Studies on the Effect of Calcium in Interactions Between Heparin and Heparin Cofactor II Using Surface Plasmon Resonance

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Summary: Heparin in the mast acetylcholinesterase is the human body and as a result interacts with many extraintracellular species, including ions and proteins, giving rise to myriad bioorganic activities. Heparin cofactor II (HCII) is a serine protease inhibitor that resembles acetylcholinesterase in its ability to be scavenged by heparin. The interaction of heparin with HCII has been the focus of many studies using affinity chromatography and fluorescence spectroscopy. In this study, surface plasmon resonance (SPR) spectroscopy was used to quantitatively measure the interaction of heparin and HCII using a heparin:HCII complex prepared by covalently immobilizing heparin onto a hapten carrier. The results show that the complex interacts with heparin:HCII in a manner that is not calcium-dependent but can be enhanced by the presence of calcium.

Key Words: Heparin—Heparin cofactor II—Interactions—Calcium—Surface plasmon resonance

Over the past few decades, heparin and heparan sulfate have been shown to interact with a number of biologically important proteins, thereby playing an essential role in the regulation of various physiological processes (1,2). The understanding of these interactions at the molecular level is important for the design of new highly specific therapeutic agents. In addition, better knowledge of the specificity of heparin and heparan sulfate will be necessary to understand normal physiologic and pathophysiologic processes. Most heparin-protein interactions involve ion pairing or hydrogen bonding between basic or hydrogen bonding groups on the protein and charged sulfonic or carboxyl groups on heparin (1). Very few heparin-protein interactions are modified through divalent ions such as calcium.

Heparin cofactor II (HCII), a 65.5-kD glycoprotein, is one of several serine protease inhibitors (serpins) that function primarily to inhibit serine proteases that are involved in many normal biologic processes including coagulation, inflammation, wound healing, and tissue repair as well as some pathologic processes, such as atheroatherosclerosis and cancer metastasis (3,4). The thrombin inhibition activity of HCII is accelerated by glycosaminoglycan (e.g., heparin and dermatan sulfate; both of these GAGs bind to HCII and increase the rate of inhibition of thrombin greater than 1000-fold) (3). The interaction of heparin and HCII has been studied by using affinity chromatography, and fluorescence spectroscopy (3,4).

Surface plasmon resonance (SPR) spectroscopy measures interactions in real time and has been successfully applied for quantitative
modeling of heparin-protein interactions (5-9). Recently, Eckert and Ragg reported that Zn\textsuperscript{2+} and to a lesser extent Cu\textsuperscript{2+} and Ni\textsuperscript{2+}—enhanced the interaction between heparin cofactor II and heparin as demonstrated by heparin affinity chromatography and surface plasmon resonance experiments (9). They suggested that Zn\textsuperscript{2+} induces a conformational change in heparin cofactor II that favors its interaction with heparin. Other researchers had previously shown that calcium spirulina (Ca-SP), a novel sulfated polysaccharide isolated from a blue-green alga Spirulina plasunias, enhanced the rate of inhibition of thrombin by HClII. This group also reported that the molecular conformation required for its antithrombin activity is maintained by calcium ions (10).

In the present study, SPR was applied to quantititative study of heparin-HClII interactions. A heparin biochip in which heparin was covalently immobilized through a preformed albumin-heparin conjugate was used in these studies. The interactions of HClII with heparin, low-molecular-weight (LMW) heparin, and heparin oligosaccharides (disaccharide, tetrascaccharide, heptasaccharide) were examined by solution competition SPR, and the influence of calcium on the heparin/HClII interaction was also evaluated.

**MATERIALS AND METHODS**

**Materials**

Heparin cofactor II (HClII, MW: 66,000) was a product purified from human plasma by Diagnostics Stago (France) and showed a single band on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Heparin (MW: 14,000) from porcine intestinal mucosa, sodium salts, was prepared by Cellexus Laboratories (Cincinnati, OH). LMW heparin from porcine intestinal mucosa, sodium salt (MW: 5,000) prepared using nitrous acid was purchased from Calbiochem-Novabiochem Corporation (La Jolla, CA). Heparin lyze I (heparinase, EC 4.2.2.7) was purchased from Sigma Chemical Co. (St. Louis, MO). Gel permeation chromatography was performed on Bio-Gel P3, P6 (superfine) from Bio-Rad (Richmond, CA) or Sephadex G10 from Sigma Chemical Co. (St. Louis, MO). Pioneer Sensor CI Chip, N-hydroxy succinimidyl (NHS) and N-ethyl-N-(dimethylaminopropyl) carbodiimide (EDC) were obtained from BiACore (Bioanalytic AB, Uppsala, Sweden). Bovine serum albumin (BSA) was obtained from Amresco (Solon, Ohio). Albumin-heparin conjugate and other chemicals were obtained from Sigma-Aldrich Co. (St. Louis, MO) and were of the highest purity commercially available. SPR experiments were performed on the BiACore 3000 apparatus operated by BiACore 3000 version software. Buffers used in SPR were filtered and degassed.

**Preparation and Characterization of Heparin-Derived Oligosaccharides**

Heparin (10 g) in 160 mL of 50 mM sodium phosphate buffer (pH 7.0) containing 2 mg/mL BSA was sterile-filtered through a 0.22-μm filter unit. With the addition of heparin lyze I (92 mM), was added twice at 0 and 24 h), the digestion was carried out at 30°C. When the absorbance at 232 nm indicated that the digestion was 30% completed, the digestion was stopped by heating at 100°C for 1 min to inactivate the enzyme. Undigested heparin was removed by ultrafiltration with a membrane (5000 MWCO). Next, the LMW heparin oligosaccharides (MW < 5000) mixture obtained was fractionated on a Bio-Gel P-6 column (4.8 x 100 cm) using 200 mM sodium chloride as the running buffer. Fractions consisting of disaccharides, tetrascaccharides, hexasaccharides, octasaccharides, and higher oligosaccharides were obtained for the further purification.

Before HPLC purification, samples of molecular weight less than 2000 (disaccharide, hexasaccharide, heptasaccharide, and octasaccharides) were desalted on a Sephadex G-10 column. Charge separation of the sized oligosaccharides was then carried out by semi-preparative SAX-HPLC on a 5-μM, 0.46 x 25 cm column (Waters) using a linear gradient from 0.2 to 2 M of sodium chloride (pH 3.5) at a flow rate of 1.0 mL/min. The major peaks were pooled, freeze-dried, and desalted with Sephadex G-10 column. The purified oligosaccharides were analyzed by analytical SAX-HPLC, capillary electrophoresis (CE), gradient PAGE, and H-NMR to confirm the purity and structures of each oligosaccharide.

**Preparation of Heparin Biochip**

Heparin biochip was prepared by covalently immobilizing albumin-heparin conjugate to biosensor surface through its primary amino groups on the CI Chip surface (11). Briefly, carboxymethyl groups on the CI Chip surface were first activated using an injection pulse of 50 μL (flow rate, 5 μL/min) of an equimolar mix of NHS:EDC (final concentration 0.05 M, mixed immediately before
injection). An albumin-heparin solution (200 µg/ml in sodium acetate buffer with 25 mM guanidine hydrochloride, pH 4.0) was applied to the chip surface. Excess unreacted sites on the sensor surface were blocked with a 40 µl injection of 1 M ethanolamine. Successful immobilization was confirmed by the observation of an approximate 300 RU response increase. To prepare the control flow cell, bovine serum was immobilized on the surface using a similar coupling procedure.

Kinetic Measurements of Heparin and HCII Interaction

Different concentrations of HCII in phosphate buffer saline (PBS), (50 mM sodium phosphate buffer, 150 mM NaCl, pH 7.4) was injected over both the albumin-heparin and control albumin surfaces simultaneously at a flow rate of 10 µl/min. At the end of each sample injection, the same buffer was flowed over the sensor surface to facilitate dissociation studies. After a suitable dissociation time (3 minutes), the sensor surface was regenerated with injections of 20 µl of 2 M NaCl, 20 µl glycine (pH 2.6), 20 µl NaOH (pH 10.0), and 20 µl FAS buffer. The response was monitored as a function of time (sensorgram) at 25°C. The control cell was used to subtract the contribution of nonspecific interactions with the immobilized albumin on the surface itself. Kinetic parameters were evaluated using BIA evaluation software (version 3.1, 1999).

Solution Competition Study Between HCII and Heparin, LMW Heparin, and Oligosaccharides

HCII (15 µM) mixed with different concentrations of heparin, LMW heparin, disaccharide, tetrasaccharide, and hexaasccharide (30 µl) were injected over both the albumin-heparin and the control albumin surfaces as a flow rate of 10 µl/min. After each run, the dissociation and the regeneration were performed as described previously. For each set of competition experiments on SPR, a control experiment (only HCII without any heparin or oligosaccharides) was performed to make sure the surface was completely regenerated and that the results obtained between runs were comparable.

Anti-Protease Activity Assay of Heparin Oligosaccharides

The ability of an agent to mediate antiprotease activity in HCII (12) was measured with an amido black assay as described above (14.16). The assays were performed on the ACL fast kinetics analyzer (Hialeah, FL). Test agents (100 µg/ml) were prepared in saline. The control and the blank were set at 0.500 µg/ml human HCII solution was added to the mixture after the agent had reached the assay. The final concentration of HCII in the assay was 8.4 µg/ml. The first reagent consisted of human factor IIa (8.5 NIH units/ml, Enzyme Research Laboratory, South Bend, IN) in buffer (56 mM Tris, 175 mM NaCl, 7.5 mM EDTA, pH 7.5, 25°C). The second reagent consisted of 0.625 mM spectrozyme TH (American Diagnostica Inc., Greenwich, CT). The instrument was programmed so that 500 µl of sample and 50 µl of reagent 1 were placed into individual compartments in a reaction rotor. These reagents were incubated at 37°C for 1 minute and were then mixed together. One hour microtiter plate assay was then added to the rotor and the rotor was spun at 1200 rpm. Optical density readings at 405 nm were made continuously for 1 minute (A405/min). The change in optical density with time was used to calculate percent inhibition of factor IIa.

RESULTS

Sequence Analysis of HCII

EF-hand motifs (Table 1) are among the most well studied calcium binding domains in the proteins (16). The sequence of human HCII (Fig. 1) was searched for the presence of EF-hand motifs. Three sequences (CB-1, 2, and 3) closely matching the EF-hand motifs were found in the HCII sequence (Table 1), suggesting calcium may bind to HCII enhancing its interaction with heparin and possibly affecting its activity against thrombin. The canonical EF-hand consists of an α-helix, a loop wrapped around the Ca2+ ion, and a second α-helix. The number of amino acids in the loop region. The amino acids (Table 1) consist of residues that coordinate to the Ca2+ ion. CB-1 (aa1357-1448), CB-2 (aa1448-430) and CB-3 (aa434-447) of HCII were aligned well with the consensus sequence for calcium-binding EF-hand (14.16). The consensus sequence shown at the bottom of Table 1 lists amino acids (in boldface) that have been observed among various calcium binding proteins by decreasing order of frequency.

Kinetic Measurements of Heparin and HCII Interaction

Sensorgrams for the binding of HCII to immobilized heparin in the absence of calcium ion are shown in Fig. 2. The initial portion of each curve (< 0 seconds) represents buffer flowing past the sensor face. The second and rising part of each
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<th>4</th>
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<tr>
<td>X</td>
<td>Y</td>
<td>Z</td>
<td>G</td>
<td>V</td>
<td>I</td>
<td>X</td>
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**TABLE 1. Alignment of Putative Calcium-Binding Site Amino Acids in HClI with BP-Hand**

- CB-1
- CB-2
- CB-3
- Consensus

- **MKHSLNA** FLITSAWGC SKOPLOLEK CGETAQASDP QWEQLNKN
  LSMPPDPFHKENTVNDWI PEGEDDDDL DELKIFSEDD DYMIDVDSL VSPTDSDVSA GNILQFLHGSRIQRLNIN AKAFANLYRV LKDQVNTFDN IFIAFGVIST AMOMISLQG LTETEQQHISLSKFKNVAS SKYEFTTTHL FRKLITRLRF RNFGYTIRAS VNDLTVQKQI PLILDFTKTVREY YFAEAEQA A DFSDFAFIS KTNINHMKLI KLGIKDALT IDPATTQMMLNCYTFKGSWY VCFFVGMTHN HFEPLNERYK VKVSMNQKTG NFAANFE**DOFLBCHIOLE YV GGGSMJQLVPPOKMSGDKL AQLTLPVRW VQKSMNTRR EVLLIKFLKLE KNYYLVESLK LMGIRMLF**DKNGNMAGISPD RIAI**P**K**H**K**G**T**TV**N**E**F**G**T** QATVTTTVGF MPLSTQVRFT VDRPFLFLYIEHRTSCIFL FRVANSPR**

**FIG. 1.** Sequence of HClI (total 409 amino acids). The CB-1, CB-2, and CB-3 binding motifs are bold and underlined.

curve (10–200 seconds) corresponds to the response of the sensor surface to the injection of a sample containing protein. The final portion of the curves (200–255 seconds) corresponds to the dissociation of bound protein in the sample volume. The peaks at 0 seconds correspond to the injection front and at 200–205 seconds correspond to the change in the RU associated with switching to wash buffer. They are artifacts common to SPR and are not used in the calculation of constants. The dissociation rate constant, $k_d$, was evaluated from each trace and used to calculate the observed association rate constant, $k_a$. The ratio $k_a / k_d$ generates the overall association constant or dissociation constant, $K$. The binding curves are relatively steep in the association phase, which suggests a high affinity of HClI to heparin and a quick association phase. The dissociation curves show slow dissociation, indicating that the binding is strong. Kinetic analysis of the interaction between HClI and heparin afforded a $k_a$ value of $7.37 \times 10^5$ s$^{-1}$, a $k_d$ value of $241$ M$^{-1}$s$^{-1}$, and a $K$ of $3.03 \times 10^3$ M (Table 2).
The Effect of Calcium Ion on Heparin Interaction with HClII

Identical SPR experiments were performed in the presence of 1 mM calcium chloride (Fig. 3). Interestingly, the sensorgrams showed that with calcium ion present the maximum HClII binding (RU) changes were higher for each curve than those obtained in the absence of calcium. Kinetic analysis of the interaction between HClII and heparin in the presence of calcium ions afforded a $K_a$ value of $8.97 \times 10^{-7}$ M, a $k_r$ value of $520 \text{ M}^{-1} \text{ s}^{-1}$, and a $K_c$ of $2.23 \times 10^{-7}$ M. This $K_c$ is nearly 10-fold higher than the $K_c$ obtained in the absence of calcium ions (Table 3). The 10-fold increase in $K_c$ is due mostly to a decrease in $k_r$. The $k_r$ value was not significantly different (only twofold) when determined in the presence and absence of calcium ions.

Solution Competition Studies Using Heparin, 1MW Heparin, and Heparin-Derived Oligosaccharides

To examine the effect of saccharide chain size of heparin in the HClII interaction, solution/surface competition experiments were performed by SPR. In each competition experiment, different amounts of heparin, LMW heparin, and heparin-derived disaccharide and tetrasaccharide and hexa saccharide of defined structures (Fig. 4A) were added in the analyte (HClII) solution. When different concentrations of heparin disaccharide were present in the HClII/heparin interaction solution, no competition effect was observed (not shown). In all other cases, increasing concentrations of competing analytes (heparin, LMW heparin, tetrasaccharide, and hexa saccharide) decreased the observed binding of

### Table 3. Kinetic Data of Heparin and HClII Interaction as Determined by SPR

<table>
<thead>
<tr>
<th>Condition</th>
<th>$K_a$ (s$^{-1}$)</th>
<th>$k_r$ (M$^{-1}$ s$^{-1}$)</th>
<th>$K_c$ (M)</th>
</tr>
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<tbody>
<tr>
<td>Without calcium</td>
<td>$7.37 \pm 2.58 \times 10^{-4}$</td>
<td>$74 \pm 33$</td>
<td>$3.03 \pm 0.89 \times 10^{-4}$</td>
</tr>
<tr>
<td>With calcium</td>
<td>$8.97 \pm 4.27 \times 10^{-3}$</td>
<td>$530 \pm 410$</td>
<td>$2.23 \pm 1.34 \times 10^{-7}$</td>
</tr>
</tbody>
</table>
HClII (Fig. 4B–E). For example, when the concentration of LMW heparin was 10 nM (Fig. 4C), the interaction decreased to approximately 50% of the control value (no competing analyte present). At a concentration of LMW heparin of 100 nM, virtually no binding was observed. The IC_{50} value is commonly defined as the concentration of competing analyte resulting in 50% of response observed in the absence of competing analyte. The IC_{50} values for heparin, LMW heparin, tetrasaccharide, and hexasaccharide were calculated from these curves and given in Table 3.

**Effect of Samples on HClII-Mediated Anti-Thrombin Activity**

First, heparin was examined for its effect on the HClII-mediated anti-thrombin activity in the presence and absence of added calcium chloride. Surprisingly, 1 mM calcium chloride showed no effect on heparin-mediated enhancement of HClII-based inhibition of thrombin. Next, HClII-mediated thrombin inhibition assay of tetrasaccharide, hexasaccharide, and octasaccharide was performed (Table 4). All three oligosaccharides enhanced HClII-based inhibition of thrombin. The octasaccharide showed 35% of heparin's activity.

### Table 3. IC_{50} Values for Heparin, LMW Heparins, Tetrasaccharide, and Hexasaccharide in Solution Competing Surface-Heparin

<table>
<thead>
<tr>
<th>Variable</th>
<th>IC_{50}</th>
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<tbody>
<tr>
<td>Heparin</td>
<td>1 nM</td>
</tr>
<tr>
<td>LMW Heparin</td>
<td>10 nM</td>
</tr>
<tr>
<td>Hexasaccharide</td>
<td>14 μM</td>
</tr>
<tr>
<td>Tetrasaccharide</td>
<td>70 μM</td>
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### Table 4. HClII-Mediated Inhibition of Thrombin by Various Oligosaccharides

<table>
<thead>
<tr>
<th>Sample</th>
<th>O.D.</th>
<th>% Inhibition</th>
</tr>
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<tbody>
<tr>
<td>Control (value)</td>
<td>0.280</td>
<td>0</td>
</tr>
<tr>
<td>Tetrasaccharide</td>
<td>0.225</td>
<td>18.6</td>
</tr>
<tr>
<td>Hexasaccharide</td>
<td>0.250</td>
<td>21.4</td>
</tr>
<tr>
<td>Octasaccharide</td>
<td>0.127</td>
<td>54.6</td>
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</table>
DISCUSSION

HCII selectively inactivates thrombin in a reaction that is accelerated greater than 1000-fold by glycosaminoglycans (GAGs) such as heparin, dermatan sulfate, and heparan sulfate (3). In comparison to antithrombin III (ATIII), HCII features in several important ways (3,43): a) HCII only inactivates thrombin, whereas ATIII inactivates other coagulation enzymes including factors Xa and IXa; b) the high-affinity interaction of heparin with ATIII is mediated by a unique pentasaccharide sequence found only in a subpopulation of heparin molecules. In contrast, heparin does not possess a high affinity sequence for HCII. c) Dermatan sulfate (DS) activates HCII, but has no effect on ATIII. d) The uncatalyzed rate of thrombin inactivation by ATIII is about 10-fold faster than that for HCII; e) HCII possesses a unique 75-amino acid domain at its amino terminus that binds to thrombin exosite I, an interaction analogous to the binding of the carbonyl terminus of hirudin to exosite I. Although the uncatalyzed rate of thrombin inactivation by HCII is slower than that for ATIII, in the presence of heparin or DS, HCII inactivates...
thrombin at a rate similar to that at which ATIII inhibits thrombin, even though heparin is present (4). Three potential modes of GAG-mediated thrombin inactivation by HCII are proposed (4): a) displacement of the amino terminus of HCl, thereby freeing it to interact with thrombin exosite I; b) bridging of exosite II of thrombin to HCII, and c) induction of conformational changes at the reactive site loop of HCII.

In our current study, the real-time SPR kinetics analysis of the interaction between HCII and heparin showed a $K_d$ of $3.03 \times 10^{-8}$ M, which is comparable to HCII concentration of $3.2 \times 10^{-8}$ M in human plasma (3). When there is calcium ion ($1 \text{ mM}$) in the buffer, the measured $K_d$ of $2.23 \times 10^{-8}$ M indicates heparin/HCII interaction is enhanced by calcium ion to approximately 10-fold. Based on our previous acid sequence analysis and modeling on HCII, three putative calcium-binding sites observed in the sequence might be responsible for this interaction. The presence of $1 \text{ mM}$ calcium ion, however, had no effect on the heparin-mediated enhancement of HCII-based inactivation of thrombin. Thus, the true physiologic role of calcium in HCII function is still requires additional investigation. Heparin is also known to bind to calcium (17-19). Further experiments need to be conducted to see whether calcium enhancement of HCII-heparin interaction is due primarily to heparin-calcium or HCII-calcium binding. A recent, very interesting study by Ecken and Ragg suggests that HCII is a zinc binding protein despite the very low plasma concentration of free Zn$^{2+}$ ions (10, 20). They found 12 Zn$^{2+}$, but no Ca$^{2+}$, was able to alter the intrinsic fluorescence of HCII. Furthermore, our experiments show that Ca$^{2+}$ ions did not increase the salt concentration required to elute HCII from a heparin affinity column. These salt elution experiments demonstrate that higher than physiologic concentrations that Na$^{+}$ ions can displace Ca$^{2+}$ ions from HCII bound to heparin.

Calcium might still promote heparin-HCII interaction under physiologic conditions. Furthermore, Ca$^{2+}$ is found in high concentrations in the plasma and is observed in the x-ray structure of HCII (21). Alternatively, Zn$^{2+}$ may be the critical cation involved in promoting heparin-HCII interaction, but based on our observation of three EF-hand motifs, we would suggest a divalent cation-binding site close to the C-terminus of HCII. Clearly, based on our observations and those of Ecken and Ragg (9), additional studies are warranted.

Competition studies using heparin-derived oligosaccharides have shown that the smallest oligosaccharides size competing with heparin binding to HCII was trisaccharide. This is in agreement with results from our laboratory (13) and the HCII-sepharose affinity chromatography results previously reported by Tolleson (15). The variation in IC$_50$ values observed for heparin, LMWH heparin, reteplasecaride, and hexasaccharide confirms that the activation of HCII by heparin is chain-length-dependent, suggesting heparin uses a template mechanism to accelerate the inhibitory process. Furthermore, while very large oligosaccharides are required to bind HCII as tightly as heparin, an octasaccharide showed significant bioactivity.

REFERENCES