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Molecular Mapping of the Thrombin-Heparin Cofactor II Complex*

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We used 55 Ala-scanned recombinant thrombin molecules to define residues important for inhibition by the serine protease inhibitor (serpin) heparin cofactor II (HCII) in the absence and presence of glycosaminoglycans. We verified the importance of numerous basic residues in anion-binding exosite-1 (exosite-1) and found 4 additional residues, Gln^{24}, Lys^{65}, His^{66}, and Tyr^{71} (using the thrombin numbering system), that were resistant to HCII inhibition with and without glycosaminoglycans. Inhibition rate constants for these exosite-1 (Q24A, K65A, H66A, Y71A) thrombin mutants (0.02–0.38 × 10^8 M^{-1} min^{-1} for HCII-heparin when compared with 2.36 × 10^8 M^{-1} min^{-1} with wild-type thrombin and 0.03–0.53 × 10^8 M^{-1} min^{-1} for HCII-dermatan sulfate when compared with 5.23 × 10^8 M^{-1} min^{-1} with wild-type thrombin) confirmed that the structural integrity of thrombin exosite-1 is critical for optimal HCII-thrombin interactions in the presence of glycosaminoglycans. However, our results are also consistent for HCII-glycosaminoglycan-thrombin ternary complex formation. Ten residues surrounding the active site of thrombin were implicated in HCII interactions. Four mutants (Asp^{51}, Lys^{52}, Lys^{145}/Thr^{147}/Trp^{148}, Asp^{234}) showed normal increased rates of inhibition by HCII-glycosaminoglycans, whereas four mutants (Trp^{50}, Glu^{202}, Glu^{229}, Arg^{233}) remained resistant to inhibition by HCII with glycosaminoglycans. Using 11 exosite-2 thrombin mutants with 20 different mutated residues, we saw no major perturbations of HCII-glycosaminoglycan inhibition reactions. Collectively, our results support a "double bridge" mechanism for HCII inhibition of thrombin in the presence of glycosaminoglycans, which relies in part on ternary complex formation but is primarily dominated by an allosteric process involving contact of the "hirudin-like" domain of HCII with thrombin exosite-1.

The blood coagulation cascade is highly regulated with the serine protease thrombin playing an essential role in both procoagulant and anticoagulant pathways. As a procoagulant, thrombin activates platelets, cleaves fibrinogen to fibrin, resulting in the formation of a fibrin clot, activates factors V, VII, and XI via a feedback mechanism, and also activates factor XIII (plasma transglutaminase) (for a review, see Ref. 1 and references cited therein). By contrast, when thrombin binds to the endothelial cell surface receptor thrombomodulin, thrombin activates the anticoagulant zymogen, protein C (for a review, see Ref. 2 and references cited therein). Macromolecular substrate recognition by thrombin is partly mediated by two clusters of positively charged basic amino acids located on opposite sides of the active site, referred to as anion-binding exosite-1 and anion-binding exosite-2 (exosite-1 and exosite-2, respectively)[1]. Exosite-1 binds fibrinogen, fibrin, heparin cofactor II (HCII) (3), and hirudin (4), whereas exosite-2 binds heparin, heparan sulfate, and dermatan sulfate (5–7).

Glycosaminoglycan-accelerated inhibition by the serine protease inhibitors (serpins) antithrombin (AT; systematic name *SERPINC1*) (8), heparin cofactor II (HCII; systematic name *SERPIND1*) (9), protein C inhibitor (also known as plasminogen activator inhibitor-3; systematic name *SERPINA5*) (10), and protease nexin-1 (systematic name *SERPINE2*) (11) is one mechanism for thrombin regulation. Besides thrombin, AT inhibits several proteases in the blood coagulation pathway, including factor IXa, Xa, XIa, XIIa, plasmin, and kallikerin; however, HCII is specific for thrombin in this group of serine proteases. Although both AT and HCII inhibition of thrombin are accelerated by heparin and heparan sulfate, HCII inhibition of thrombin is also accelerated by dermatan sulfate (12, 13). The glycosaminoglycan-binding site for AT and HCII is localized to the D-helix region of these serpins (for a review, see Refs. 8 and 14–20 and references cited therein). Recent studies show that HCII acts as a thrombin inhibitor in the arterial circulation (21) and that dermatan sulfate proteoglycans are modified during atherosclerotic lesion development to give reduced HCII-thrombin inhibition activity (22), suggesting that HCII is an extravascular thrombin inhibitor.

Based on both the crystal structure of the thrombin-HCII complex (23) and biochemical/molecular biology studies (24–28), a more detailed understanding of thrombin inhibition by HCII has emerged. In the presence of glycosaminoglycans, thrombin should bind preferentially to the glycosaminoglycan and may open the active site for better substrate binding (29). Binding of HCII to the glycosaminoglycan frees the amino-terminal acidic domain to be appropriately positioned for

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[1] The abbreviations and trivial names used are: exosite-1, anion-binding exosite-1; exosite-2, anion-binding exosite-2; serpin, serine protease inhibitor; HCII, heparin cofactor II; AT, antithrombin; Tos, tosyl (*p*-toluenesulfonate).

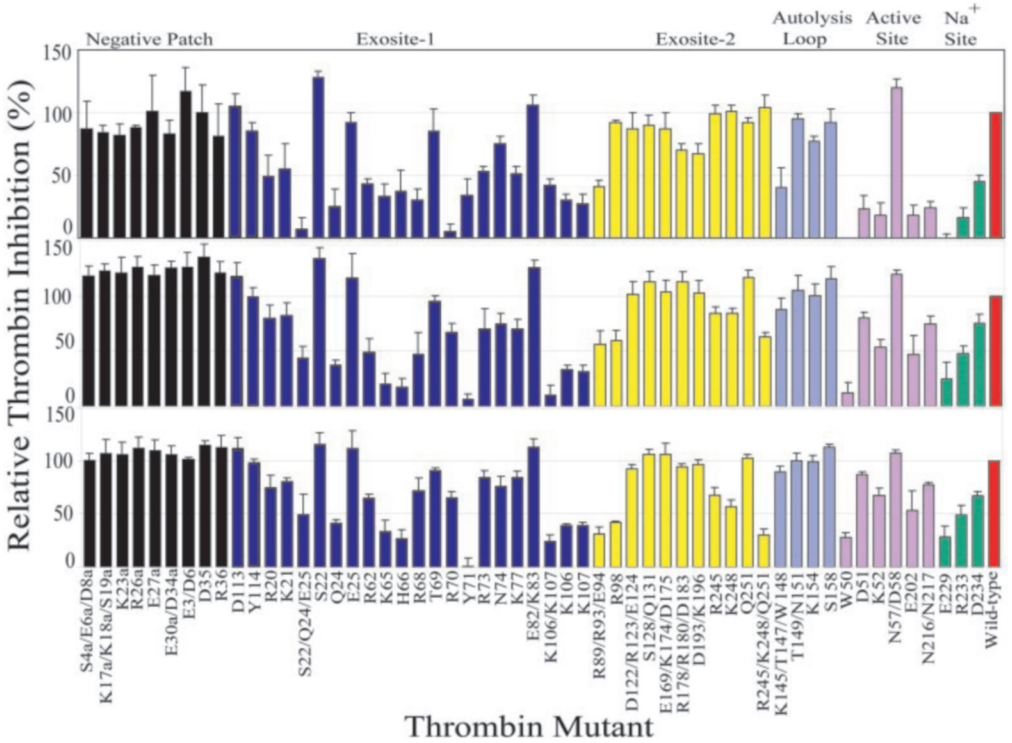

FIG. 1. **Inhibition of thrombin mutants by HCII in the absence and presence of glycosaminoglycans.** Thrombin (wild-type) and the thrombin mutants were incubated with 350 nM HCII in the absence of glycosaminoglycans (*upper panel*), 10 nM HCII in the presence 10 μg/ml dermatan sulfate (*middle panel*), or 5 μg/ml heparin (*lower panel*). Under the conditions used in these assays, inhibition of wild-type thrombin by HCII either in the absence of or in the presence of glycosaminoglycans was 50%. All thrombin mutants were normalized to wild-type thrombin (set to 100%). These data represent the average of 3–5 independent experiments performed in triplicate.

thrombin interaction (15–17, 28, 30). Thrombin exosite-1 binds to the first acidic region of HCII to facilitate proteolytic attack of the P1-P1′ Leu-Ser of HCII by the active site of thrombin. Thrombin is now covalently trapped in a 1:1 stable bimolecular complex with HCII (23). Although this information further illustrates the importance of both the acidic domain of HCII and the exosite-1 of thrombin, the molecular details and the role of individual surface-derived residues of thrombin are incomplete. In the current study, we used 55 recombinant thrombin mutants in which solvent-accessible residues were substituted with Ala to determine the residues important for the thrombin-HCII interaction in the absence and presence of glycosaminoglycans. Our results suggest that thrombin has several unique determinants for distinguishing HCII from the prototypical thrombin inhibitor AT, both in the absence of and in the presence of glycosaminoglycans. In addition, our data support a mechanism for thrombin inhibition in the presence of glycosaminoglycan that involves binding of both proteins to the glycosaminoglycans but also relies on the interaction of the amino-terminal acidic domain of HCII with exosite-1 for maximal rates of inhibition.

EXPERIMENTAL PROCEDURES

Materials—Human α-thrombin and HCII were purified to homogeneity as described previously (31, 32). Expression and purification of wild-type and recombinant mutant thrombins using Chinese hamster ovary cells have been described previously (33, 34). Heparin was obtained from Diosynth BV (Oss, the Netherlands), and dermatan sulfate was purchased from Calbiochem. To remove heparin contaminants, dermatan sulfate was nitrous acid-treated (35). The thrombin chomogenic substrate, Tos-Gly-Pro-Arg-*p*-nitroanilide, was purchased from Centerchem, Inc. (Norwalk, CT).

Protease Inhibition—All assays were performed at room temperature in 96-well microtiter plates. The rates were measured under pseudo-first order kinetic reaction conditions as described previously (26). Screening assays were used to compare all the Ala-scanned thrombin mutants for HCII inhibition. In the absence of glycosaminoglycans, 1 nM wild-type or mutant recombinant thrombin (diluted into 20 mM HEPES, 150 mM NaCl, 0.1% polyethylene glycol 8000, 0.05% sodium azide at pH 7.4 (HNPN buffer) containing 1 mg/ml ovalbumin) was incubated with 350 nM HCII in the presence of 100 μg/ml Polybrene in HNPN buffer, pH 7.4. In the presence of glycosaminoglycans (5 μg/ml heparin or 10 μg/ml dermatan sulfate), 1 nM thrombin was incubated with 10 nM HCII in HNPN buffer, pH 7.4. Incubation times were established for wild-type thrombin with HCII under these conditions to give 50% thrombin inhibition (in the absence and presence of glycosaminoglycans), and all the thrombin mutants were assessed under the identical reaction inhibition conditions. Residual thrombin activity was measured by the addition of 150 μM Tos-Gly-Pro-Arg-*p*-nitroanilide containing 2 mg/ml polybrene for reactions in the presence of glycosaminoglycans. Substrate cleavage was measured by color development at 405 nm on a V_{max} kinetic microplate reader (Molecular Devices). Assays were performed at least in triplicate on three or more occasions with the thrombin mutants. Second order inhibition rate constants (k_2) were calculated as described (25).

A selection of thrombin mutants was also evaluated over longer time courses of inhibition by HCII in the absence and presence of GAG as described previously. In the presence of glycosaminoglycans (0.006–2 mg/ml for heparin and 0.05–2 mg/ml for dermatan sulfate), 1 nM thrombin was incubated with 10 nM HCII in HNPN buffer, pH 7.4. At various time intervals, residual thrombin activity was measured by the addition of 150 μM Tos-Gly-Pro-Arg-*p*-nitroanilide containing 2 mg/ml polybrene for reactions in the presence of glycosaminoglycans.

Molecular Modeling—The file 1JMO (23) was retrieved from the Research Collaboratory for Structural Bioinformatics Protein Data base (www.rcsb.org (36)). All manipulations were done using Insight II software (Accelrys, Inc.; San Diego, CA).

RESULTS

Inhibition of Thrombin Mutants by HCII in the Absence of Glycosaminoglycans—We used an Ala-scanning site-directed mutagenesis approach to map the residues on thrombin essential for HCII inhibition. In an initial single time point screening assay, 25 of 55 thrombin mutants were significantly more resistant to inhibition by HCII when compared with wild-type thrombin (≤50% thrombin inhibition; Fig. 1, *upper panel*, and

TABLE I

Thrombin mutants with ≤50% thrombin inhibition during HCII inhibition in the absence and presence of glycosaminoglycans

The data summarized below are the relative thrombin inhibition activity for the various thrombin mutants compared to wild-type recombinant thrombin in the absence and presence of glycosaminoglycans (using a one-point time assay that gave 50% thrombin inhibition, which was then normalized to 100%). The values represent the mean ± S.D. of three to five separate determinations performed in triplicate. All assays were performed as described under "Experimental Procedures."

Mutations (thrombin numbering) [a]	Mutations (using chymotrypsinogen numbering) [a]	Thrombin location	% Relative thrombin inhibition by HCII in screening assay		
			No GAG [c]	+ Heparin	+ Dermatan sulfate
R20A	R35A	Exosite-1	49 ± 17	76 ± 12	79 ± 12
K21A	K36A	Exosite-1	49 ± 19	89 ± 15	82 ± 11
S22A/Q24A/E25A	S37/Q38/E39	Exosite-1	14 ± 5.7	49 ± 3.9	43 ± 10
Q24A	Q38A	Exosite-1	32 ± 6.1	40 ± 5	37 ± 4.4
R62A	R67A	Exosite-1	43 ± 3.8	64 ± 6	48 ± 12
K65A	K70A	Exosite-1	33 ± 9.6	30 ± 8	20 ± 9
H66A	H71A	Exosite-1	36 ± 17	27 ± 7	17 ± 7
R68A	R73A	Exosite-1	30 ± 8.5	61 ± 8	47 ± 19
R70A	R75A	Exosite-1	5.4 ± 0.6	56 ± 4	66 ± 9
Y71A	Y76A	Exosite-1	34 ± 12	0.5 ± 0.07	9 ± 3
R73A	$R77^{A}A$	Exosite-1	50 ± 2.9	82 ± 5	70 ± 18
K77A	K81A	Exosite-1	50 ± 6.0	87 ± 9	70 ± 9
K106/K107	K109A/K110A	Exosite-1	42 ± 5.6	23 ± 4	13 ± 9
K106	K109A	Exosite-1	30 ± 4.8	44 ± 3	33 ± 4
K107	K110A	Exosite-1	27 ± 7.7	38 ± 3	31 ± 6
R89A/R93A/E94A	R93A/R97A/97^{A}A	Exosite-2	40 ± 5.6	31 ± 8	56 ± 12
R98A	R101A	Exosite-2	92 ± 2	44 ± 4.4	59 ± 9
R245A/K248/Q251A	R233A/K236A/Q239A	Exosite-2	103 ± 10	30 ± 7	63 ± 4
E229	E217A	Na^{+}-binding site	<0.01	37 ± 12	15 ± 2
R233A	R221A	Na^{+}-binding site	16 ± 8.2	52 ± 7	47 ± 7
D234A	D222A	Na^{+}-binding site	45 ± 5.2	75 ± 5	75 ± 9
W50A	$W60^{D}$A	60-insertion loop	<0.01	35 ± 5	12 ± 9
D51A	$D60^{E}$A	60-insertion loop	18 ± 2.7	101 ± 7	80 ± 5
K52A	$K60^{F}$A	60-insertion loop	22 ± 6.5	70 ± 9	53 ± 7
E202	E192A	Active site region	18 ± 8.0	30 ± 4	47 ± 16
K145A/T147A/W148A	K145A/T147A/W148A	Autolysis loop	40 ± 16	87 ± 8	87 ± 10
N216A/N217A	$N204^{B}$A/N205A	Loop 203–206	24 ± 5.3	78 ± 6	74 ± 7

[a] The numbering of thrombin is based on α-thrombin. The letter "a" denotes the A-chain, and shown above are residues located in the B-chain.
[b] The numbering system is compared to the crystallographic convention with respect to bovine chymotrypsinogen (Bode's reference).
[c] GAG, glycosaminoglycan.

Table I). 15 mutants were mapped to thrombin exosite-1 (R20A, K21A, S22A/Q24A/E25A, Q24A, R62A, K65A, H66A, R68A, R70A, Y71A, R73A, K77A, K106A/K107A, K106A, K107A). Five mutants were localized to residues surrounding the active site region (E202A) including the 60-insertion loop (W50A, D51A, K52A) and the autolysis loop (K145A/T147A/W148A). One mutant was mapped from the 203–206-loop (N216A/N217A), one thrombin mutant was mapped to exosite-2 (R89A/R93A/E94A), and three thrombin mutants were associated with the sodium-binding site (E229A, R233A, D234A).

Full time courses of inhibition by HCII were performed on seven thrombin mutants (Q24A, K65A, R62A, H66A, Y71A, R89A/R93A/E94A, and E229A) found resistant to HCII in the initial screen. The selection of these specific thrombin mutants is representative of the regions identified to be important for HCII interaction, and these residues have never been characterized for their involvement in HCII inhibition (either in the absence of or in the presence of glycosaminoglycans). Inhibition rate constants for these thrombin mutants (k_2 values) were reduced from 2–6-fold when compared with wild-type thrombin (k_2 = 2.38 ± 0.26 M^{-1} min^{-1}; Table II). These results suggest that a substantial number of thrombin residues are involved in the recognition of HCII in the absence of glycosaminoglycans, in contrast to AT inhibition of thrombin (37).

Inhibition of Thrombin Mutants by HCII in the Presence of Glycosaminoglycans—We next studied thrombin inhibition in a screening assay using HCII with heparin or dermatan sulfate at a fixed concentration. Fourteen of 25 thrombin mutants identified above also showed ≤50% thrombin inhibition activity when compared with wild-type thrombin inhibition by HCII in the presence of glycosaminoglycan. They were mapped to exosite-1 (S22A/Q24A/E35A, Q24A, R62A, K65A, H66A, R68A, Y71A, K106A/K107A, K106A, K107A); the active site region (W50A, E202A); and the sodium-binding site (E229A, R233A) (Fig. 1, *middle* and *bottom panels*; and Table I). Interestingly, 10 of the 25 mutants (R20A, K21A, D51A, K52A, R70A, R73A, K77A, K145A/T147A/W148A, N216A/N217A, D234A) were "compensated" to >50% of wild-type thrombin inhibition activity in the presence of HCII and glycosaminoglycans (Fig. 1).

A subset of thrombin mutants was then further assayed to examine k_2 values for inhibition by HCII when compared with wild-type thrombin over a range of heparin and dermatan sulfate concentrations (Fig. 2 and Table II). Wild-type recombinant thrombin showed the typical bell-shaped dependence on glycosaminoglycan concentrations for the k_2 values of HCII inhibition for both heparin (~10,000-fold acceleration of the inhibition rate) and dermatan sulfate (~20,000-fold acceleration of the inhibition rate).

Four exosite-1 thrombin mutants (Q24A, K65A, H66A, and Y71A) gave maximal k_2 values that were all substantially decreased and ranged from a low of 200-fold (heparin) and 300-fold (dermatan sulfate) accelerated k_2 values for Y71A to Q24A that had 5000- (heparin) and 7000-fold (dermatan sulfate) accelerated k_2 values (Fig. 2 and Table II). The sodium-binding mutant E229A thrombin had 9500- and 11,000-fold accelerated k_2 values for inhibition by HCII with heparin and dermatan sulfate, respectively (Fig. 2 and Table II).

In the absence of glycosaminoglycan, exosite-2 mutant R89A/R93A/E94A thrombin had a 2-fold reduction in inhibition rate by HCII but showed essentially normal rate acceleration in the presence of heparin (11,000-fold) and dermatan sulfate (28,000-fold) (Fig. 2 and Table II). Intriguingly, exosite-2 mutant

TABLE II

Effect of thrombin mutants during HCII inhibition in the absence and presence of glycosaminoglycans

All assays were performed as described under "Experimental Procedures." The k_2 values represent the mean ± S.D. of at least three separate determinations performed in triplicate.

Mutations (thrombin numbering)	Thrombin location	$k_2 \times 10^4$ (no GAG)[a]	k_2 mutant/k_2 rIIa	$k_2 \times 10^8$ + Heparin[b]	Heparin acceleration	k_2 mutant/k_2 rIIa	$k_2 \times 10^8$ + DS[a]	DS Acceleration	k_2 mutant/k_2 rIIa
		$M^{-1}min^{-1}$		$M^{-1}\ min^{-1}$	*-fold*		$M^{-1}\ min^{-1}$	*-fold*	
Wild-type		2.38 ± 0.26	1.00	2.36 ± 0.33	9916	1.00	5.23 ± 0.38	21975	1.00
Q24A	Exosite-1	0.75 ± 0.11	0.32	0.38 ± 0.05	5067	0.16	0.53 ± 0.08	7067	0.10
R62A	Exosite-1	1.4 ± 0.09	0.58	0.91 ± 0.10	6500	0.39	0.62 ± 0.2	4429	0.12
R62Q	Exosite-1	0.88 ± 0.08	0.37	0.011 ± 0.02	125	0.005	<0.01	71	0.001
K65A	Exosite-1	0.96 ± 0.04	0.40	0.20 ± 0.03	2083	0.08	0.18 ± 0.04	1875	0.03
H66A	Exosite-1	0.95 ± 0.11	0.40	0.18 ± 0.04	1894	0.08	0.31 ± 0.01	3263	0.06
Y71A	Exosite-1	0.97 ± 0.13	0.41	0.02 ± 0.008	206	0.01	0.03 ± 0.005	309	0.01
R89A/R93A/E94A	Exosite-2	1.12 ± 0.08	0.47	1.26 ± 0.06	11250	0.53	3.18 ± 0.2	28393	0.61
R245A/K248A/Q251A	Exosite-2	2.71 ± 0.44	1.14	2.98 ± 0.3	10996	1.26	8.82 ± 0.24	32546	1.68
E229A	Na^+ site	0.37 ± 0.11	0.16	0.35 ± 0.05	9459	0.14	0.41 ± 0.25	11081	0.16

[a] GAG, glycosaminoglycan.

[b] Rates at peak concentration of heparin or dermatan sulfate.

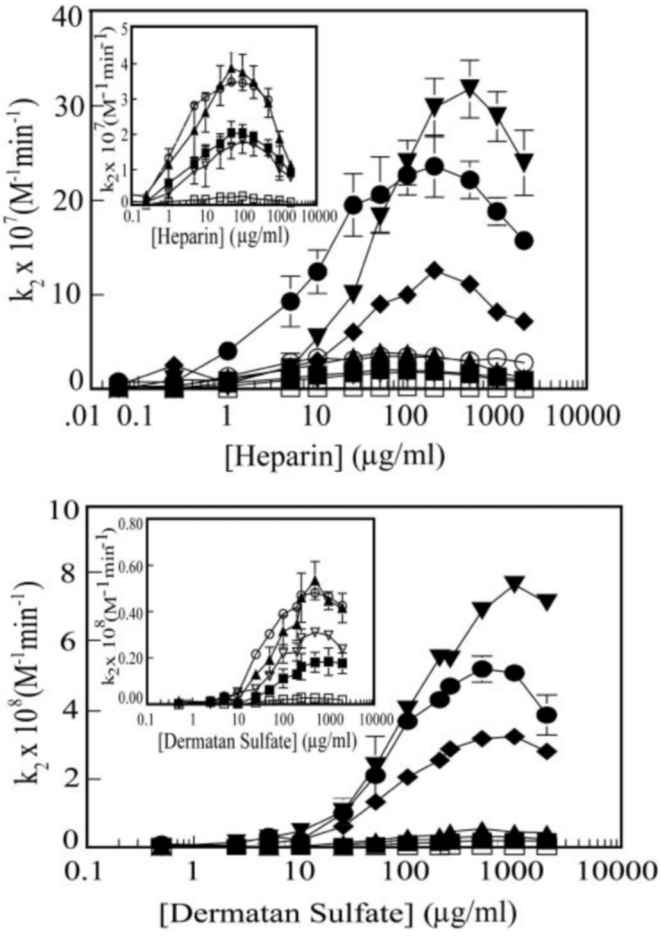

FIG. 2. **Inhibition of selected thrombin mutants by HCII in the presence of heparin and dermatan sulfate.** Wild-type thrombin (●) or thrombin mutants (Q24A (▲), K65A (■), H66A (△), Y71A (□), R89A/R93A/E94A (◆), R245A/D248/Q251 (▼), E229 (○)) were incubated with 10 nM HCII at increasing amounts of heparin (*top panel*) and dermatan sulfate (*bottom panel*). The *insets* represent HCII-heparin and HCII-dermatan sulfate inhibition curves for thrombin mutants (Q24A, K65A, H66A, and Y71A) with a smaller range of *y* axis values. These data represent the average of at least 3 independent experiments done in triplicate.

R245A/K248A/Q251A thrombin remained slightly more active than wild-type thrombin both in the absence of and in the presence of glycosaminoglycan (Fig. 2 and Table II).

We also compared R62A with R62Q mutant thrombin since Arg^{62} (as R62Q) had previously been shown to be a key residue for the HCII-thrombin inhibition (38). There was a more pronounced loss of heparin- and dermatan sulfate-catalyzed HCII-thrombin inhibition with R62Q mutant thrombin when compared with R62A mutant thrombin (Fig. 3, *top* and *middle panels*; Table II). By contrast, heparin-catalyzed AT inhibition of R62Q and R62A mutant thrombins was similar, confirming that exosite-1 is not critical for thrombin inhibition by AT (Fig. 3, *bottom panel*).

Collectively, these results indicate a more extensive requirement of thrombin exosite-1 for HCII inhibition than previously known. None of the exosite-2 mutants examined were drastically resistant during glycosaminoglycan-dependent HCII inhibition reactions, and although the sodium-binding site mutations perturb the active site of thrombin, the glycosaminoglycan-dependent acceleration of the HCII-thrombin inhibition reaction is separate and independent from the active site defect.

DISCUSSION

Use of Ala-scanned Mutants for Thrombin Structure-Activity Relationships—Thrombin is a multifunctional serine protease that is critical for hemostasis and inflammatory processes. In the present study, we used 55 thrombin mutants in which ~2/3 of the surface-accessible residues were mutated to alanine. Recently, these and other Ala-scanned thrombin mutants have proven invaluable in understanding the interaction of thrombin with macromolecular substrates, including: fibrinogen (39); AT and AT-heparin (37); thrombomodulin (33, 40); protein C (33); thrombin-activable fibrinolysis inhibitor (33); factor V (34); protease-activated receptors (41); glycoprotein Ibα (42); factor VIII (43); factor XI (44); and the sodium-binding site (45). We used this library of thrombin mutants to identify surface residues important for the thrombin-HCII interaction both in the presence of and in the absence of glycosaminoglycans (Fig. 4). The HCII inhibition mechanism of thrombin is novel for a serpin-protease interaction in part due to its unique amino-terminal acidic domain and to its atypical (for a thrombin inhibitor) Leu^{444}-reactive center residue. Combined with its putative pathophysiological role in arterial thrombosis (21) and extravascular thrombin regulation (22, 46–48), our results described here offer a biochemical perspective to the HCII-thrombin inhibition reaction.

HCII-Thrombin Exosite-1 Interactions—As anticipated, many of the residues that displayed the most dramatic effects were localized to thrombin exosite-1. Our results complement a study that showed that the interaction between HCII and thrombin used the basic residues of exosite-1 for "electrostatic steering" interactions for complex formation (38). We also show a role for several non-charged residues in exosite-1 that could

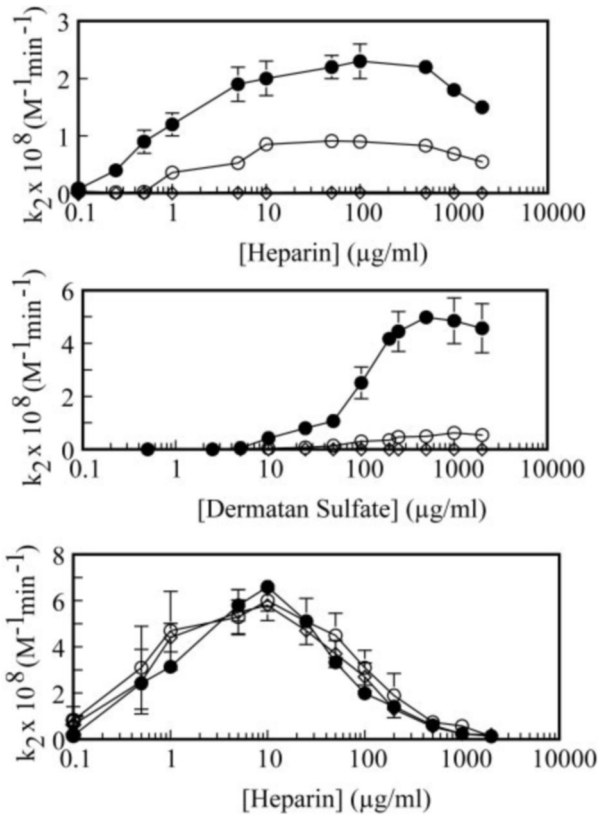

FIG. 3. **Inhibition of R62A and R62Q thrombin mutants by HCII and AT in the presence of glycosaminoglycans.** Wild-type thrombin (●), thrombin mutant R62A (○), or R62Q (◇) was incubated with 10 nM HCII and increasing amounts of heparin (*top panel*) and dematan sulfate (*middle panel*) or with AT and increasing concentrations of heparin (*bottom panel*). These data represent the average of at least 3 independent experiments done in triplicate.

allow for short range hydrophobic interactions between thrombin and HCII as proposed for another exosite-1-dependent thrombin inhibitor, hirudin (49).

The role of thrombin exosite-1 has been partially characterized using dysthrombin Quick 1 (R62C) (50), γ_T- and ϵ-thrombins (27, 30, 51), exosite-1 mutants R68E and R70E (5), and 10 basic exosite-1 residues changed to Gln (38). In the current study, we measured the inhibition of 22 different Ala-scanned exosite-1 thrombin mutants. Remarkably, 15 mutants were significantly resistant to inhibition by HCII in the absence of glycosaminoglycans, and 9 of them remained significantly resistant when in the presence of HCII and glycosaminoglycans. Of notable interest, a large decrease in k_2 value was found for Y71A (118-fold for heparin, 174-fold for dermatan sulfate), H66A (13-fold for heparin, 17-fold for dermatan sulfate), K65A (12-fold for heparin, 29-fold for dermatan sulfate), and Q24A (6-fold for heparin, 10-fold for dermatan sulfate) during HCII-glycosaminoglycan inhibition when compared with wild-type thrombin. These results confirm that the structural integrity of exosite-1 is critical for interactions between the acidic domain of HCII and thrombin (Fig. 4). All four exosite-1 thrombin mutants retained some modest HCII-glycosaminoglycan-accelerated inhibition rates (bell-shaped logarithmic curves of inhibition with respect to k_2 values and glycosaminoglycan concentrations), which supports the concept that glycosaminoglycan binding to both reactants is a feature of the inhibition mechanism (especially without a fully functional exosite-1 region). These exosite-1 residues are located in the same plane of exosite-1, spatially close to one another and very near Arg^{62} and Arg^{73} previously identified to be involved in the exosite-1 inhibition process for HCII-thrombin (38). Inspection of the HCII-thrombin encounter complex structure shows that Tyr^{71} and Gln^{24} of exosite-1 "sandwich" Leu^{61} of the HCII acidic domain, which suggests that their mutation to Ala directly alters an HCII-thrombin interaction. In contrast, Lys^{65} and His^{66} are on the backside of this portion of exosite-1; it is likely that these mutations alter the overall loop structural dynamics during the recognition of the acidic domain of HCII. Finally, our results show a slightly larger alteration of the inhibition rates in the presence of dermatan sulfate when compared with heparin, implying a larger dependence on exosite-1 for the HCII-dermatan sulfate inhibition reaction.

HCII-Thrombin Exosite-2 Interactions—An important and major distinction between AT and HCII has been data showing the critical role of thrombin exosite-2 for glycosaminoglycan-accelerated inhibition of thrombin by AT and less of a role for exosite-2 during HCII inhibition reactions. Some exosite-2 thrombin mutants have been examined previously for effects on HCII glycosaminoglycan-dependent inhibition reactions, including R89E, R245E, K248E, K252E, and R89A/R93A/R98A (5, 52, 53). In the current study, we measured the inhibition of 11 different exosite-2 thrombin mutants (containing 20 different mutations) with HCII in the absence and presence of glycosaminoglycans. Only three exosite-2 thrombin mutants showed major differences during the HCII-thrombin inhibition reaction, and in the absence of glycosaminoglycan, only mutant R89A/R93A/E94A thrombin was resistant to inhibition. At a fixed dose of glycosaminoglycan, thrombin mutants R89A/R93A/E94A, R98A, and R245A/K248A/Q251A had reduced thrombin inhibition by HCII-heparin/dermatan sulfate. Closer examination of the reaction properties of R89A/R93A/E94A and R245A/K248A/Q251A thrombin mutants showed that at optimal concentrations of glycosaminoglycans with HCII, R89A/R93A/E94A (2-fold decreased k_2 for heparin, 1.6-fold for dermatan sulfate) was only slightly less active than wild-type thrombin. The R245A/K248A/Q251A mutant was slightly more active than wild-type thrombin (1.3-fold increased k_2 for heparin, 1.7-fold for dermatan sulfate), although the maximal acceleration occurred at a higher glycosaminoglycan concentration, indicating a decreased affinity of glycosaminoglycan. This is in stark contrast to the vital importance of these residues and others in exosite-2 during the AT-heparin-thrombin inhibition reaction (5–7, 37, 54, 55). Interestingly, Arg^{89}, Arg^{93}, and Glu^{94} are located immediately adjacent to the 60-insertion loop of thrombin, and these residues may influence 60-insertion loop interaction with HCII-thrombin and AT-thrombin and possibly explain the diminished thrombin inhibition rate in the absence of glycosaminoglycan (Fig. 4). By contrast, the region of exosite-2 defined by Arg^{245}, Lys^{248}, and Gln^{251} is quite distant from Arg^{89}, Arg^{93}, and Glu^{94}, and although these residues are important for the AT-heparin-thrombin inhibition reaction, they are apparently not essential for any HCII-thrombin interaction. Overall, the exosite-2 residues that are essential for glycosaminoglycan-accelerated inhibition by AT are apparently not required for the HCII-glycosaminoglycan-catalyzed inhibition reaction. These results also imply that the residues to support thrombin-glycosaminoglycan binding for ternary complex formation with HCII remain to be identified.

HCII-Thrombin Active Site Region Interactions—Residues located around or near the active site, particularly residue Trp^{50} (56, 57), are critical for the thrombin-HCII interaction (this study), thrombin-AT interaction (37, 56, 57), activation of factor V (34), protein C activation (33), and TAFI activation and the thrombin-thrombomodulin interaction (33). Logically, these results suggest that mutating residues in the active site region disrupts the interaction of thrombin with macromolec-

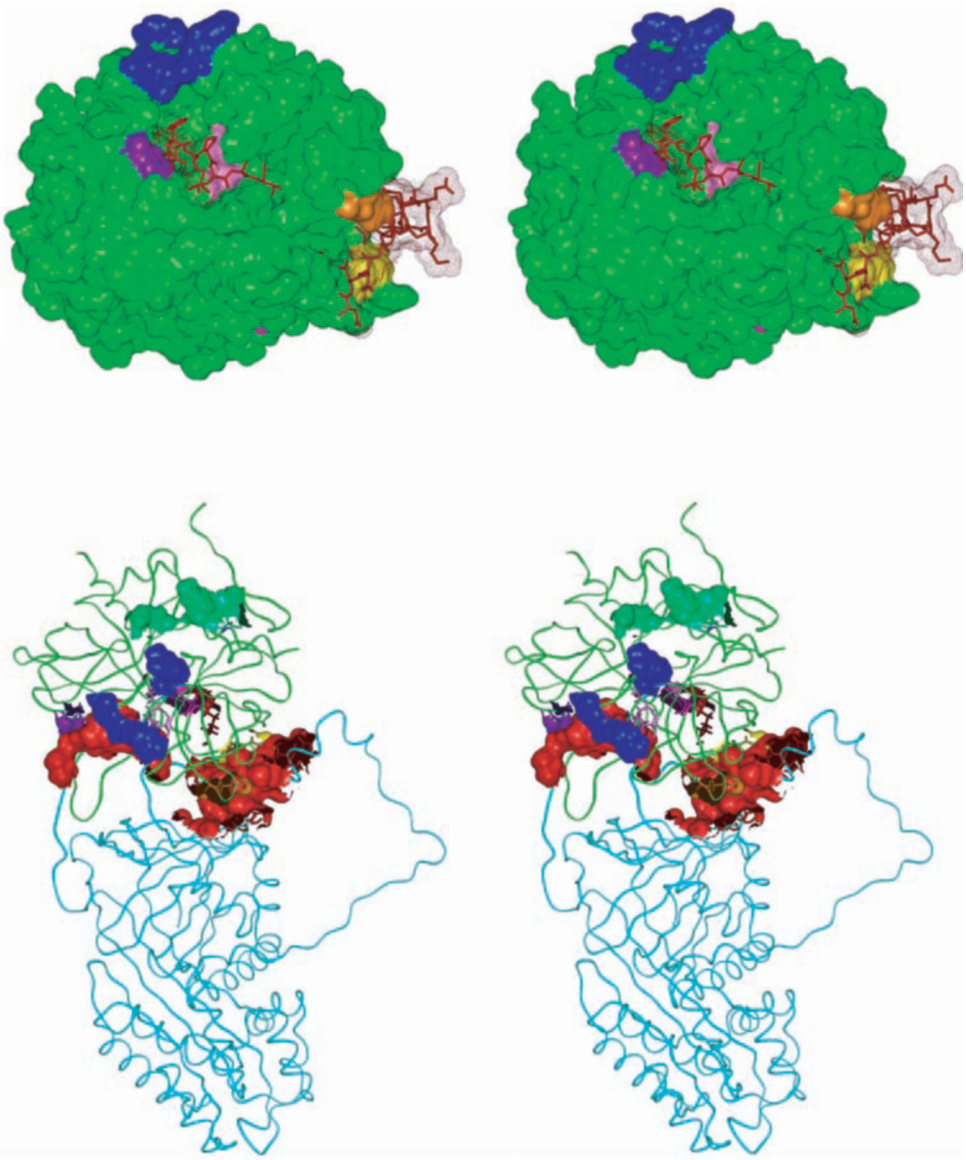

FIG. 4. **Stereo views of the thrombin-HCII encounter complex interactions.** *Upper panel*, the Connolly solvent-accessible surface of thrombin (in *green* except for the following residues: *pink*, active site triad; *orange*, Gln^{24}; *yellow*, Tyr^{71}; *dark red*, Lys^{65}; *dark pink*, His^{66}; *dark blue*, $Arg^{89}/Arg^{93}/Glu^{94}$; *light green*, $Arg^{245}/Lys^{248}/Gln^{251}$; *purple*, Glu^{229}) is shown as *solid*. The active site cleft is in the *center* of the molecule, and exosite-1 is on the *lower right*. *Stick renditions* of residues Asp^{57}-Phe^{67} and Val^{439}-Thr^{446} of HCII are shown in *red*, and the Connolly solvent-accessible surfaces of these residues are shown as *lines*. *Lower panel*, the backbones of α-thrombin (*green*) and HCII (*cyan*) are depicted as *ribbons*, and the residues are colored as listed above. The residues listed above are shown as *sticks*, and the Connolly solvent-accessible surfaces of these residues are shown as *solid*. The molecules are rotated forward 90° around the *x* axis when compared with the *upper panel*.

ular substrates. Other residues in or near the 60-insertion loop (Asp^{51} and Lys^{52}) and Glu^{202} (located above and below the active site, respectively, as depicted in Fig. 4) are also critical for the rate of thrombin inhibition by HCII in the absence but not in the presence of glycosaminoglycans, suggesting that glycosaminoglycans are able to compensate for this altered 60-insertion loop/active site interaction (Fig. 4). These results further suggest the intimate role of the 60-insertion loop above the active site cleft to regulate macromolecular substrates and their attempt to dock/interact with the active site catalytic triad, and our data show that HCII is similar to most thrombin substrates.

HCII-Thrombin Interactions and the Sodium-binding Site of Thrombin—We studied the role of the sodium-binding site of thrombin (for a review, see Refs. 45 and 58 and references cited therein) during HCII inhibition in the absence and presence of glycosaminoglycans using three sodium-binding site thrombin mutants. As seen previously with other macromolecular substrates, E229A, R233A, and D234A thrombin mutants were all resistant to HCII in the absence of glycosaminoglycans, but E229A remained greatly resistant to inhibition by HCII in the presence of heparin or dermatan sulfate. However, a detailed inhibition profile of E229A thrombin with HCII-glycosaminoglycans showed an almost normal -fold acceleration with heparin (~9500-fold) and dermatan sulfate (11,000-fold), yet the absolute k_2 values were greatly reduced when compared with wild-type thrombin. An inspection of the HCII-thrombin encounter complex reveals that Glu^{229} of thrombin is in proximity to Phe^{441} of the reactive site loop of HCII, showing that this region of HCII directly encounters elements of the sodium-binding site of thrombin (Fig. 4). The results imply that disruption of the sodium-binding site does not alter the integrity of thrombin exosite-1 since inhibition by HCII is normally accelerated in the presence of glycosaminoglycans. The functional independence of the exosite-1 of thrombin from its sodium-binding site is consistent with previous findings showing

that E229A thrombin binds normally to fibrin clot and interacts normally with the DNA-based thrombin aptamer, both of which are mediated by a similar subset of residues within exosite-1 that is important in HCII interaction (39, 59).

Mechanism for HCII-Thrombin Inhibition in the Presence of Glycosaminoglycans—The mechanism of glycosaminoglycan-accelerated thrombin inhibition by HCII is unique among thrombin-inhibiting serpins (5, 9, 13, 15, 16, 23–28, 60, 61). HCII appears to use a novel allosteric process in which glycosaminoglycan binding to the D-helix region allows a more permissive acidic domain to act as a "tethered ligand" for binding to exosite-1 of thrombin (5, 9, 16, 24, 30, 38, 51, 52, 61, 62). There have been two similar mechanisms proposed, with the only difference being the role of glycosaminoglycan acting as a secondary bridge between HCII and thrombin (28, 30, 38, 51, 62) *versus* a purely allosteric model without the need for ternary complex formation between HCII-glycosaminoglycan-thrombin (5, 6, 23–26, 52).

The thrombin mutant data from the current study reinforce the view that the interface between HCII and thrombin is quite different and more complex than other serpin-protease complexes (23). As shown in the HCII-thrombin encounter complex (23), our results with this library of thrombin mutants demonstrate that thrombin offers important contact points with HCII in at least three regions of the protease (Fig. 4): (*a*) a dominant role of exosite-1 (both in the absence of and in the presence of glycosaminoglycans); (*b*) an active site/60-insertion loop region/sodium-binding site (in some instances, this loss is compensated by glycosaminoglycans); and (*c*) an autolysis loop (in the absence of glycosaminoglycans). By contrast, none of the exosite-2 thrombin mutants are drastically reduced in activity during HCII-glycosaminoglycan interactions. However, inspection of the thrombin structure shows exosite-2 to be quite expansive with at least 12 basic amino acid residues available to bind negatively charged ligands (data not shown). Although our results indicate that no specific region of thrombin exosite-2 is critical for maximal glycosaminoglycan-accelerated inhibition by HCII (at least from studying 20 different amino acid mutations), this does not eliminate ternary complex formation as a component of the thrombin inhibition in the presence of glycosaminoglycans. In fact, the distinct bell-shaped logarithmic curves in the Fig. 2 *inset* for the exosite-1 thrombin mutants and the rates of inhibition given in Table II imply ternary complex formation, even if the allosteric component is defective.

Taking into account the inhibition data for the library of thrombin mutants with HCII-glycosaminoglycans, our results support the double bridge mechanism that is composed of a lesser function of ternary complex formation (1–2 orders of magnitude acceleration) mediated by glycosaminoglycans binding to both HCII and thrombin (presumably via exosite-2) coupled with a dominant role for the allosteric process (2–3 orders of magnitude acceleration) driven through HCII binding to thrombin exosite-1 (30, 38). Thus, our results emphasize the vital importance of the allosteric mechanism for HCII-thrombin inhibition in the presence of glycosaminoglycans and imply that this process is promoted both by electrostatic steering of the basic exosite-1 region optimally aligning the HCII acidic domain and by hydrophobic contacts, which is reminiscent of the hirudin carboxyl-terminal tail/thrombin exosite-1 interaction (63).

REFERENCES

1. Coleman, R. W., Hirsh, J., Marder, V. J., Clowes, A. W., and George, J. N. (2001) *Hemostasis and Thrombosis: Basic Principles and Clincal Practice* (4th Ed.), Lippincott Williams and Wilkins, Philadelphia
2. Esmon, C. T. (2003) *Chest* **124,** Suppl. 4, 26S-32S
3. Bode, W., Turk, D., and Karshikov, A. (1992) *Protein Sci.* **1,** 426–471
4. Stone, S. R., Braun, P. J., and Hofsteenge, J. (1987) *Biochemistry* **26,** 4617–4646-4624
5. Sheehan, J. P., Wu, Q., Tollefsen, D. M., and Sadler, J. E. (1993) *J. Biol. Chem.* **268,** 3639–3645
6. Sheehan, J. P., and Sadler, J. E. (1994) *Proc. Natl. Acad. Sci. U. S. A.* **91,** 5518–5522
7. Gan, Z. R., Li, Y., Chen, Z., Lewis, S. D., and Shafer, J. A. (1994) *J. Biol. Chem.* **269,** 1301–1305
8. Schedin-Weiss, S., Arocas, V., Bock, S. C., Olson, S. T., and Bjork, I. (2002) *Biochemistry* **41,** 12369–12376
9. Tollefsen, D. M. (1997) *Adv. Exp. Med. Biol.* **425,** 35–44
10. Suzuki, K., Nishioka, J., and Hashimoto, S. (1983) *J. Biol. Chem.* **258,** 163–168
11. Scott, R. W., Bergman, B. L., Bajpai, A., Hersh, R. T., Rodriguez, H., Jones, B. N., Barreda, C., Watts, S., and Baker, J. B. (1985) *J. Biol. Chem.* **260,** 7029–7034
12. Tollefsen, D. M., Pestka, C. A., and Monafo, W. J. (1983) *J. Biol. Chem.* **258,** 6713–6716
13. Pratt, C. W., Whinna, H. C., and Church, F. C. (1992) *J. Biol. Chem.* **267,** 8795–8801
14. Blinder, M. A., Andersson, T. R., Abildgaard, U., and Tollefsen, D. M. (1989) *J. Biol. Chem.* **264,** 5128–5133
15. Blinder, M. A., and Tollefsen, D. M. (1990) *J. Biol. Chem.* **265,** 286–291
16. Ragg, H., Ulshofer, T., and Gerewitz, J. (1990) *J. Biol. Chem.* **265,** 22386–22391
17. Whinna, H. C., Blinder, M. A., Szewczyk, M., Tollefsen, D. M., and Church, F. C. (1991) *J. Biol. Chem.* **266,** 8129–8135
18. Desai, U., Swanson, R., Bock, S. C., Bjork, I., and Olson, S. T. (2000) *J. Biol. Chem.* **275,** 18976–18984
19. Ersdal-Badju, E., Lu, A., Zuo, Y., Picard, V., and Bock, S. C. (1997) *J. Biol. Chem.* **272,** 19393–19400
20. Arocas, V., Turk, B., Bock, S. C., Olson, S. T., and Bjork, I. (2000) *Biochemistry* **39,** 8512–8518
21. He, L., Vicente, C. P., Westrick, R. J., Eitzman, D. T., and Tollefsen, D. M. (2002) *J. Clin. Invest.* **109,** 213–219
22. Shirk, R. A., Parthasarathy, N., San Antonio, J. D., Church, F. C., and Wagner, W. D. (2000) *J. Biol. Chem.* **275,** 18085–18092
23. Baglin, T. P., Carrell, R. W., Church, F. C., Esmon, C. T., and Huntington, J. A. (2002) *Proc. Natl. Acad. Sci. U. S. A.* **99,** 11079–11084
24. Liaw, P. C. Y., Austin, R. C., Fredenburgh, J. C., Stafford, A. R., and Weitz, J. I. (1999) *J. Biol. Chem.* **274,** 27597–27604
25. Bauman, S. J., and Church, F. C. (1999) *J. Biol. Chem.* **274,** 34556–34565
26. Mitchell, J. W., and Church, F. C. (2002) *J. Biol. Chem.* **277,** 19823–19830
27. Han, J.-H., Cote, H. C. F., and Tollefsen, D. M. (1997) *J. Biol. Chem.* **272,** 28660–28665
28. Verhamme, I. M., Bock, P. E., and Jackson, C. M. (2004) *J. Biol. Chem.* **279,** 9785–9795
29. Huntington, J. A., and Esmon, C. T. (2003) *Structure* (*Camb.*) **11,** 469–479
30. van Deerlin, V. M. D., and Tollefsen, D. M. (1991) *J. Biol. Chem.* **266,** 20223–20231
31. Church, F. C., and Whinna, H. C. (1986) *Anal. Biochem.* **157,** 77–83
32. Griffith, M. J., Noyes, C. M., Tyndall, J. A., and Church, F. C. (1985) *Biochemistry* **24,** 6777–6782
33. Hall, S. W., Nagashima, M., Zhao, L., Morser, J., and Leung, L. L. (1999) *J. Biol. Chem.* **274,** 25510–25516
34. Myles, T., Yun, T. H., Hall, S. W., and Leung, L. L. (2001) *J. Biol. Chem.* **276,** 25143–25149
35. Teien, A. N., Abildgaard, U., and Höök, M. (1976) *Thromb. Res.* **8,** 859–867
36. Berman, H. M., Westbrook, J., Feng, Z., Gilliland, G., Bhat, T. N., Weissig, H., Shindyalov, I. N., and Bourne, P. E. (2000) *Nucleic Acids Res.* **28,** 235–242
37. Tsiang, M., Jain, A. K., and Gibbs, C. S. (1997) *J. Biol. Chem.* **272,** 12024–12029
38. Myles, T., Church, F. C., Whinna, H. C., Monard, D., and Stone, S. R. (1998) *J. Biol. Chem.* **273,** 31203–31208
39. Tsiang, M., Jain, A. K., Dunn, K. E., Rojas, M. E., Leung, L. L., and Gibbs, C. S. (1995) *J. Biol. Chem.* **270,** 16854–16863
40. Pineda, A. O., Cantwell, A. M., Bush, L. A., Rose, T., and Di Cera, E. (2002) *J. Biol. Chem.* **277,** 32015–32019
41. Ayala, Y. M., Cantwell, A. M., Rose, T., Bush, L. A., Arosio, D., and Di Cera, E. (2001) *Proteins* **45,** 107–116
42. De Cristofaro, R., and De Filippis, V. (2003) *Biochem. J.* **373,** 593–601
43. Myles, T., Yun, T. H., and Leung, L. L. (2002) *Blood* **100,** 2820–2826
44. Yun, T. H., Baglia, F. A., Myles, T., Navaneetham, D., Lopez, J. A., Walsh, P. N., and Leung, L. L. (2003) *J. Biol. Chem.* **278,** 48112–48119
45. Pineda, A. O., Carrell, C. J., Bush, L. A., Prasad, S., Caccia, S., Chen, Z. W., Mathews, F. S., and Di Cera, E. (2004) *J. Biol. Chem.* **279,** 31842–31853
46. Shirk, R. A., Church, F. C., and Wagner, W. D. (1996) *Arterioscler. Thromb. Vasc. Biol.* **16,** 1138–1146
47. Whinna, H. C., Choi, H. U., Rosenberg, L. C., and Church, F. C. (1993) *J. Biol. Chem.* **268,** 3920–3924
48. Hoffman, M., Loh, K. L., Bond, V. K., Palmieri, D., Ryan, J. L., and Church, F. C. (2003) *Exp. Mol. Pathol.* **75,** 109–118
49. Myles, T., Le Bonniec, B. F., Betz, A., and Stone, S. R. (2001) *Biochemistry* **40,** 4972–4979
50. Phillips, J. E., Shirk, R. A., Whinna, H. C., Henriksen, R. A., and Church, F. C. (1993) *J. Biol. Chem.* **268,** 3321–3327
51. Rogers, S. J., Pratt, C. W., Whinna, H. C., and Church, F. C. (1992) *J. Biol. Chem.* **267,** 3613–3617
52. Sheehan, J. P., Tollefsen, D. M., and Sadler, J. E. (1994) *J. Biol. Chem.* **269,** 32747–32751
53. Cooper, S. T., Rezaie, A. R., Esmon, C. T., and Church, F. C. (2002) *Thromb. Res.* **107,** 67–73

54. Ye, J., Rezaie, A. R., and Esmon, C. T. (1994) *J. Biol. Chem.* **269,** 17965–17970
55. He, X., Ye, J., Esmon, C. T., and Rezaie, A. R. (1997) *Biochemistry* **36,** 8969–8976
56. Rezaie, A. R. (1996) *Biochemistry* **35,** 1918–1924
57. Rezaie, A. R., and Olson, S. T. (1997) *Biochemistry* **36,** 1026–1033
58. Carter, W. J., Myles, T., Gibbs, C. S., Leung, L. L., and Huntington, J. A. (2004) *J. Biol. Chem.* **279,** 26387–26394
59. Hall, S. W., Gibbs, C. S., and Leung, L. L. (2001) *Thromb. Haemostasis* **86,** 1466–1474
60. Tollefsen, D. M. (1995) *Thromb. Haemostasis* **74,** 1209–1214
61. Ragg, H., Ulshofer, T., and Gerewitz, J. (1990) *J. Biol. Chem.* **265,** 5211–5218
62. Holland, C. A., Henry, A. T., Whinna, H. C., and Church, F. C. (2000) *FEBS Lett.* **484,** 87–91
63. Rydel, T. J., and Tulinsky, A. (1991) *J. Mol. Biol.* **221,** 583–601