Activation of Heparin Cofactor II by Calcium Spirulan*

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Heparin cofactor II (HCII) is a plasma serine protease inhibitor whose ability to inhibit α-thrombin is accelerated by a variety of sulfated polysaccharides in addition to heparin and dermatan sulfate. Previous investigations have indicated that calcium spirulan (Ca-SP), a novel sulfated polysaccharide, enhanced the rate of inhibition of α-thrombin by HCII. In this study, we investigated the mechanism of the activation of HCII by Ca-SP. Interestingly, in the presence of Ca-SP, an N-terminal deletion mutant of HCII (rHCII-D74) inhibited α-thrombin, as native recombinant HCII (native rHCII) did. The second-order rate constant for the inhibition of α-thrombin by rHCII-D74 was 2.0 × 10^9 M^−1 min^−1 in the presence of 50 μg/ml Ca-SP and 10,000-fold higher than in the absence of Ca-SP. The rates of native rHCII and rHCII-D74 for the inhibition of γ-thrombin were increased only 20- and 120-fold, respectively. Our results suggested that the anion-binding exosite I of α-thrombin was essential for the rapid inhibition reaction by HCII in the presence of Ca-SP and that the N-terminal acidic domain of HCII was not required. Therefore, we proposed a mechanism by which HCII was activated allosterically by Ca-SP and could interact with the anion-binding exosite I of thrombin not through the N-terminal acidic domain of HCII. The Arg103 → Leu mutant bound to Ca-SP-Toyopearl with normal affinity and inhibited α-thrombin in a manner similar to native rHCII. These results indicate that Arg103 in HCII molecule is not critical for the interaction with Ca-SP.

Thrombin is considered to be the pivotal enzyme in the coagulation pathway (1). Besides its critical role in hemostasis, thrombin elicits cellular responses through the thrombin receptor that may contribute to inflammation, wound healing, and atherosclerosis (2). Heparin cofactor II (HCII) is an important plasma protein that selectively inhibits thrombin (3). A number of sulfated polysaccharides have been identified as HCII inhibitors, and the binding site of HCII for Ca-SP is neither the heparin- nor dermatan sulfate-binding site (4–6). Based on alignment of the amino acid sequence, HCII contains Arg103 in a position homologous to Arg47 of antithrombin (AT) (21). In the present study, we have demonstrated that the anion-binding exosite I of α-thrombin plays an important role in the stimulation of the thrombin inhibition by Ca-SP.

The results of previous experiments using recombinant variant forms of HCII have suggested that the binding sites for heparin and dermatan sulfate are overlapped in part but are not identical (20–22). Our previous report has indicated that the binding site of HCII for Ca-SP is neither the heparin- (Lys173) nor dermatan sulfate-binding site (24). Based on the alignment of the amino acid sequence, HCII contains Arg103 in a position homologous to Arg106 of antithrombin (AT) (23). Naturally occurring mutations at Arg103 have been discovered in AT, and the mutant molecules lose the abilities to bind to heparin and accelerate the thrombin inhibition by heparin (24). However, it has been reported that Arg103 of HCII does not play a major role in the binding of heparin and dermatan sulfate to HCII (21). In the present study, we have demonstrated that Arg103 in HCII molecule is not required for Ca-SP binding and Ca-SP-dependent thrombin inhibition.

EXPERIMENTAL PROCEDURES

Materials—Human α-thrombin was purchased from Sigma. Human γ-thrombin was from Hematologic Technologies, Inc. (Essex Junction, VT). Heparin (bovine lung) and dermatan sulfate (pig skin) were from Seikagaku Kogyo Co. Ltd. (Tokyo, Japan). Chromogenic substrate S-2238 was from Kabi-Vitrum (Stockholm, Sweden). Calcium spirulan was iso-

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§ The abbreviations used are: HCII, heparin cofactor II; rHCII, recombinant HCII; AT, antithrombin; PAGE, polyacrylamide gel electrophoresis; Ca-SP, calcium spirulan.
lated from *S. platensis* and purified as described previously (25). The purified Ca-SP was homogenous on high performance liquid chromatography analysis and composed of rhamnose, 3-O-methylrhamnose, 2,3-di-O-methylrhamnose, 3-O-methylxylene, uronic acids, and sulfate. The backbone of Ca-SP consisted of 1,3-linked rhamnose and 1,2-linked 3-O-methylrhamnose units with some sulfate substitution at the 4-position.

**Expression and Purification of Reombinant HCII Variants**—We previously described the construction of the vector pYT/WT for the expression of native recombinant HCII (26). Deletion mutation was constructed in native pYT/WT vector using the polymerase chain reaction-based QuikChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). The primers (obtained from Grainer Japan, Tokyo, Japan) used for deletion were as follows, 5'-CAGGAAAACATGTGGCAGACTGTGG-3' and 5'-CACACTGTCGACGAGACCCCTAAGGCTTTGCAG-3'. The obtained deleted plasmid was named pYM74. The native pYT/WT was performed mutagenesis for Arg103 → Leu using the QuikChange Site-directed Mutagenesis Kit; mutagenic primers used in this procedure were 5'-CATGGCAAGGCTGATCAGGCTCTAAC-3' and 5'-GTGAAACGCTCGAACGAGACCCCTAAGGCTTTGCAG-3'. The nucleotides in italics indicate the site of codon substitution. The final constructs were confirmed by sequencing using the chain termination method of Sanger et al. (27). Recombinant HCII was expressed in *Escherichia coli* MV1184 cells and purified from the cell lysates as described previously (10). The purity of the final preparations was assessed by SDS-PAGE on 10% polyacrylamide gels stained with Coomassie Blue. The protein concentration was determined by Bio-Rad Protein Assay Kit.

**Inhibition of Thrombin by HCII**—Thrombin inhibition assay was performed at room temperature in 50 mM Tris-HCl, pH 8.4, 0.15 M NaCl, and 0.1% BSA (assay buffer). The second-order rate constant ($k_2$) of thrombin inhibition by HCII was determined as follows. In the case of $k_2 < 1 \times 10^3 \text{ M}^{-1} \text{s}^{-1}$, HCII (150–600 nM native rHCII or rHCII-74) and thrombin (1.9 nM α-thrombin or 13 nM γ-thrombin) were incubated with or without heparin, dermatan sulfate, or Ca-SP in 1 ml of assay buffer. Samples (each 200 μl) were removed at appropriate intervals, and then 50 μl of 1 mg/ml S-2238 was added. The residual thrombin activity was determined by measuring the change in the absorbance at 405 nm. The apparent first-order rate constants ($k_{\text{app}}$) for thrombin inhibition were determined by fitting the data to the following equation: $k_{\text{app}} = k_2[E_0]/[E]_t$, in which $[E_0]$ is the initial thrombin activity and $[E]_t$ is the thrombin activity at time $t$. The second-order rate constants ($k_2$) were calculated by dividing $k_{\text{app}}$ by the initial concentration of HCII. In the case of $k_2 \approx 1 \times 10^3 \text{ M}^{-1} \text{s}^{-1}$, $k_2$ values for thrombin inhibition by HCII were calculated by a single incubation time point (20 s incubation), as described previously (10). The concentration of HCII used in this experiment was 15 nM.

**Preparation of Ca-SP-immobilized Toyopearl and Ca-SP-Toyopearl Chromatography**—A suspension of 2 g of AF-amino Toyopearl 650M gel (Tohos Corp., Tokyo, Japan) in 2 ml of 0.2 M K$_2$HPO$_4$ containing 60 mg of Ca-SP and 20 mg of NaCNBr$_2$ (Sigma) was incubated at room temperature for 3 days with shaking. After this coupling reaction, the gel was washed with H$_2$O, 0.5 M NaCl/0.1 M NaHCO$_3$, 0.5 M NaCl/acetate buffer, pH 4, and H$_2$O, successively. To remove free amino groups remaining in the gel, the gel was acetylated with acetic anhydride in 0.2 M sodium acetate. The relative affinity of native HCII and Arg103 → Leu mutant for immobilized Ca-SP was determined using the Ca-SP-Toyopearl column. The samples in 50 mM Tris-Cl, pH 7.4, 50 mM NaCl were loaded onto the column and eluted with a linear NaCl gradient of 50 mM to 0.5 M. Fractions of 1 ml were collected and were analyzed by thrombin inhibition assays in the presence of 2.5 μg/ml Ca-SP, as described above.

**RESULTS**

**Inhibition of α-thrombin by the Deletion Mutant of Recombinant HCII in the Presence of Calcium Spirulan**—Native recombinant HCII (native rHCII) and the N-terminal deletion mutant (rHCII-74) were purified from bacterial lysates and subjected to SDS-PAGE (Fig. 1). Each purified rHCII molecule subjected to SDS-PAGE (Fig. 1). Each purified rHCII molecule showed a single band corresponding to the predicted size of the non-glycosylated polypeptide (~60 kDa for native rHCII and 45 kDa for rHCII-74). As reported previously, Ca-SP markedly enhanced the antithrombin activity of HCII (plasma HCII and native rHCII) (10). In the presence of Ca-SP, rHCII-74 inhibited α-thrombin in a dose-dependent manner, whereas it could hardly inhibit α-thrombin in the presence of heparin or dermatan sulfate under the experimental conditions (Fig. 2). We then measured the second-order rate constant for inhibition of α-thrombin by rHCII-74 in the presence of Ca-SP. Fig. 3 shows that rHCII-74 exhibits typical bell-shaped curves for inhibition of α-thrombin in the presence of increasing concentration of Ca-SP. The optimal Ca-SP concentration was 50 μg/ml, which was identical for native rHCII (10). In the Ca-SP concentration, the rate constant for the inhibition of α-thrombin by native rHCII was 4.3 × 10$^3$ M$^{-1}$ s$^{-1}$ (see Table I). The rate constant by rHCII-74 was 2.0 × 10$^3$ M$^{-1}$ s$^{-1}$ and was 10,000-fold higher than in the absence of Ca-SP. In contrast to the effect of Ca-SP, heparin or dermatan sulfate increased only 55- and 4-fold, respectively, at their optimal concentration. The concentration of Ca-SP that gave 50% inhibition of α-thrombin (IC$_{50}$) with rHCII-74 was approximately 0.1 μg/ml, similar to that of native rHCII (Fig. 4). Thus, rHCII-74 behaved in a manner similar to native rHCII in the presence of Ca-SP. These results indicate that Ca-SP-accelerated thrombin inhibition is not mediated through the N-terminal acidic domain of HCII.

**Inhibition of γ-Thrombin by Native Recombinant HCII and the Deletion Mutant in the Presence of Calcium Spirulan**—γ-
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FIG. 3. Ca-SP-accelerated α-thrombin inhibition by rHCII-Δ74. α-Thrombin (1.9 nM) was incubated with 15 nM rHCII-Δ74 for 20 s at room temperature in the presence of increasing concentrations of Ca-SP. The inhibition rate constants (k₂) were measured in triplicate, and the above curve represented the mean values.

Table I
Second-order rate constants (k₂) for inhibition of α-thrombin and γ-thrombin by native rHCII and rHCII-Δ74 in the presence and absence of heparin, dermatan sulfate, or Ca-SP

| Glycosaminoglycan | Native rHCII | rHCII-Δ74 | Native rHCII | rHCII-Δ74 |
|-------------------|--------------|-----------|--------------|-----------|
| No GAG*           | 1.4 × 10⁶    | 1.8 × 10⁶ | 1.4 × 10⁴    | 1.6 × 10⁴ |
| Heparin (25 μg/ml)| 1.9 × 10⁶    | 1.0 × 10⁶ | 1.0 × 10⁵    | 2.0 × 10⁵ |
| DS (250 μg/ml)    | 9.1 × 10⁴    | 7.4 × 10⁴ | 2.9 × 10⁴    | 2.9 × 10⁴ |
| Ca-SP (50 μg/ml)  | 4.3 × 10⁶    | 2.0 × 10⁶ | 1.1 × 10⁶    | 1.9 × 10⁶ |

* Glycosaminoglycan.

** The optimal concentration is shown in parentheses.

FIG. 4. Effects of Ca-SP concentration on α-thrombin inhibition by native rHCII and rHCII-Δ74. α-Thrombin (1.9 nM) was incubated with 15 nM native rHCII (●) or rHCII-Δ74 (○) in the presence of increasing concentrations of Ca-SP. After 5 min, the residual thrombin activity was measured by adding S-2238 and expressed as percentage of the control (in the absence of Ca-SP).

FIG. 5. Ca-SP-Toyopearl chromatography of native rHCII and the Arg₁₀³ → Leu variant. Purified native rHCII or the Arg₁₀³ → Leu variant (30 μg) was applied to a Ca-SP-Toyopearl column and eluted with a linear gradient of NaCl. An aliquot of each fraction was incubated with rHCIIs (20 nM) as indicated in the legend to Fig. 4. ■ native rHCII; ○ rHCII (Arg₁₀³ → Leu). A representative experiment is shown.

FIG. 6. Effects of Ca-SP concentration on the inhibition of α-thrombin by the Arg₁₀³ → Leu variant. Incubations were performed with rHCIIs (20 nM) as indicated in the legend to Fig. 4. ● native rHCII; ○ rHCII (Arg₁₀³ → Leu). A representative experiment is shown.

CII-Δ74 inhibited γ-thrombin at a rate constant similar to that for α-thrombin. These results suggest that, without glycosaminoglycan, native rHCII interacts weakly with anion-binding exosite I. The rate of inhibition of α-thrombin by native rHCII and rHCII-Δ74 increased 3000–10,000-fold in the presence of Ca-SP. By contrast, the rate of inhibition of γ-thrombin was increased only 80–120-fold by Ca-SP. Thus, in the presence of Ca-SP, deletion of the N-terminal acidic domain of HCII does not affect the maximum rate of reaction, but deletion of anion-binding exosite I of thrombin greatly reduces the reaction rate. These experiments indicate that anion-binding exosite I is required for rapid inhibition of α-thrombin by native rHCII and rHCII-Δ74 in the presence of Ca-SP.

Binding to Ca-SP-Toyopearl and Inhibition of α-Thrombin by the Arg₁₀³ → Leu Variant of HCII in the Presence of Calcium Spirulan—In order to investigate the binding site of HCII to Ca-SP, we prepared the Arg₁₀³ → Leu mutant of HCII. Native rHCII and the Arg₁₀³ → Leu mutant were subjected to Ca-SP-Toyopearl chromatography to determine their relative affinities for Ca-SP. Native rHCII and the Arg₁₀³ → Leu mutant were eluted from the column with a peak at 0.2 M NaCl, suggesting that the positive charge of this residue is not necessary for the interaction with Ca-SP (Fig. 5). In the absence of glycosaminoglycan, the Arg₁₀³ → Leu variant had a second-order rate constant for α-thrombin inhibition of 1.6 × 10⁵ M⁻¹min⁻¹ which was similar to that of native rHCII. The effect of Ca-SP
on the α-thrombin inhibition by the Arg103 → Leu variant is shown in Fig. 6. The mutation Arg103 → Leu did not affect the ability of Ca-SP to accelerate the inhibition of α-thrombin. This result is consistent with the data from the affinity chromatography which indicate that the variant binds normally to Ca-SP. Thus, Arg103 in HCII molecule is probably not critical for both Ca-SP binding and Ca-SP-dependent thrombin inhibition.

**DISCUSSION**

It has been known that the major effect of glycosaminoglycan binding to HCII is to promote the inhibition of thrombin allosterically (11). The HCII-glycosaminoglycan complex interacts with thrombin anion-binding exosite I, probably through the N-terminal acidic domain of HCII (14). However, the fact that rHCII-Δ74 inhibits α-thrombin in the presence of Ca-SP indicates that Ca-SP-accelerated thrombin inhibition is not mediated through the N-terminal acidic domain of HCII. The experiments with γ-thrombin indicate that anion-binding exosite I on thrombin plays an important role in the mechanism of inhibition of thrombin by HCII in the presence of Ca-SP. Therefore, we propose that the stimulation of the thrombin-HCII reaction by Ca-SP can occur by the following mechanism. HCII is activated allosterically by Ca-SP, and the HCII-Ca-SP complex interacts with thrombin anion-binding exosite I. This interaction could facilitate the formation of a stable complex by binding the active site of thrombin into approximation with the reactive site of HCII. However, the interaction is independent on the N-terminal acidic domain of HCII.

γ-Thrombin is a proteolytically cleaved form of α-thrombin that has a reduced affinity for hirudin but retains the ability to hydrolyze synthetic peptide substrates and to bind heparin or dermatan sulfate, Ca-SP may form a better template. The binding site of HCII for Ca-SP is not included in the glycosaminoglycan-binding site (the Lys165 to Phe195 region). Furthermore, our previous report has indicated that the binding site of HCII for Ca-SP is neither the heparin- (Lys173) nor dermatan sulfate-binding site (Arg189) (10). AT does not contribute the binding site of HCII for Ca-SP and thrombin inhibition may be superior for the inhibition of clot-associated coagulant activity (33). Since the inhibition of thrombin by HCII activated with Ca-SP does not require the N-terminal acidic domain, Ca-SP-activated HCII may play an important role in the inhibition of fibrin-bound thrombin. Proteolyzed forms of α-thrombin have been proposed to exist in vivo and could participate in either physiological or pathological events (34). As has been shown here, Ca-SP stimulates the inhibition of γ-thrombin by HCII as compared with heparin or dermatan sulfate. Thus, the revelation of the mechanism of activation of HCII by Ca-SP may provide new avenues for development of antithrombotic agents.

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