Heparin and dermatan sulfate activate heparin cofactor II (HCII) comparably, presumably by liberating the amino terminus of HCII to bind to exosite I of thrombin. To explore this model of activation, we systematically substituted basic residues in the glycosaminoglycan-binding domain of HCII with neutral amino acids and measured the rates of thrombin inactivation by the mutants. Mutant D, with changes at Arg184, Lys185, Arg189, Arg192, Arg195, demonstrated a ~130-fold increased rate of thrombin inactivation that was unaffected by the presence of glycosaminoglycans. The increased rate reflects displacement of the amino terminus of mutant D because (a) mutant D inactivates γ-thrombin at a 65-fold slower rate than α-thrombin, (b) hirudin-(54–65) decreases the rate of thrombin inactivation, and (c) deletion of the amino terminus of mutant D reduces the rate of thrombin inactivation ~100-fold. We also examined the contribution of glycosaminoglycan-mediated bridging of thrombin to HCII to the inhibitory process. Whereas activation of HCII by heparin was chain-length dependent, stimulation by dermatan sulfate was not, suggesting that dermatan sulfate does not utilize a template mechanism to accelerate the inhibitory process. Fluorescence spectroscopy revealed that dermatan sulfate evokes greater conformational changes in HCII than heparin, suggesting that dermatan sulfate stimulates HCII by producing more effective displacement of the amino terminus.

Heparin cofactor II (HCII), a serpin found in human plasma at a concentration of 1.2 μM, selectively inactivates thrombin in a reaction that is accelerated >1000-fold by glycosaminoglycans (GAGs) such as heparin, dermatan sulfate, and heparan sulfate (1). A second serpin, antithrombin (AT), also inactivates thrombin but differs from HCII in four important ways. First, whereas HCII only inactivates thrombin, AT inactivates other coagulation enzymes including factors Xa and IXa (2). Second, the high affinity interaction of heparin with AT is mediated by a unique pentasaccharide sequence found only in a subpopulation of heparin molecules (3–5). In contrast, heparin does not possess a high affinity sequence for HCII (6). Furthermore, dermatan sulfate (DS), a GAG found in the extracellular matrix of connective tissue (7, 8), activates HCII, but has no effect on AT (1). Third, the uncatalyzed rate of thrombin inactivation by AT is about 10-fold faster than that for HCII, probably reflecting differences in the amino acid residue at their P-1 position, with AT containing an Arg residue and HCII a Leu (9). Fourth, 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 carboxyl terminus of hirudin to exosite I (1).

Although the uncatalyzed rate of thrombin inactivation by HCII is slower than that for AT, in the presence of heparin or DS, HCII inactivates thrombin at a rate similar to that at which AT inactivates thrombin when heparin is present (1, 10). The current model to explain GAG-mediated catalysis of HCII inactivation of thrombin suggests that binding of polyanionic GAGs to the electropositive GAG-binding domain on HCII disrupts the intramolecular ionic interaction between the amino-terminal acidic domain of HCII and basic residues in the GAG-binding domain (11–13). Once the amino terminus of HCII is no longer conformationally restrained, the region encompassing residues 54–75 (14) interacts with exosite I on thrombin, thereby facilitating enzyme-inhibitor complex formation.

Release of the amino-terminal domain upon GAG binding to HCII is believed to account for most, but not all, of the stimulatory effect of heparin or DS. Studies with exosite II variants of thrombin with reduced heparin affinity suggest that some acceleration in the rate of thrombin inactivation results from simultaneous binding of heparin to exosite II on thrombin and the GAG-binding domain on HCII (13, 15). In this way, heparin acts as a template for surface approximation of enzyme and inhibitor, analogous to its role in catalysis of AT-mediated inactivation of thrombin (16). Whether DS also serves a template function is unclear. In addition to inducing conformational changes in the amino-terminal domain of HCII, DS also may evoke allosteric changes in the reactive site loop or elsewhere because it produces a 3-fold increase in the rate of thrombin inactivation by an HCII variant lacking the amino-terminal domain (12).

The current model of thrombin inactivation by HCII reveals three potential modes of GAG-mediated activation: displacement of the amino terminus of HCII, thereby freeing it to...
interact with thrombin exosite I; bridging of exosite II of thrombin to HClII; and induction of conformational changes at the reactive site loop of HClII. To examine the relative importance of each of these mechanisms and to explore the possibility that heparin and DS have different modes of action, we first eliminated the GAG dependence of the HClII inactivation reaction by substituting basic residues in the GAG-binding domain of HClII with neutral amino acids. By measuring the rates of thrombin inactivation by these HClII mutants, we determined the importance of binding of the amino-terminal domain of HClII to exosite I on thrombin in isolation from other GAG-induced effects. To examine the contribution of GAG-mediated bridging of HClII to exosite II on thrombin, we compared the effect of high and low molecular weight heparin and DS fractions on the rates of thrombin inactivation by HClII. Finally, to explore the possibility that GAGs elicit conformational changes at the reactive site loop of HClII, we measured the effect of heparin and DS on the rate of thrombin inactivation by an HClII variant lacking the amino-terminal domain.

**EXPERIMENTAL PROCEDURES**

**Materials—**Oligonucleotides were synthesized by the Institute for Molecular Biology and Biotechnology at McMaster University, Hamilton, ON, Canada. Human HClII and AT, isolated from plasma by affinity chromatography, and monospecific polyclonal IgG against human HClII and human AT were from Affinity Biotechnologies Inc. (Hamilton, ON). Polyclone was obtained from Aldrich (Milwaukee, WI). Heparin, hirudin-(54–65), antithrombin III, factor Xa, factor IXa, factor XIa, and dithiothreitol, 0.5 mM of each deoxynucleotide, 37.5 units of RNAguard transcriptase. cDNA synthesis was performed at 42 °C for 60 min. The mixture on ice. The volume was brought to 20 

60–18 (29), a 29-nucleotide single-stranded DNA aptamer that interacts with heparin to HCII. Heparin or DS was present at 3 μM. All reactions were terminated by the addition of 10 mM of each deoxynucleotide, 5 mM EDTA, 0.1 M NaCl, pH 7.4 (TBS). Bound protein was eluted with 200 

ml of TBSP containing 10 mg/ml Polybrene. Residual thrombin activity was calculated by measuring absorbance at 405 nm for 5 min using a Molecular Devices plate reader. The second-order rate constant (k2) for thrombin inactivation was determined by fitting the data to the equation, 

\[
k_2 \ln([P_i]/[P_f]) = \ln k_2 t + \ln k_1,
\]

where \([P_i]\) is initial thrombin activity and \([P_f]\) is thrombin activity at time \(t\) (16). The second-order rate constant, \(k_2\), was then calculated by dividing \(k_1\) by the HClII concentration.

**Heparin-Sepharose Affinity Chromatography—**Heparin-Sepharose affinity chromatography was used to compare the binding of the HClII variants to heparin. 0.2 ml of purified HClII, at a concentration of 10 μg/ml, was batch adsorbed with 0.2 ml of heparin-Sepharose resin for 1 h at 4 °C. Adsorbed proteins were eluted in a stepwise fashion with 1 ml of 20 mM HEPES, pH 7.4, 0.1% polyethylene glycol 8000 containing NaCl in concentrations ranging from 30 mM to 1 M. Aliquots from the flow-through and eluates were analyzed by SDS-PAGE followed by immunoblotting with sheep anti-HClII antibody. Protein elution profiles were obtained by laser densitometry scans of immunoblots using the UltraScanXL TM XL laser densitometer (Amersham Pharmacia Biotech). To verify the sequence of the HClII cDNA (21) and the authenticity of the variants.

**Rates of Thrombin Inactivation by Wild-type and Variant HClII—**The second-order rate constants (k2) for inactivation of thrombin by the various HClII variants were determined under pseudo-first-order conditions. Heparin and/or DS (25). DNS-HCII was prepared by reacting HCII with a 2-fold molar excess of DNS-Cl in 0.1 M Na2HPO4, pH 8.0, as described (25).
DNS-HCII had activity similar to that of native plasma HCII (pHCII) as assessed by measuring the second-order rate constants for thrombin inactivation both in the absence and presence of GAGs. Fluorescence studies were performed on 2 ml of 50 mM DNS-HCII in a 1 × 1-cm quartz cuvette using a Perkin-Elmer LSS0B luminescence spectrometer. The temperature of the cuvette was maintained at 33 °C with a circulating water bath connected to the cell holder and the sample was stirred continuously with a magnetic stirrer. Excitation and emission wavelengths were set to 335 and 520 nm, respectively, with excitation and emission slit widths of 15 and 20 nm, respectively, and an emission filter of 430 nm. After readings were taken of DNS-HCII alone (I₀), known quantities of either heparin or DS were then added to the cuvette and while stirring, the change in fluorescence was monitored (I). Kₑ values were calculated by plotting I/I₀ versus GAG concentration. The parameters Kₑ and α were calculated by nonlinear regression using the equation, 

\[ \frac{I}{I_0} = \left( \frac{1}{K_e + [\text{GAG}]/[\text{DNS-HCII}]} \right) \left( 1 + \frac{[\text{GAG}]/[\text{DNS-HCII}]^2}{(1 + \frac{[\text{GAG}]/[\text{DNS-HCII}]^2}{a^2} + 1) \cdot (a^2 + 1)} \right) \]

where α is the maximum fluorescence change and assuming a stoichiometry of 1:2 (26).

The association of pHCII with heparin or DS also was monitored by the GAG-dependent intrinsic fluorescence intensity change of HCII. Fluorescence of 2 ml of 1 μM pHCII was monitored with excitation and emission wavelengths set to 280 and 340 nm, respectively, excitation and emission slit widths set to 6 nm, and an emission filter of 290 nm. Known quantities of either heparin or DS were then added to the cuvette and the change in fluorescence was monitored. The Kₑ values were calculated by plotting I/I₀ versus GAG concentration, as described above.

RESULTS

Purification of HCII Variants—Human HCII is a 480-amino acid, single-chain glycoprotein with a molecular mass of ~66 kDa (27). The functional domains of HCII and the amino acid sequence of its GAG-binding domain are shown schematically in Fig. 1. The GAG-binding domain of HCII has been identified by sequence homology with AT and by analysis of natural (28) and recombinant (11, 29, 30) variants of HCII. In this study, wild-type (wt) human HCII cDNA was cloned from HepG2 cells and site-directed mutagenesis was used to generate recombinant HCII molecules with Arg₁₈₄, Lys₁₈₅, Arg₁₉₀, and Arg₁₉₃ replaced with Gln, and Arg₁₉₃ replaced with Asn, neutral residues previously shown to reduce the affinity of HCII for heparin-Sepharose (11). The recombinant HCII (rHCII) variants, denoted Mut A, B, C, and D, possess 2, 4, or 5 mutations and are listed in Table I. In addition, the 74-amino-terminal residues were deleted from wild-type (wt-del74) and Mut D (Mut D-del74) HCII. Sequence analysis was used to verify the authenticity of the wild-type (21) and mutated sequences. cDNAs encoding the wild-type and variant forms of HCII were expressed in BHK cells. The apparent molecular masses of the recombinant proteins, as determined by SDS-PAGE and immunoblot analysis, are consistent with their predicted molecular masses (data not shown).

Heparin-Sepharose Affinity Chromatography of GAG-binding Domain Variants—To compare the heparin binding properties of wt-rHCII with the rHCII variants, the proteins were subjected to heparin-Sepharose affinity chromatography and eluted with increasing concentrations of NaCl. As shown in Table I, both pHCII and wt-rHCII are retained on the heparin-Sepharose column at NaCl concentrations up to 180 mM. Mutation of positively charged residues at positions 184 and 185 (Mut A) or at positions 192 and 193 (Mut B) reduces the NaCl concentration necessary for elution to 130 and 150 mM, respectively. Mutation of positively charged residues at positions 184, 185, 192, and 193 (Mut C) or at positions 184, 185, 189, 192, and 193 (Mut D) in the GAG-binding domain further reduces the NaCl concentration needed for elution to 100 mM.

Since the amino-terminal acidic domain of HCII is believed to bind intramolecularly to the basic GAG-binding domain, deletion of the 74-residue amino-terminal acidic (del74) domain should unmask the GAG-binding domain, thereby increasing the binding of HCII to heparin. This concept is supported by the observation that the NaCl concentration required to elute wt-rHCII-del74 is 3.8-fold higher than that needed for wt-rHCII (700 and 180 mM NaCl, respectively; Table I). In contrast, to elute Mut D-del74, which not only lacks the amino-terminal acidic domain but also has mutations in the GAG-binding domain, only a 1.4-fold higher NaCl concentrations is needed (250 mM). These findings corroborate previous results that the heparin-binding domain is unmasked upon deletion of the amino-terminal 74 residues (12). However, since Mut D-del74 is retained on the heparin-Sepharose column at higher concentrations of NaCl than is wt-rHCII, it is possible that additional residues may be involved in heparin binding. A potential candidate is Lys¹⁷³, a residue that has been shown to contribute to binding of heparin, but not DS (31).

Thrombin Inactivation by HCII GAG-binding Domain Variants—The second-order rate constants for the inactivation of thrombin by the affinity-purified HCII mutants were determined in the absence of presence of 5 μM heparin or DS under pseudo first-order conditions (Fig. 2). This concentration of heparin and DS was chosen because, in preliminary studies, it produced maximal stimulation of thrombin inactivation by pH-CII (data not shown). Mut A and B did not display elevated rates of thrombin inactivation in the absence of GAG, likely reflecting only partial disruption of the GAG-binding domain. Our results with Mut A and Mut B differ from those of Ragg et al. (11) in which their double point mutants exhibited slightly enhanced levels of thrombin inhibitory activity in the absence of GAGs as analyzed by SDS-polyacrylamide gels. This may reflect differences in the expression systems because we used BHK cells whereas Ragg and colleagues (11) used COS cells.
Alternatively, endogenous GAGs may account for the increased activities reported by Ragg et al. (11) because their recombinant HCII variants were obtained directly from conditioned media without subsequent purification steps. In contrast, in the current study, mutants were isolated by immunoaffinity chromatography. When this step was omitted, we also detected increased thrombin inhibitory activity in some instances.

The rate of thrombin inactivation by Mut A is not increased by heparin or DS addition. In contrast, the rate of thrombin inactivation by Mut B increases 470-fold (from $5.1 \times 10^5$ m$^{-1}$ min$^{-1}$ to $2.4 \times 10^7$ m$^{-1}$ min$^{-1}$) in the presence of heparin, but only 5-fold in the presence of DS (from $5.1 \times 10^4$ m$^{-1}$ min$^{-1}$ to $2.4 \times 10^5$ m$^{-1}$ min$^{-1}$). These findings are consistent with various reports and highlight the observations that Arg189 and Arg183 are key contributors to DS, but not heparin, binding to HCII, whereas Arg184 and Lys185 are important for the binding of both heparin and DS (11, 30). The observation that, in the absence of GAGs, Mut A and B inactivate thrombin at rates similar to those of wt-HCII and pHCII indicates that limited mutation in the GAG-binding domain is insufficient to release the amino-terminal domain from its intramolecular interactions. Consequently, we focused on the variants with more extensive mutations.

The rates of thrombin inactivation by Mut C and Mut D in the absence of GAG are $6.2 \times 10^6$ m$^{-1}$ min$^{-1}$ and $6.0 \times 10^6$ m$^{-1}$ min$^{-1}$, respectively, values that are about 140-fold higher than those of pHCII and wt-rHCII (3.9 $\times 10^4$ m$^{-1}$ min$^{-1}$ and 4.6 $\times 10^4$ m$^{-1}$ min$^{-1}$, respectively). Neither heparin nor DS addition significantly increases the rate of thrombin inactivation by Mut C or Mut D. By contrast, the rates of thrombin inactivation by pHCII and wt-rHCII increase 2000–4000-fold in the presence of these GAGs. Although Mut C and Mut D displayed similar rates of thrombin inactivation and binding to heparin-Sepharose, Mut D was selected for detailed analysis because it also has Arg189 mutated to Gln and this residue has been proposed to contribute to GAG binding (31).

Substrate Specificity of Mut D—Based on immunoblot analyses, Mut D forms SDS-stable complexes with thrombin both in the absence and presence of heparin or DS (not shown). In contrast, under the same conditions, pHCII and wt-rHCII only form enzyme-inhibitor complexes in the presence of either GAG. Mut D retains its selectivity for thrombin, and like pHCII and wt-rHCII, does not form complexes with factors IXa, Xa, or XIa.

Elucidation of the Mechanism of Action of Mut D—To determine whether the increased thrombin inhibitory activity of Mut D in the absence of GAGs reflects interaction of its aminoterminal acidic domain with exosite I on thrombin, two sets of experiments were performed. First, we compared the rates at which Mut D inactivates $\gamma$-thrombin, a proteolytic derivative of thrombin lacking exosite I, and RA-thrombin, a recombinant thrombin variant containing three point mutations in exosite II that result in a 20-fold decrease in heparin affinity (18). We chose these thrombin variants because previous studies with $\gamma$-thrombin and RA-thrombin have demonstrated a strict requirement for binding of pHCII to exosite I, but not exosite II, on thrombin, even in the presence of GAG (Fig. 3A) (12, 15, 32, 33). As shown in Fig. 3B, Mut D inactivates $\gamma$-thrombin at a
66-fold slower rate than thrombin (6.8 × 10^4 M^-1 min^-1 versus 6.5 × 10^4 M^-1 min^-1, respectively). In contrast, the rate of inactivation of RA-thrombin by Mut D is similar to that of thrombin. Second, we examined the effect of hirudin-(54–65) on the rate of thrombin inactivation by Mut D. This peptide interacts with exosite I of thrombin (34) and it has been shown previously to slow the rate of thrombin inactivation by pHCII in the presence of GAGs (12). The addition of 20 μM hirudin-(54–65) produces a 33-fold decrease in the rate of thrombin inactivation by Mut D (from 6.5 × 10^6 to 4.3 × 10^4 M^-1 min^-1). These results highlight the importance of thrombin exosite I in mediating the increased thrombin inhibitory activity of Mut D.

To demonstrate that the increased basal rate of thrombin inactivation by Mut D reflects release of its amino-terminal acidic domain from intramolecular interactions, we examined the ability of Mut D-del74, a variant of Mut D that lacks the amino-terminal acidic domain, to inactivate thrombin in the absence and presence of GAGs. As shown in Fig. 2, the rate of thrombin inactivation by Mut D-del74 is 109-fold lower than that for Mut D (5.5 × 10^4 M^-1 min^-1 and 6.5 × 10^6 M^-1 min^-1, respectively). Like Mut D, neither heparin nor DS increases the rate of thrombin inactivation by Mut D-del74. In contrast, heparin produces a 5-fold (from 8.8 × 10^4 M^-1 min^-1 to 4.7 × 10^5 M^-1 min^-1) increase in the rate of thrombin inactivation by wt-del74 rHCII, which possesses an intact GAG-binding domain. Unlike heparin, DS has no effect on the rate of thrombin inactivation by wt-del74 rHCII (Fig. 2), even when the DS concentration is increased from 3 to 30 μM (data not shown).

**Contribution of the Template Mechanism to the GAG-catalyzed Inhibitory Process**—To assess the importance of GAG-mediated bridging of HCII to thrombin, we examined the rates of thrombin inactivation as a function of heparin or DS chain length. As shown in Table II, heparin fractions of 6 kDa or less (which correspond to approximately 30 and 60 saccharide residues in HCII) increase the rate of thrombin inactivation as a function of heparin or DS chain length. As shown in Table II, heparin fractions confirm previous reports that the minimum molecular mass of heparin required for catalysis via the template mechanism is between 6 and 9 kDa (6, 35). The data with DS suggest that GAG-mediated bridging does not play a role in DS-mediated catalysis of thrombin inactivation by HCII.

We also examined the ability of HD22, a single-stranded DNA aptamer that binds exosite II of thrombin (20), to compromise the GAG-catalyzed inhibitory process. In confirmation of its specificity for exosite II, thrombin-bound FITC-HD22 (K_D of 10 nM) is displaced by DS or heparin (data not shown). HD22 produces a concentration-dependent, 10-fold reduction in the rate of thrombin inactivation by HCII in the presence of 120 nM heparin (K_D of 6 nM) (Fig. 5). In contrast, the aptamer has no effect on the rate of inactivation in the presence of either 1 μM (Fig. 5) or 3 μM (not shown) DS. These findings support the concept that, unlike heparin, DS does not serve a template function.

**Effect of GAG Binding on the Conformation of HCII**—To explore the possibility that DS and heparin evoke distinct structural changes in HCII, we labeled pHCII with DNS-Cl, a sensitive probe of protein conformation (36). As shown in Fig. 5A, titration of DNS-pHCII with DS results in a 6% decrease in fluorescence intensity, an indication of an increase in solvent hydrophilicity around the fluorophore. In contrast, when DNS-pHCII is titrated with heparin, there is only a minor 1% decrease in the fluorescence intensity. Based on nonlinear regression analysis of the binding curve, DS binds to HCII with a K_D value of 5.1 μM.

We also compared the fluorescence emission of tryptophan residues in HCII in the presence of GAGs with that in their absence (Fig. 5B). DS addition to 1 μM pHCII results in a decrease in protein fluorescence, with a maximum decrease of 13% at DS concentrations of ≥150 μM. As observed for DNS-HCII, heparin at concentrations up to 250 μM failed to significantly change the fluorescence intensity of pHCII. Based on analysis of these results, DS binds to pHCII with a K_D of 25.3 μM. Although these findings do not identify which of the four tryptophan residues is responsive to DS binding, nor the nature of the conformational changes, they are consistent with the hypothesis that the allosterically transmitted conformational changes evoked by DS and heparin binding are distinct.

**TABLE II**

| Heparin Fraction | k_D (10^6 M^-1 min^-1) |
|------------------|------------------------|
| 4 kDa            | 2.4 ± 0.6 × 10^7       |
| 6 kDa            | 2.0 ± 0.8 × 10^7       |
| 9 kDa            | 4.5 ± 0.5 × 10^4       |
| 18 kDa           | 1.2 ± 0.1 × 10^8       |
| 2.4 ± 0.1 × 10^8 |
| 1.7 ± 0.2 × 10^7 |
| 5.6 ± 0.8 × 10^4 |
| 6.9 ± 1.7 × 10^5 |
| 4.7 ± 1.5 × 10^5 |
Heparin-catalyzed inactivation of thrombin by pHCII. The rates of inactivation of 10 nM thrombin by 100 nM pHCII in the presence of 2 μg/ml heparin (●) or 20 μg/ml DS (○) were determined in the presence of increasing concentrations of the HD22 aptamer. The data represent the mean of two determinations and the bars signify the S.E.

The discrepancy of the $K_d$ values for DS obtained by these two analyses may reflect the different conditions under which the experiments were performed or the fact that different reporter groups were monitored.

DISCUSSION

The current model of the mechanism of action of HCII suggests that the amino-terminal acidic domain, which is freed from intramolecular interactions upon GAG binding, interacts with exosite I on thrombin (1). The essential role of the amino-terminal domain of HCII in the inhibitory process has been revealed through deletion or mutation of this region (11, 12). Although previous work has identified individual residues constituting the GAG-binding domain (11, 28, 30), in this study we attempted to neutralize the GAG-binding domain with the aim of rendering HCII GAG independent. We have demonstrated that charge negation at residues 184, 185, 189, 192, and 193 (Mut D) enables HCII to react over 100 times more efficiently with thrombin, without the participation of a GAG.

Although interaction of the amino-terminal domain of HCII with exosite I on thrombin is considered to be the requisite step in the inactivation reaction, by analogy to other serpins GAGs may also bridge the inhibitor to the enzyme or induce conformational changes in the reactive site loop of the inhibitor. To address these possibilities, we used low molecular weight fractions of heparin and DS to examine the extent to which GAG-mediated bridging of HCII to thrombin contributes to the inhibitory process. We also used fluorescence studies to determine whether heparin and DS evoke distinct structural changes when they bind to HCII. Because our studies address different aspects of the mechanism of action of HCII, each will be discussed individually.

Role of Amino-terminal Domain—Three lines of evidence suggest that the increased thrombin inhibitory activity of Mut D reflects release of the amino-terminal acidic domain from intramolecular interactions, enabling the domain to bind to thrombin exosite I. First, in the absence of GAGs, Mut D exhibits a 140-fold elevated rate of inactivation of thrombin, but only a 2-fold increase with γ-thrombin, a trypsinized derivative of thrombin that lacks exosite I. Since γ-thrombin displays normal reactivity with AT (32) but not Mut D, the possibility that the mutations introduced into Mut D conformationally activates its reactive site loop can be elimi-

nated. That exosite II does not contribute to the elevated activity of Mut D is demonstrated by its comparable rates of inactivation of native thrombin and RA-thrombin, a thrombin variant with three point mutations in exosite II that endow it with a 20-fold lower affinity for heparin (18). Second, the rate of thrombin inactivation by Mut D is decreased in the presence of hirudin-(54–65), an analogue of the carboxyl terminus of hirudin that binds exosite I of thrombin (34). Previous studies have shown that this peptide reduces the rate of inactivation of thrombin by pHCII, but not by AT, revealing its specificity for exosite I (12, 33). Third, deletion of the amino-terminal domain of Mut D (Mut D-del74) reduces its rate of inactivation of thrombin to that exhibited by pHCII with native thrombin in the absence of GAG or by Mut D with γ-thrombin. Furthermore, the extent of stimulation of wt-del74 by heparin or DS is over 200-fold less than that of wt-rHCII. These studies, therefore, provide independent confirmation that the amino-terminal domain makes a significant contribution to the inhibitory mechanism of HCII. Moreover, they indicate that release of the amino-terminal domain alone is insufficient to fully promote inactivation of thrombin by HCII, suggesting that GAG binding to HCII may stimulate its inhibitory activity through additional mechanisms.

Role of Heparin Bridging—Numerous studies have demonstrated a dose-dependent reduction in the maximal rate of thrombin inactivation at high concentrations of heparin, consistent with a template mechanism whereby heparin bridges thrombin to HCII (32, 33, 37, 38). Notably, a deletion mutant lacking the ability to bind to exosite I via the amino-terminal domain (wt-del71) displays a biphasic heparin stimulation response (12). Our studies, however, suggest that heparin bridging makes a relatively minor contribution to the overall catalysis of the thrombin inhibitory reaction provided by heparin. This is revealed by the demonstration that thrombin inactivation by wt-del74 is stimulated by heparin only 6-fold and by the finding that the exosite II-binding aptamer reduces the magnitude of heparin catalysis by only 10-fold. In contrast, DS does not serve a template role since it does not stimulate wt-del74-mediated inactivation of thrombin and DS catalysis of HCII is not affected by the exosite II aptamer.

Further support for a template mechanism involving heparin is the observation that the minimal heparin chain length required for catalysis of inactivation by wt-del74 is between 20 and 30 saccharide units (which corresponds to a molecular mass between 6 and 9 kDa), a chain-length requirement comparable to that observed for pHCII (Fig. 4, Table II). These findings are consistent with the results of other investigators who demonstrated that only heparin chains comprised of 24 or more saccharide units produce maximal catalysis of thrombin inactivation by HCII (6, 35).

The mutations introduced into the GAG-binding domain of Mut D reduce its affinity for heparin so that GAG-mediated templating cannot occur. Consequently, we postulate that the 140-fold increase in the basal rate at which Mut D inactivates thrombin reflects displacement of the amino terminus of HCII similar to that induced by the binding of shorter heparin chains to native HCII. In support of this concept, the rate of thrombin inactivation by Mut D in the absence of GAG is only 2.8-fold slower than the rate at which wt-rHCII inactivates thrombin in the presence of a 6-kDa heparin fraction ($6.0 \times 10^5$ M$^{-1}$ min$^{-1}$ and $1.7 \times 10^7$ M$^{-1}$ min$^{-1}$, respectively), a heparin chain that is too short to bridge HCII to thrombin.

Allosteric Effects—In contrast to heparin, DS appears to accelerate thrombin inactivation by HCII exclusively through induction of allosteric changes in the amino-terminal acidic domain because (a) low molecular weight DS fractions increase
the rate of thrombin inactivation by pHCII to the same extent as unfractionated DS (Fig. 4), and (b) unfractionated DS, comprised of more than 30 saccharide units, does not increase the rate of thrombin inactivation by wt-del74 rHCII (Fig. 2). Whereas unfractionated DS, desmin, and low molecular weight fractions of desmin increase the rate of thrombin inactivation by pHCII > 1000-fold, Mut D inactivates thrombin at a rate only 130-fold greater than the basal rate of thrombin inactivation by pHCII. These observations raise the possibility that the allosteric changes induced by the binding of unfractionated and low molecular weight DS to HCII are more extensive than those produced by heparin or by the mutations introduced into the GAG-binding domains of Mut D. This concept is supported by the observation that a reactive site HCII variant with a Leu$^{444}$ → Arg mutation is stimulated by DS to a greater extent than by heparin (15, 39). Further support comes from our findings that the heparin- and DS-binding sites on HCII are not identical. Mutation of Arg$^{192}$ and Arg$^{193}$ to Gln and Asn, respectively (Mut B), decreases the stimulatory activity of DS, as unfractionated DS (Fig. 4), and (b) inactivation (Fig. 2). In contrast, substitution of Arg$^{184}$ and Lys$^{185}$ with Gln residues (Mut A) abolishes the ability of both GAGs to enhance thrombin inactivation.

The results of fluorescence spectroscopy studies (Fig. 6) support the concept that heparin and DS induce different conformational changes upon binding to HCII. When DNS-pHCII is titrated with DS or heparin, the changes in extrinsic fluorescence evoked by DS are greater than those produced by heparin. Likewise, when intrinsic fluorescence is monitored, titration with DS also produces greater changes than titration with heparin. These findings are consistent with our hypothesis that the conformational changes in the amino terminus evoked by DS optimize its interaction with exosite I on thrombin to a greater extent than those induced by heparin. Differences in the allosteric changes in the amino-terminal acidic domain induced by unfractionated or low molecular weight DS relative to heparin may reflect the more extensive contacts that the former GAGs make with HCII.

The fact that neither DS nor desmin accelerates thrombin inactivation by wt-del74 rHCII makes it unlikely that these GAGs induce major conformational changes at the reactive center of HCII that render the Leu$^{444}$-Ser$^{445}$ peptide bond a more favorable site for thrombin cleavage. Therefore, GAG-induced conformational activation of the reactive site loop, while contributing significantly to the inactivation of factor Xa by AT (40), may serve a lesser role with HCII. This is corroborated by reports that heparin produces little stimulation in the rate of inactivation of γ-thrombin (32, 33) or chymotrypsin (41) by HCII. Furthermore, because conversion of Leu$^{444}$ to Arg makes HCII 100-fold more efficient at inactivating thrombin (15, 39), it is possible that the reactive site loop of HCII is in a more accessible conformation than that of AT. These observations suggest that displacement of the amino-terminal domain is the predominant mechanism by which HCII is allosterically activated by GAGs.

The results of this study advance our knowledge in a number of important ways. First, we have identified the residues in HCII that physically impair the ability of its amino terminus to ligate exosite I on thrombin in the absence of GAGs. Charge negation of these residues eliminates the GAG dependence for thrombin inactivation, presumably by releasing the amino terminus from intramolecular ionic bonds. Second, we explored the extent to which heparin and DS utilize the allosteric and template mechanisms in the catalysis of enzyme-inhibitor complex formation. Our findings suggest that DS activates HCII exclusively through release of the amino terminus. In contrast, whereas most of the stimulatory effect of heparin is mediated by the amino terminus of HCII, heparin also serves a template function by simultaneously interacting with the GAG-binding domain of HCII and exosite II on thrombin. Third, release of the amino-terminal domain of HCII, through charge neutralization at residues 184, 185, 189, 192, and 193, is insufficient to fully stimulate inactivation of thrombin. These findings raise the possibility that there are additional intramolecular interactions that constrain the amino terminus. One candidate is Arg$^{200}$, since its mutation to Gln increases the rate of thrombin inactivation 5-fold even though it resides outside of the GAG-binding domain (37). In addition, GAG binding to HCII may not only release the amino terminus, but may also alter its conformation or that of the reactive site loop thereby optimizing the interaction of HCII with thrombin.

Acknowledgments—We acknowledge Dr. Charles Esmon for critical reading of the manuscript and for many helpful discussions, and Janice Rischke for high performance liquid chromatography analyses.

REFERENCES

1. Tollefsen, D. M. (1995) Thromb. Haemostasis 74, 1209–1214
2. Rosenberg, R. D. (1987) in Haemostasis and Thrombosis: Basic Principles and Clinical Practice (Colman, R. W., Hirsh, J., Marder, V. J., and Salzman, E. W., eds) pp. 1373–1382, J. B. Lippincott, Philadelphia
3. Lindahl, U., Backstrom, G., Thunberg, L., and Leder, I. G. (1980) Proc. Natl. Acad. Sci. U. S. A. 77, 6561–6567
4. Casu, B., Orante, P., Torri, G., Zoppetti, G., Choay, J., Lormeau, J. C., Petitou, M., and Sina, P. (1981) Biochem. J. 197, 599–609
5. Choay, J., Petitou, M., Lormeau, J. C., Sihay, P., Casu, B., and Gatti, G. (1983) Biochem. Biophys. Res. Commun. 116, 492–499
6. Sia, P., Petitou, M., Lormeau, J. C., Dupoux, D., Boneu, B., and Choay, J. (1988) Biochim. Biophys. Acta 966, 188–195
7. Rosenberg, L. C., Choi, H. U., Tang, L. H., Johnson, T. L., Pal, S., Weber, C., Reiner, A., and Poole, A. R. (1985) Biochem. J. 260, 6304–6313
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8. Choi, H. U., Johnson, T. L., Pal, S., Tang, L. H., Rosenberg, L., and Neame, P. J. (1989) J. Biol. Chem. 264, 2876–2884
9. Griffith, M. J., Noyes, C. M., Tyndall, J. A., and Church, F. C. (1985) Biochemistry 24, 6777–6782
10. Olsen, S. T., and Björk, I. (1994) Semin. Thromb. Hemostasis 20, 373–409
11. Ragg, H., Ulshofer, T., and Gerewitz, J. (1990) J. Biol. Chem. 265, 2876–2884
12. Van Deerlin, V. M. D., and Tollefsen, D. M. (1991) J. Biol. Chem. 266, 2876–2884
13. Sheehan, J. P., Tollefsen, D. M., and Sadler, J. E. (1994) J. Biol. Chem. 269, 32747–32751
14. Hortin, G. L., Tollefsen, D. M., and Benutto, B. M. (1989) J. Biol. Chem. 264, 13979–13982
15. Ciaccia, A. V., Willemze, A. J., and Church, F. C. (1997) J. Biol. Chem. 272, 8988–8993
16. Olsen, S. T., Björk, I., and Shore, J. D. (1993) Methods Enzymol. 222, 525–560
17. Cosmi, B., Fredenburgh, J. C., Rischke, J., Hirsh, J., Young, E., and Weitz, J. I. (1997) Circulation 95, 118–124
18. Ye, J., Rezaie, A. R., and Esmon, C. T. (1994) J. Biol. Chem. 269, 17965–17970
19. Harenberg, J., Jeschek, M., Acker, M., Malsch, R., Huhle, G., and Heene, D. L. (1996) Blood Coagul. Fibrinolysis 7, 49–56
20. Tasset, D. M., Kubik, M. F., and Steiner, W. (1997) J. Mol. Biol. 272, 688–698
21. Blinder, M. A., Marasa, J. C., Reynolds, C. H., Deaven, L. L., and Tollefsen, D. M. (1988) Biochemistry 27, 752–759
22. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) in Molecular Cloning: A Laboratory Manual (Sambrook, J., Fritsch, E. F., and Maniatis, T., eds) pp. 13.1–13.102, Cold Spring Harbor Laboratory Press, New York
23. Cuatrecasas, P. (1970) J. Biol. Chem. 245, 3059–3065
24. Laemmli, U. K. (1970) Nature 227, 680–685
25. Weitz, J., Young, E., Johnston, M., Stafford, A. R., Fredenburgh, J. C., and Hirsh, J. (1999) Circulation 99, 682–689
26. Fredenburgh, J. C., Stafford, A. R., and Weitz, J. I. (1997) J. Biol. Chem. 272, 25495–25499
27. Tollefsen, D. M., Majorus, D. W., and Blank, M. K. (1982) J. Biol. Chem. 257, 2162–2169
28. Blinder, M. A., Andersen, T. R., Abildgaard, U., and Tollefsen, D. M. (1989) J. Biol. Chem. 264, 5128–5133
29. Ragg, H., Ulshofer, T., and Gerewitz, J. (1990) J. Biol. Chem. 265, 22386–22391
30. Blinder, M. A., and Tollefsen, D. M. (1990) J. Biol. Chem. 265, 32748–32751
31. Whinna, H. C., Blinder, M. A., Szewczyk, M., Tollefsen, D. M., and Church, F. C. (1991) J. Biol. Chem. 266, 8129–8135
32. Becker, D. L., Fredenburgh, J. C., Stafford, A. R., and Weitz, J. I. (1999) J. Biol. Chem. 274, 6226–6233
33. Rogers, S. J., Pratt, C. W., Whinna, H. C., and Church, F. C. (1992) J. Biol. Chem. 267, 3613–3617
34. Rydel, T. J., Tulinsky, A., Bode, W., and Huber, R. (1991) J. Mol. Biol. 221, 583–601
35. Bray, B., Lane, D. A., Freyssinet, J. M., Pejler, G., and Lindahl, U. (1989) Biochem. J. 262, 225–232
36. Nesheim, M. E., Prendergast, F. G., and Mann, K. G. (1979) Biochemistry 18, 996–1003
37. Ciaccia, A. V., Monroe, D. M., and Church, F. C. (1997) J. Biol. Chem. 272, 14074–14079
38. Myles, T., Church, F. C., Whinna, H. C., Monard, D., and Stone, S. R. (1998) J. Biol. Chem. 273, 31203–31208
39. Han, J. H., Van Deerlin, V. M., and Tollefsen, D. M. (1997) J. Biol. Chem. 272, 8243–8249
40. Rezaie, A. R. (1998) J. Biol. Chem. 273, 16824–16827
41. Church, F. C., Noyes, C. M., and Griffith, M. J. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 6431–6434