and E207A/D212A showed no further reduction in enzymatic activity than the corresponding single mutants D210A and D212A. Similarly, the triple mutant E207A/D210A/D212A was not significantly different from the double mutant D210A/D212 (Table II). This suggests that Glu207 individually or jointly is not essential for enzymatic activity. Since Asp210, Asp212, Gly378, and Thr382 individually affected enzymatic activity, we looked at the effect of combined mutations of these amino acids. The double mutations D210A/D212A and G213A/T216A produced a more pronounced reduction in enzymatic activity (an \(5\)-fold reduction in \(k_{\text{cat}}\) values) than any of the individual mutations (Table II). The triple mutant D212A/G213A/T216A also decreased the enzymatic activity \(5\)-fold. However, no further reduction in enzymatic activity was observed for the double mutant D212A/T216A as compared with the T216A single mutation.

All mutations in CB-1 resulted in increases in \(K_{0.5}\) values (Table II). Furthermore, there was a strong correlation between loss of enzymatic activity and an increase in \(K_{0.5}\) (Fig. 2). These results suggest that CB-1 mutants lower the enzymatic activity of heparinase I primarily through lowering its calcium affinity. Moreover, this result is consistent with what is seen when comparing the exhaustive heparin digests of recombinant heparinase I and the mutants E207A/D210A, E207A/D212A, and D212A/G213A/T216A. Mutations in CB-1 did not affect the product profile of heparinase I, but simply slowed the enzyme's catalytic turnover rate (Fig. 3, A–D).

**Mutagenesis of CB-2—**CB-2 was initially screened by targeting key amino acids within the site and creating double mutations. For the G378A/Y379A double mutant, the enzymatic activity was reduced significantly (10-fold reduction in \(k_{\text{cat}}\) values) and the calcium concentration at which half-maximal activity was observed \(K_{0.5}\), was decreased by 9-fold. Since the joint alteration of Gly378 and Tyr379 as well as Glu381 and Thr382 affected heparinase I activity drastically, we subsequently investigated the effect of individually altering these residues to alanines to examine whether one mutation had a more pronounced effect than the other on heparinase I activity. When Gly378 and Thr382 were individually changed to alanines, their \(k_{\text{cat}}\) values decreased only by about half (Table II). However, the Y379A
and E381A single mutations decreased enzymatic activity \( (k_{cat}) \) by \(-10\)-fold, suggesting that these residues are important for calcium binding and/or heparinase I activity. Since Thr\(^{373}\), Asn\(^{375}\), and Ser\(^{377}\) also have oxygen-containing side chains and conform to the EF-hand motif consensus sequence (Table I), we studied the effect of individually changing these amino acids on heparinase I activity. For the T373A and S377A mutants, no significant decrease in enzymatic activity was observed (Table II). The N375A mutation decreased heparinase I activity \( (k_{cat}) \) by \(-9\)-fold.

Unlike the CB-1 mutants, all CB-2 mutants showed decreased \( k_{cat} \) values (Table II). These results were puzzling in light of what was seen for CB-1 and led us to complete additional experiments to understand the role of CB-2 in calcium binding and heparinase I enzymatic activity (see below and “Discussion”).

First, we carefully analyzed the exhaustive digest product profiles for the mutants N375A, S377A, G378A/Y379A, and E381A/T382A, the mutants that had a marked effect on activity. The digest profiles were similar to that of \(-L\) recombinant heparinase I, but, unlike CB-1 mutants, there was a lower amount of the major products (essentially di- and tetrasaccharides) and a greater fraction of digestion fragments larger than a hexasaccharide (Fig. 4, A–D) (4). The presence of digestion fragments larger than a hexasaccharide argues for a role for CB-2 in the processivity of heparinase I (see “Discussion”).

**Heparin Affinity Chromatography**—In light of the differences in the heparin digest product profiles of CB-1 versus CB-2 mutants, we sought to understand whether calcium plays a role in heparin binding to heparinase I. In this case, mutations that affected calcium binding might also affect the binding of the substrate heparin to the enzyme. We have shown previously that, in the absence of calcium, native heparinase I from *F. heparinum* binds a heparin-POROS column and can be eluted at a salt concentration of \(-500\) mM (10). This technique has also been used to investigate whether mutations in heparinase I affect binding of heparin to the enzyme (12). Thus, heparin-

**Fluorescence Competition**—The results of the kinetic analysis of CB-1 and CB-2 mutants raised the question of whether the mutant enzymes had altered salt (NaCl) elution profiles. As shown in Table II, wild-type recombinant heparinase I eluted at a salt concentration of \(-482\) mM. For CB-1, all mutants except for Gly\(^{213}\) and Thr\(^{216}\) eluted at a higher salt concentration. Interestingly, a direct correlation was observed between the salt concentration eluted and loss of negative charge of carboxyl groups in both CB-1 and CB-2 as shown in Fig. 5. This trend in the elution profile is expected since the interaction between heparinase I and heparin includes both a nonspecific ionic component and a specific heparin-heparinase component (12). For the nonspecific ionic component, neutralizing repulsive negative charges through mutagenesis creates a more favorable interaction, whereas converting a neutral amino acid to alanine has no effect. Most of the CB-2 mutants (T373A, N375A, S377A, G378A, Y379A, T382A, and G378/Y379A), which involve no charge neutralization, eluted at salt concentrations comparable to wild-type recombinant heparinase I (Table II). Therefore, all of the mutations involved in this study resulted in little to no change in the ability of heparinase I to bind heparin specifically, consistent with the earlier observation that heparinase I is able to bind heparin in the absence of calcium (10). Together with the observation that all mutant enzymes retained enzymatic activities to various extents, these results suggest that the structure of the enzyme was unlikely to be perturbed upon site-specific mutagenesis, justifying the comparison of kinetic parameters of mutant enzymes and wild-type \(-L\) recombinant heparinase I.

**Fluorescence Competition**—The results of the kinetic analysis of CB-1 and CB-2 mutants raised the question of whether mutations in CB-2 mediate their effect through decreasing the enzyme activity as a function of calcium concentration, we tested the ability of heparinase I and selected mutants to bind calcium using a fluorescence titration assay. Thus, the rationale for the fluorescence competition study was to determine, using the calci-
um-chelating probe rhod-5N, whether mutations in CB-1 or CB-2 affect the ability of heparinase I to bind calcium. In this study, we expected heparinase I to bind and compete for calcium with the fluorescence probe rhod-5N, and this competition was expected to lower the apparent affinity of rhod-5N for calcium. This decrease was reflected in an increase in the apparent $K_d$ values. Those mutants (E207A/D210A/D212A and D210A/G213A/T216A in CB-1 and N375A, E381A, and G378A/Y379A in CB-2) that showed a significant decrease in enzymatic activity were chosen for the fluorescence competition study.

Table III shows the data derived from the fluorescence competition study. As expected, wild type –L recombinant heparinase I bound calcium and competed the calcium off the fluorescence probe, resulting in a significantly increased $K_d$ value. Triple mutants in CB-1 had a diminished ability to compete for calcium, and only led to a minor increase in $K_d$. The calcium binding ability of the CB-2 mutants lies between that of the wild-type enzyme and the CB-1 mutants, suggesting a moderate reduction in the calcium binding ability. This result confirmed that both CB-1 and CB-2 are involved in calcium binding, albeit differently (see “Discussion”).

**DISCUSSION**

**Calcium Binding to Heparinase I: CB-1 and CB-2**—The data presented in this study confirm that both calcium-binding sites in heparinase I, identified in the preceding paper (19), are involved in calcium binding and enzymatic activity, albeit differently. Site-directed mutagenesis studies in CB-1 identified Asp$^{210}$, Asp$^{212}$, Gly$^{213}$, and Thr$^{216}$ as important residues in calcium binding and enzymatic activity; kinetic studies showed that these corresponding mutants, individual or combined, decreased the $k_{cat}$ value for the degradation of heparin by heparinase I and increased the $K_0.5$ value for calcium (Table II). In addition, examining the $k_{cat}$ and $K_0.5$ values for CB-1 mutants indicated an inverse correlation between the two values, suggesting that these mutants lower the enzymatic activity of heparinase I through decreasing the binding of calcium to the enzyme. Fluorescence studies further confirmed that mutation of these residues to alanines led to a decreased calcium binding affinity in the CB-1 mutant enzymes (Table III). Thus, taken together, these studies show that CB-1 binds calcium and that mutations in CB-1 mediate their effect, either entirely or in part, through decreasing the affinity of heparinase I for calcium.

One important observation of this study is that the latter half of CB-1 (including Gly$^{213}$ and Thr$^{216}$) appears to be more important than its first half (Glu$^{207}$, Asp$^{210}$, and Asp$^{212}$) in calcium binding and enzymatic activity. The $k_{cat}$ values of G213A, T216A, and G213A/T216A gave $k_{cat}$ values of 28, 50, and 19 s$^{-1}$ compared with 92, 74, 65, and $25$ s$^{-1}$ obtained from E207A, D210A, D212A, and E207A/D210A/D212A, respectively. A similar trend was observed for the $K_0.5$ values for calcium binding of these mutant enzymes (Table II).

A second observation derived from this study is that both CB-1 and CB-2 are involved in calcium binding; however, CB-2 plays a more prominent role in heparinase I activity. As shown in the fluorescence competition study, mutations in both CB-1 and CB-2 decreased the calcium binding affinity of heparinase I. On the other hand, mutations in CB-2 (N375A, Y379A, E381A, G378A/Y379A, and E381A/T382A) decreased enzymatic activity drastically ($k_{cat}$ values were decreased by $\sim$10-fold), whereas none of the mutations in CB-1 reduced enzymatic activity by $>$5-fold. Together with the $K_0.5$ data (see below), these results indicate that mutations in CB-2 exert a more pronounced effect on heparinase I, and thereby, the residues in CB-2 mediate their effect on heparinase I activity through interactions that are more complex compared with CB-1.

If CB-2 is involved in calcium binding to heparinase I, why does a mutation in CB-2 result in a decrease in $K_0.5$? One interpretation of these results is that both CB-1 and CB-2 bind calcium; CB-1, which conforms more readily to the calcium-chelating consensus motif, is a high affinity site. On the other hand, CB-2, which conforms less readily to the calcium-chelating consensus motif, is presumably a lower affinity calcium-binding site (Table I). Mutations in CB-1 result in a CB-1 site with decreased affinity for calcium; however, selected mutations in CB-2 completely eliminate its ability to bind calcium. In this case, the $K_0.5$ for CB-2 mutants is reflective of calcium binding to site 1.

This interpretation is consistent with three observations. First, in the fluorescence competition experiments, mutations in CB-2 resulted in an enzyme that was more like wild-type heparinase I as compared with CB-1 mutants in competing calcium away from rhod-5N. This points to the fact that CB-1 binds calcium better than CB-2. Second, there is very little variation in the $K_0.5$ value for the CB-2 mutants, consistent with the hypothesis that any mutation in CB-2 eliminates the ability of CB-2 to bind calcium. Also, the $K_0.5$ value for the CB-2 mutants ($50–90$ $\mu$m) is probably reflective of the affinity of calcium for CB-1. Finally, the heparin binding properties of the CB-2 mutants suggests that other possible effects, including unfolding of the protein, are not likely to occur here. These results, coupled with the biochemical studies of the previous paper (19), point to two sites in heparinase I that bind calcium, a high affinity site (CB-1) and a lower affinity site (CB-2).
Role of Calcium in Heparinase I Activity—Although we have shown that CB-1 and CB-2 of heparinase I are essential for maximum heparinase I activity, it remains to be seen by what mechanism CB-1 and CB-2 mediate a role for calcium in heparinase activity. Since calcium has been best known for its ability to induce a conformational change upon binding to many calcium-binding proteins (16, 17), one would expect calcium to play a similar role in heparinase I. However, CD studies with heparinase I in the presence and absence of calcium revealed no conformational change. In addition, based on our previous study (19), which posited the importance of a ternary complex between heparin, heparinase I, and calcium in terms of the enzymatic activity of heparinase I, it would seem likely that calcium plays a more fundamental role in the enzymatic mechanism of heparinase I.

There are potentially two roles that calcium binding can play in the enzymatic activity of heparinase I. One possibility is that calcium is directly involved in the active-site chemistry of heparinase I. Based on a general mechanism proposed by Gerlt and Gassman (18) for enzyme-catalyzed β-elimination reactions of carboxylic acid-containing substrates, a cation, such as calcium, can interact directly with the anionic carboxylate group of the substrate and sufficiently decrease the pKa of the carbon acid such that abstraction of the α-proton can be achieved by a basic amino acid of the enzyme.

We proposed that, in heparinase I, Cys135 exists as a thiolate anion in the active-site pocket of heparinase I and initiates the abstraction of the C-5 proton of uronate (9). In addition, His203 and Lys199 have been shown to play a role in catalysis (11, 12). In extending the above-mentioned theory of a concerted acid-base catalysis to the β-elimination reaction of heparinase I, the abstraction of the C-5 proton by the base, presumed to be the thiolate anion of Cys135, would require a general acid catalyst acting on the carboxyl group. Ca2+ could potentially satisfy such a requirement by acting as a Lewis acid (Fig. 6). Alternatively, Lys199 could act as an acid catalyst to protonate the carbonyl oxygen in the carboxyl group, and Ca2+ could act to stabilize either deprotonated Lys199 or Cys135 (Fig. 6). The polarization of the carboxyl group by Ca2+ or Lys199 would acidify the α-proton at C-5 and facilitate the abstraction by Cys135 of heparinase I. Histidine could act as a second acid catalyst to protonate the leaving β-substituent.

An additional possible role for calcium is that it can be involved in the processivity of heparinase I and/or modulate the exolytic preference of heparinase I. Recently, we have shown that heparinase I is an exolytic/processive enzyme (13). Thus, heparinase I preferentially cleaves at the nonreducing end of the HLGAG substrate, but instead of releasing the products, heparinase I retains the product, moving to the next scissile bond and clipping the substrate. In this case, the mode of action of heparinase I is characterized as processive, starting from the nonreducing end and clipping the substrate toward the reducing end. Instead of being directly involved in the catalytic mechanism as outlined above, calcium might be involved in “shuttling” the HLGAG substrate through the active-site core of heparinase I without release of the substrate. If calcium is involved in this activity, then the presence of a low affinity calcium-binding site, such as CB-2 in heparinase I, would be essential for proper shuttling of the substrate. Such a role for CB-2 is also consistent with the observation that loss of activity is associated with the production of higher order digestion fragments such as would be formed if the processivity of heparinase I were affected (Fig. 4, A–D). Experiments are in progress to address this issue especially whether calcium is involved in the processivity of heparinase I.

In summary, this study confirms the accompanying biochemical investigation (19) into calcium binding to heparinase I. Furthermore, we have identified key residues in CB-1 and CB-2 that are critical for proper functioning of heparinase I. Within CB-1, the latter half of the calcium-chelating sequence (including Gly213, and Thr216) is more critical for activity than the former half (19). The contribution of selected residues within this sequence affects both enzymatic activity and calcium binding activity by heparinase I. Mutations within the second binding site (CB-2) have a greater effect on the enzymatic activity of heparinase I, arguing for a more pronounced role for CB-2 as compared with CB-1 in the enzymatic activity of heparinase I. It remains to be seen if calcium plays a direct role in the catalytic reaction mechanism or in the exolytic/processive preference of heparinase I, or both.

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Fig. 6. Schematic model of the catalytic domain of heparinase I. Cys135, His203, and Lys199 were identified as catalytically critical residues in previous studies (9, 11, 12). We propose that Ca2+ functions as a Lewis acid acting on the carboxyl group. Lys199 could act as an acid catalyst to protonate the carbonyl oxygen in the carboxyl group. Ca2+ could act to stabilize either deprotonated Lys199 or Cys135. The polarization of the carboxyl group by Ca2+ or Lys199 would acidify the α-proton at C-5 and facilitate the abstraction by Cys135 of heparinase I. Histidine could act as a second acid catalyst to protonate the leaving β-substituent.

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