THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 266, No. 13, Issue of May 5, pp. 8129-8135, 1991
Printed in U.S.A.

Role of Lysine 173 in Heparin Binding to Heparin Cofactor II*

(Received for publication, December 12, 1990)

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Heparin cofactor II (HC) is a plasma serine proteinase inhibitor (serpin) that inhibits α-thrombin in a reaction that is dramatically enhanced by heparin and other glycosaminoglycans/polyanions. We investigated the glycosaminoglycan binding site in HC by: (i) chemical modification with pyridoxal 5′-phosphate (PLP) in the absence and presence of heparin and dermatan sulfate; (ii) molecular modeling; and (iii) site-directed oligonucleotide mutagenesis. Four lysyl residues (173, 252, 343, and 348) were protected from modification by heparin and to a lesser extent by dermatan sulfate. Heparin-protected PLPHC retained both heparin cofactor and dermatan sulfate cofactor activity while dermatan sulfate-protected PLPHC retained some dermatan sulfate cofactor activity and little heparin cofactor activity. Molecular modeling studies revealed that Lys^{173} and Lys^{252} are within a region previously shown to contain residues involved in glycosaminoglycan binding. Lys^{343} and Lys^{348} are distant from this region, but protection by heparin and dermatan sulfate might result from a conformational change following glycosaminoglycan binding to the inhibitor. Site-directed mutagenesis of Lys^{173} and Lys^{343} was performed to further dissect the role of these two regions during HC-heparin and HC-dermatan sulfate interactions. The $Lys^{343} \rightarrow$ Asn or Thr mutants had normal or only slightly reduced heparin or dermatan sulfate cofactor activity and eluted from heparin-Sepharose at the same ionic strength as native recombinant HC. However, the $Lys^{173} \rightarrow$ Gln or Leu mutants had greatly reduced heparin cofactor activity and eluted from heparin-Sepharose at a significantly lower ionic strength than native recombinant HC but retained normal dermatan sulfate cofactor activity. Our results demonstrate that Lys^{173} is involved in the interaction of HC with heparin but not with dermatan sulfate, whereas Lys^{343} is not critical for HC binding to either glycosaminoglycan. These data provide further evidence for the determinants required for glycosaminoglycan binding to HC.

Heparin cofactor II (HC)[1] and antithrombin III (AT) are members of the family of serine proteinase inhibitors (serpins) in human plasma. Both proteins inhibit proteinases by forming a covalent complex with the proteinase active site in a 1:1 molar ratio (1-3). Although AT inhibits most of the proteinases involved in coagulation, HC exerts its anticoagulant effect by specifically inhibiting thrombin (2, 4).

Heparin is a highly negatively charged glycosaminoglycan composed of alternating residues of glucosamine and uronic acid (5). Heparin increases by several orders of magnitude the *in vitro* and *ex vivo* rates of thrombin inhibition by both AT and HC (1, 6, 7). Dermatan sulfate is another glycosaminoglycan, consisting of alternating galactosamine and uronic acid residues, which acts specifically on HC inhibition of thrombin and does not accelerate the inhibition of any coagulation proteinase by AT (4). There is extensive evidence that the effect of heparin and dermatan sulfate is mediated through the formation of a ternary complex with both the inhibitor and thrombin binding to the same glycosaminoglycan molecule (8-11).

Investigations of natural mutations as well as chemical modification studies have identified two regions in AT, from Gly^{35} to Glu^{50} and Lys^{107} to Lys^{136}, that are important for heparin binding. Included in these regions are several essential lysyl and arginyl residues which are thought to interact with the negatively charged glycosaminoglycans (12-32). Molecular modeling studies, using the crystallographic data from the related serpin α_1-proteinase inhibitor (α_1-PI; 29), have shown that these two regions of AT are also close in the tertiary structure of the molecule and may together form the heparin binding site in AT (26).

HC shows little homology to the Gly^{35} to Glu^{50} region of AT, but extensive homology to the Lys^{107} to Lys^{136} region, which extends from Lys^{165} to Phe^{195} in HC. This region has been suggested as the putative glycosaminoglycan binding site in HC (33-35). Lysyl and arginyl residues have also been shown to be essential for glycosaminoglycan binding by HC (30, 34, 36) and specific residues in this region have been shown to be important for these interactions (34, 35, 37). In this study we used pyridoxal 5′-phosphate (PLP) to selectively modify lysines in the absence and presence of heparin and dermatan sulfate in order to discover lysines in HC possibly involved in binding to these glycosaminoglycans. We evaluated these results and studied the structure of HC by computer-assisted molecular modeling. We then used site-

* This work was supported in part by Research Grants HL-32656 and HL-14147 (Specialized Center of Research in Thrombosis) from the National Institutes of Health. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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[1] The abbreviations used are: HC, heparin cofactor II; serpin, serine proteinase inhibitor; AT, antithrombin III; α_1-PI, α_1-proteinase inhibitor; PLP, pyridoxal 5′-phosphate; PLPHC, heparin cofactor II modified by pyridoxal 5′-phosphate; rHC, recombinant HC; HPLC, high-performance liquid chromatography; TPCK-trypsin, tosylphenylalanylchloromethyl ketone-treated trypsin; Chromozym TH, N^{α}-*p*-tosyl-Gly-Pro-Arg-*p*-nitroanilide.

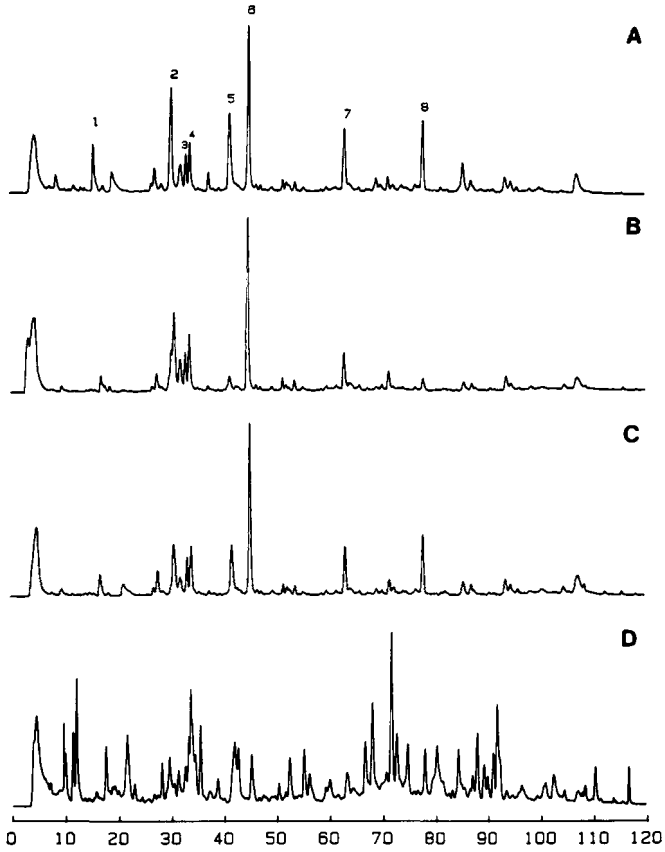

FIG. 1. **Tryptic peptide maps of PLPHC.** Approximately 1 mg of HC modified with PLP in the absence (*panel A*) and in the presence of heparin (*panel B*) or dermatan sulfate (*panel C*) were chromatographed by reverse-phase high-performance liquid chromatography as described under "Experimental Procedures." Phosphopyridoxylated lysine-containing peptides were detected by absorbance at 325 nm. *Panel D* shows detection of all peptides in unprotected HC by absorbance at 210 nM. Time scale for all panels is in minutes. Absorbance full scale is 0.1 for *panels A-C* and 1.5 for *panel D*.

directed mutagenesis to further assess the involvement of Lys^{173} and Lys^{343} in glycosaminoglycan binding.

EXPERIMENTAL PROCEDURES[2]

RESULTS

Identification of Lysyl Residues in HC Protected by Heparin and Dermatan Sulfate from Phosphopyridoxylation—In agreement with our previous observations (30), when HC was modified in the absence of glycosaminoglycan, an average of 2.7 mol of PLP were incorporated per mol of HC. In the presence of 1 mg/ml heparin or dermatan sulfate an average of 1.9 or 2.5 mol of PLP/mol of HC were incorporated, respectively. Increasing the dermatan sulfate concentration tenfold did not increase the protection (data not shown). Tryptic maps were next produced in order to identify which lysines had been modified. The tryptic peptide map of unprotected PLPHC showed eight major peaks at 325 nm where PLP absorbs (Fig. 1, *panel A*). These eight peaks yielded six peptides, the sequences of which are shown in Table I. When rechromatographed, *peaks 2–4* in Fig. 1, *panel A*, gave a mixture of the peptides containing Lys^{65} and Lys^{303}. All the other peaks gave one 325-nm peak when rechromatographed.

The tryptic maps of heparin-protected PLPHC and dermatan sulfate-protected PLPHC (Fig. 1, *panels B* and *C*, respectively) show that *peaks 1, 5, 7,* and *8* were reduced in the heparin-protected sample and to a lesser extent in the dermatan sulfate-protected sample. These results also show that Lys^{65} and Lys^{303} were modified to essentially the same extent in all three PLPHC samples. The extent of protection was determined (Table II). In heparin-protected PLPHC both Lys^{173} and Lys^{252} showed only about 20% of the modification that they showed in the unprotected sample, whereas Lys^{343} and Lys^{348} showed 31 and 57% modification, respectively. The protection was not as extensive in dermatan sulfate-protected PLPHC. Lys^{173} and Lys^{252} showed 81 and 57% modification, respectively, compared with the unprotected sample, whereas Lys^{343} and Lys^{348} showed 52 and 74% modification, respectively, compared with unprotected PLPHC.

Interaction of Modified HC with Heparin and Dermatan Sulfate—The ability of heparin and dermatan sulfate to accelerate thrombin inhibition by the modified HC species was studied. Control experiments showed that all samples had normal antithrombin activity (inhibition of thrombin by HC in the absence of glycosaminoglycans) consistent with previous results (Ref. 30; data not shown). Fig. 2 shows thrombin inhibition by the various modified HC species as a function of heparin and dermatan sulfate concentration.

Over a range of 0.1–120 μg/ml heparin the reduced control HC sample (treated with sodium borohydride but not PLP) showed a normal pattern of increasing and then decreasing inhibition, with a maximum inhibition at 10 μg/ml heparin (Fig. 2*A*). The modified inhibitors also showed this pattern, although none retained the full activity of the reduced control HC. Also, the maximum inhibition was shifted to 40 μg/ml heparin, probably due to the repulsion of the heparin by the negatively charged PLP moiety. The unprotected PLPHC retained ~20% heparin cofactor activity, which is consistent

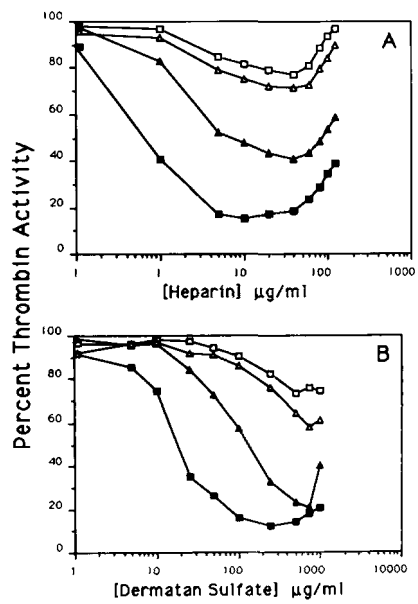

FIG. 2. **Effect of glycosaminoglycan concentration on inhibition of thrombin by PLPHC.** 5 nM thrombin was incubated with 50-nM reduced control HC (■), unprotected PLPHC (□), heparin-protected PLPHC (▲), or dermatan sulfate-protected PLPHC (△) as described under "Experimental Procedures." *Panel A*, effect of increasing heparin concentrations (0.1–120 μg/ml). *Panel B*, effect of increasing dermatan sulfate concentrations (1–1000 μg/ml). Results are expressed as percent of thrombin activity in the absence of inhibitor.

[2] Portions of this paper (including "Experimental Procedures" and Tables I-III) are presented in miniprint at the end of this paper. Miniprint is easily read with the aid of a standard magnifying glass. Full size photocopies are included in the microfilm edition of the Journal that is available from Waverly Press.

with similar chemical modification studies with AT (21). Heparin-protected PLPHC and dermatan sulfate-protected PLPHC retained ~66 and ~23% heparin cofactor activity, respectively. Over a dermatan sulfate range of 1–1000 μg/ml, the reduced control HC sample again showed a normal pattern of increasing and then decreasing inhibition, with a maximum inhibition at 250 μg/ml dermatan sulfate (Fig. 2*B*). Unprotected PLPHC retained ~20% dermatan sulfate cofactor activity and dermatan sulfate-protected PLPHC retained ~33%, relative to the reduced control HC sample. However, heparin-protected PLPHC retained 91% dermatan sulfate cofactor activity, although twice as much dermatan sulfate was necessary to reach maximum inhibition. Thus heparin (with a greater extent of lysine protection) was able to protect both heparin and dermatan sulfate cofactor activity in HC, whereas dermatan sulfate (with a lesser extent of protection) could only minimally protect dermatan sulfate cofactor activity.

Molecular Modeling of HC—We utilized computer-assisted molecular modeling in order to visualize and further compare the putative glycosaminoglycan binding regions of AT and HC. As has been done with AT (26), we modeled HC based on its serpin homologue α_1-PI. Shown in Fig. 3 are the 4 lysyl residues protected in the presence of heparin and dermatan sulfate from modification by PLP. Also shown are several residues in the Lys^{165} to the Phe^{195} region of HC known to be involved in glycosaminoglycan binding. This region includes Lys^{173} and is homologous with the proposed heparin binding region in AT. Lys^{252} is nearby with respect to tertiary structure. These residues form a region of positive charge on the surface of the molecule, which could interact with glycosaminoglycans and other polyanions. Lys^{343} and Lys^{348} are not near the putative binding site, but are in a region homologous to a region in AT in which lysines were protected by heparin, but not found to be essential for heparin binding (23). These residues may be protected by a conformational change upon glycosaminoglycan binding or by nonspecific interactions of these residues with the glycosaminoglycans.

Expression of Recombinant HC—Mutated recombinant HC (rHC) molecules were created by two distinct mutagenesis techniques resulting in a Leu or Gln substitution for Lys^{173} and Thr or Asn substitution for Lys^{343}. Each of the mutations eliminated the positive charge on the side chain of the amino acid. The final construct was expressed in *Escherichia coli* as product of the vector pMON-HCII. Besides the absence of the signal peptide, the protein product lacked the NH_2-terminal 18 amino acid residues and the post-translational modifications of plasma HC and has been characterized previously (34, 35). The rHC variants appeared to be identical in size to native rHC and had an apparent molecular weight of 55,000 determined by immunoblot analysis of concentrated lysate subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (not shown).

Binding of rHC to Heparin Sepharose—Native rHC and each of the variants were subjected to heparin-Sepharose chromatography to determine their relative affinities for heparin. Native rHC and both of the Lys^{343} substitutions were eluted from the column with a peak at ~0.35 M NaCl suggesting that the positive charge of this residue is not necessary for the interaction with heparin (Fig. 4*A*). With native rHC, ~10% of the immunoreactive protein was detected in the column flow-through, but ~25–30% was identified in the flow through of the $Lys^{343} \rightarrow$ Thr or Asn variants (data not shown). Under the same conditions, we have previously demonstrated that the column capacity had not been exceeded and that the flow through may represent denatured rHC (35). In contrast, $Lys^{173} \rightarrow$ Leu or Gln resulted in protein products that were eluted from heparin-Sepharose at a lower ionic strength (Fig. 4*B*). The peak of native rHC eluted at ~0.38 M NaCl, whereas rHC ($Lys^{173} \rightarrow$ Gln) and rHC ($Lys^{173} \rightarrow$ Leu) eluted at ~0.26 and 0.15 M NaCl, respectively. For both of these rHC variants, a small fraction of immunoreactive protein (≤10%) was detected in the column flow-through.

Glycosaminoglycan-independent Thrombin Inhibition by rHC Variants—Thrombin inhibition was measured in the absence of glycosaminoglycan for native rHC and each of the rHC variants (Table III), as described previously (47). Native rHC had a second-order rate constant of 1.1–1.6 × 10^5 M^{-1} min^{-1} which is ~4-fold lower than previously reported by Tollefsen *et al.* (7). Using the same truncated form of native rHC, Blinder and Tollefsen (35) have previously reported a

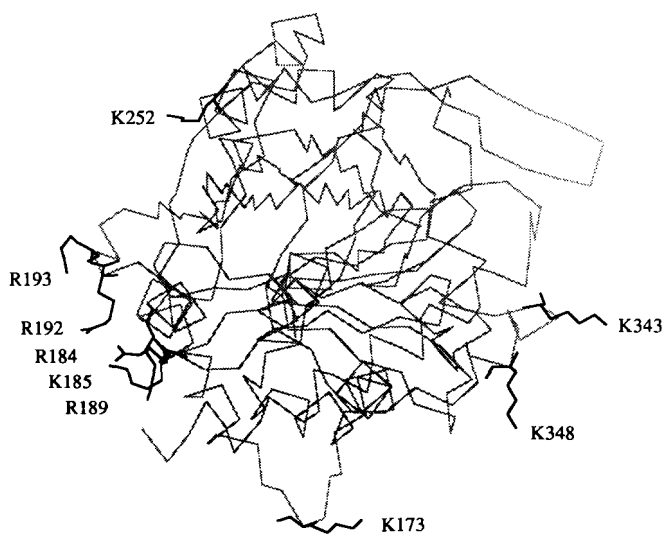

FIG. 3. **Molecular modeling of HC based on its serpin homologue α_1-PI.** The protein backbone is shown in *gray*. Shown in black are the four lysyl residues protected by heparin and dermatan sulfate from modification by PLP and residues known to be involved in glycosaminoglycan binding to HC.

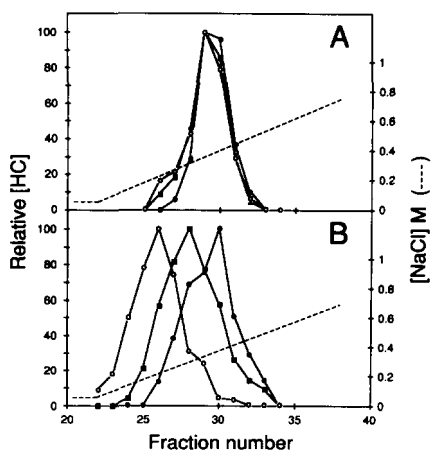

FIG. 4. **Heparin-Sepharose chromatography of rHC and rHC variants.** One ml of *E. coli* lysates containing rHC or rHC variants was applied to a 2-ml heparin-Sepharose column and eluted with a linear gradient of NaCl. An aliquot of each fraction was subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis and the rHC was detected on an immunoblot probed with ^{125}I-labeled anti-HC IgG. The amount of HC in each fraction was determined by densitometry of the immunoblot. The relative amounts were normalized against a peak fraction (=100) as shown. *Panel A*: ●, native rHC; ○, rHC ($Lys^{343} \rightarrow$ Thr); ■, rHC ($Lys^{343} \rightarrow$ Asn). *Panel B*: ●, native rHC; ○, rHC ($Lys^{173} \rightarrow$ Leu); ■, rHC $Lys^{173} \rightarrow$ Gln).

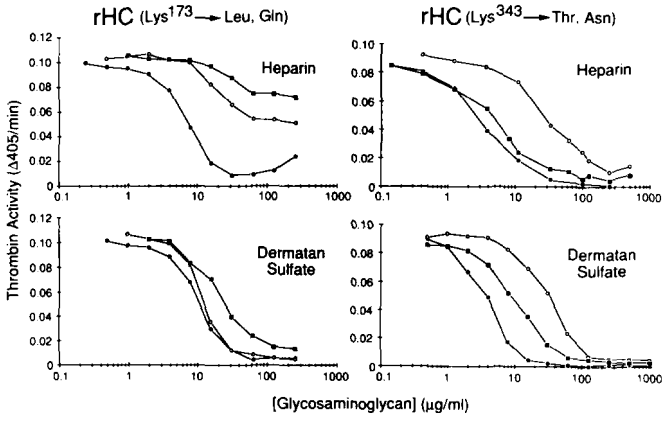

FIG. 5. **Effect of glycosaminoglycan concentration on inhibition of thrombin by rHC variants of Lys^{173} and Lys^{343}.** Incubations were performed with either 155–168 nM rHC for the experiments with variants of Lys^{343} or 40–48 nM rHC for those of variants of Lys^{173}. Thrombin (14 nM) and heparin or dermatan sulfate were added at the final concentration indicated. *Left-hand panels*: ●, native rHC; ○, rHC (Lys^{173} → Leu); ■, rHC (Lys^{173} → Gln). *Right-hand panels*: ●, native rHC; ○, rHC (Lys^{343} → Thr); ■, rHC (Lys^{343} → Asn).

value of 5.7×10^4 M^{-1} min^{-1}. Variations may result from the determinations of rHC concentration in the lysate and in the amounts of denatured protein that occur in each preparation. The mutation Lys^{343} → Thr produced only modest changes in the second-order rate constant providing evidence that the reactive site of this mutant was intact. In contrast, the Lys^{343} → Asn substitution appeared to decrease the second-order rate constant ~7-fold, suggesting that this molecule had an altered ability to inhibit proteinases in the absence of glycosaminoglycans. The Lys^{173} → Leu and Lys^{173} → Gln substitutions resulted in proteins with very similar rate constants compared with native rHC and therefore retained their ability to inhibit thrombin.

Heparin- and Dermatan Sulfate-dependent Thrombin Inhibition by rHC Variants—The effects of heparin and dermatan sulfate on the thrombin inhibition by rHC and rHC variants are shown in Fig. 5. In these experiments, native rHC inhibited 50% of the thrombin with ~3–7 μg/ml of heparin and ~4–10 μg/ml of dermatan sulfate. The Lys^{343} → Thr variant required similar heparin concentrations and only 2–3-fold more dermatan sulfate than the native rHC to inhibit 50% of the thrombin, suggesting that the charge at Lys^{343} is probably not critical for glycosaminoglycan-dependent inhibition. However, the Lys^{343} → Asn variant required ~10-fold higher concentrations of each glycosaminoglycan for 50% inhibition, which may be accounted for by the decreased rate of thrombin inhibition by this variant in the absence of glycosaminoglycan (Table III).

In contrast, the rHC molecules containing substitutions of Lys^{173} required at least a 30-fold higher concentration of heparin for 50% thrombin inhibition. With dermatan sulfate, however, the Lys^{173} → Leu variant showed normal thrombin inhibition and the Lys^{173} → Gln variant required only a 2-fold higher concentration for 50% inhibition. These observations suggest that the charge at Lys^{173} is required for heparin- but not dermatan sulfate-dependent thrombin inhibition.

DISCUSSION

The glycosaminoglycans heparin and dermatan sulfate exert their anticoagulant effect in human plasma by increasing the inhibition rate of proteinases by the serpins HC and AT by as much as 1000-fold (1, 6, 7). In both cases, binding of the glycosaminoglycan to the inhibitor is necessary for activity, and this binding is thought to occur by ionic interactions between the negatively charged glycosaminoglycan and positively charged amino acids of the inhibitor. Chemical modification, natural mutation, and site-directed mutagenesis of these basic amino acid residues result in inhibitors with decreased binding to glycosaminoglycans (12–32). The binding site of AT is very specific for a pentasaccharide sequence in heparin, and this sequence contains a unique 3-*O*-sulfated glucosamine at the third position. Glycosaminoglycans and other polyanions that do not contain these structures have almost no ability to accelerate proteinase inhibition by AT (48). HC does not require this specific pentasaccharide sequence for acceleration of thrombin inhibition; in fact, the HC/thrombin inhibition reaction can be accelerated by a wide range of polyanions (33, 49–51).

Even though they have different specificities, it is thought that the glycosaminoglycan binding sites in both HC and AT occur in similar regions. Several residues have been shown to be specifically involved in heparin binding to AT, and as a result two regions, Gly^{35} to Glu^{50} and Lys^{107} to Lys^{136}, have been designated as forming the heparin binding domain of AT (16–19, 21, 23, 25, 27, 52). HC contains a region, Lys^{165} to Phe^{195}, that shows extensive homology to the Lys^{107} to Lys^{136} region of AT (Fig. 6). This region is densely populated with positively charged amino acids that could interact with various polyanions and is the putative glycosaminoglycan binding site in HC. Indeed, recent studies have shown the involvement of: (i) Lys^{185} and possibly Arg^{184} and Arg^{193} in heparin binding and (ii) Lys^{185}, Arg^{189}, Arg^{192}, Arg^{193}, and possibly Arg^{184} in dermatan sulfate binding to HC (34, 35, 37).

In this study we have utilized several approaches to define further the determinants of the glycosaminoglycan binding domain in HC. First, we used chemical modification in the absence and presence of heparin or dermatan sulfate to identify lysyl residues that might be involved in glycosaminoglycan binding. We identified 4 lysyl residues (173, 252, 343, and 348) in HC that we were protected from modification in the presence of glycosaminoglycan.

We then used computer-assisted molecular modeling to interpret the above results. Lys^{173} was very well protected from chemical modification by heparin and less so by dermatan sulfate. Lys^{173} is homologous to a residue in AT that has been found to be involved in heparin binding to that inhibitor (21). Our model shows it to be on the surface of the molecule and thus accessible for interaction with glycosaminoglycans. Amphipathic α-helices are thought to be a possible secondary structure through which proteins bind to glycosaminoglycans (33, 53). Lys^{173} is located just before the start of such an α-helix (D-helix using the nomenclature of α_1-PI; Refs. 26 and 29) containing other residues in HC important in glycosaminoglycan binding. These results implicate Lys^{173} in glycosaminoglycan binding to HC, especially in heparin interactions.

Lys^{252} was well protected from chemical modification by heparin and fairly well by dermatan sulfate. Lys^{252} is not

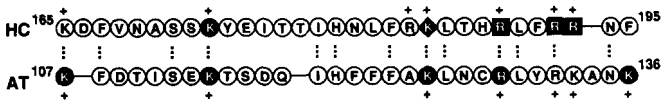

FIG. 6. **Comparison of the glycosaminoglycan binding domains of AT and HC.** Alignment of the Lys^{107} to Lys^{136} region of AT and the Lys^{165} to Phe^{195} region of HC. *Crosses* denote positively charged amino acids. Shown in *black* are residues known to be involved in heparin binding (*circles*), dermatan sulfate binding (*squares*), or both (*diamond*).

within the putative glycosaminoglycan binding of site of HC, but from our model it is close to this region in the tertiary structure of the molecule. Lys^{185}, Arg^{189}, Arg^{192}, Arg^{193}, and possibly Arg^{184} are known to be involved in glycosaminoglycan binding in HC (34, 35, 37) and can be seen in Fig. 3 to form a surface of positive charge, near Lys^{252}, that could interact with the negatively charged glycosaminoglycans. Lys^{252} may be involved in glycosaminoglycan binding, but it is also possible that interactions of other residues in the putative glycosaminoglycan binding site lead indirectly to protection of this lysyl residue from phosphopyridoxylation.

Lys^{343} and Lys^{348} are also not in the putative glycosaminoglycan binding site of HC. These residues were moderately well protected by both heparin and dermatan sulfate, with Lys^{343} being somewhat more protected by each glycosaminoglycan. In our model Lys^{343} and Lys^{348} are also shown to be on the surface of the HC molecule but removed from the other residues known to be involved in glycosaminoglycan binding. These residues are probably not involved in glycosaminoglycan binding and may be protected from chemical modification by a conformational change caused by the glycosaminoglycan binding to HC or by nonspecific ionic interactions between the glycosaminoglycan and HC.

Finally, we performed site-directed mutagenesis based on the information from the chemical modification and modeling studies. We chose Lys^{173} as the most probable residue involved in glycosaminoglycan binding and we chose to mutate Lys^{343} in order to investigate the possible involvement of the region around this residue in glycosaminoglycan binding.

The Lys^{173} variants both bound with less affinity to heparin-Sepharose than did the native rHC. These variant rHC species also showed greatly reduced heparin cofactor activity but essentially normal dermatan sulfate cofactor activity. This is in agreement with the chemical modification data for Lys^{173}. Heparin was better able to protect Lys^{173} from chemical modification than dermatan sulfate was, implying that this residue interacted more with heparin than with dermatan sulfate. Additionally, the heparin-protected PLPHC retained heparin cofactor activity, whereas the dermatan sulfate-protected PLPHC (with 60% less protection of Lys^{173}) retained almost no heparin cofactor activity. These results suggest that Lys^{173} is involved in the binding of heparin to HC but is not critical for the binding of dermatan sulfate to HC. We believe the effects of chemical modification and mutation of Lys^{173} on heparin binding are due to the loss of the positive charge of this residue and therefore the elimination of an electrostatic interaction between the side chain of Lys^{173} and heparin. However, we cannot rule out the possibility that the effects on binding result from alteration of the tertiary structure of the glycosaminoglycan binding domain in HC.

Different results were obtained with the Lys^{343} variants. Both of these variants bound to heparin-Sepharose almost identically to native rHC. The heparin cofactor and dermatan sulfate cofactor activities of the $Lys^{343} \rightarrow$ Thr variant were very similar to those of the native rHC. The $Lys^{343} \rightarrow$ Gln variant had less heparin and dermatan sulfate cofactor activity than rHC, but this can be accounted for by the decreased antithrombin activity of this variant. These results indicate that Lys^{343} is not critical for the binding of either glycosaminoglycan to HC.

A thorough understanding of the interaction of glycosaminoglycans with AT and HC is necessary because of the widespread use of heparin as an anticoagulant. Various residues in the Lys^{165} to Phe^{195} region of HC have been shown to effect both heparin and dermatan sulfate binding and the mutation of Arg^{189} results in deficient dermatan sulfate binding but normal heparin binding (34, 35, 37). In this study we showed that mutation of Lys^{173} leads to deficient heparin binding but normal dermatan sulfate binding. Thus we can state that although heparin and dermatan sulfate bind to the same region of HC, they have specific residues on the protein with which they interact. Further and precise determination of the nature of these interactions between glycosaminoglycans and AT or HC may ultimately lead to the development of better and more specific anticoagulants.

Acknowledgments—We thank the following for their contributions: Dr. David G. Klapper for sequencing the modified peptides; Dr. Mark R. Harris, Dr. Phillip Bowen, and the staff the University of North Carolina Laboratory for Molecular Modeling for their assistance and advice; and Dr. Charlotte W. Pratt for her helpful discussions and comments.

REFERENCES

1. Griffith, M. J., Noyes, C. M., and Church, F. C. (1985) *J. Biol. Chem.* **260,** 2218–2225
2. Tollefsen, D. M., and Blank, M. K. (1981) *J. Clin. Invest.* **68,** 589–596
3. Travis, J., and Salvesen, G. S. (1983) *Annu. Rev. Biochem.* **52,** 655–709
4. Parker, K. A., and Tollefsen, D. M. (1985) *J. Biol. Chem.* **260,** 3501–3505
5. Casu, B. (1985) *Adv. Carbohydr. Chem. Biochem.* **43,** 51–134
6. Bjork, I., and Lindahl, U. (1982) *Mol. Cell. Biochem.* **48,** 161–182
7. Tollefsen, D. M., Pestka, C. A., and Monafo, W. J. (1983) *J. Biol. Chem.* **258,** 6713–6716
8. Peterson, C. B., and Blackburn, M. N. (1987) *J. Biol. Chem.* **262,** 7559–7566
9. Nesheim, M., Blackburn, M. N., Lawler, C. M., and Mann, K. G. (1986) *J. Biol. Chem.* **261,** 3214–3221
10. Griffith, M. J. (1983) *Proc. Natl. Acad. Sci. U. S. A.* **80,** 5460–5464
11. Danielsson, Å., Raub, E., Lindahl, U., and Björk, I. (1986) *J. Biol. Chem.* **261,** 15467–15473
12. Rosenberg, R. D., and Damus, P. S. (1973) *J. Biol. Chem.* **248,** 6490–6505
13. Blackburn, M. N., and Sibley, C. C. (1980) *J. Biol. Chem.* **255,** 824–826
14. Blackburn, M. N., Smith, R. L., Sibley, C. C., and Johnson, V. A. (1981) *Ann. N. Y. Acad. Sci.* **370,** 700–707
15. Pecon, J. M., and Blackburn, M. N. (1984) *J. Biol. Chem.* **259,** 935–938
16. Blackburn, M. N., Smith, R. L., Carson, J., and Sibley, C. C. (1984) *J. Biol. Chem.* **259,** 939–941
17. Koide, T., Odani, S., Takahashi, K., Ono, T., and Sakuragawa, N. (1984) *Proc. Natl. Acad. Sci. U. S. A.* **81,** 289–293
18. Chang, J.-Y., and Tran, T. H. (1986) *J. Biol. Chem.* **261,** 1174–1176
19. Brunel, F., Duchange, N., Fischer, A.-M., Cohen, G. H., and Zakin, M. M. (1987) *Am. J. Hematol.* **25,** 223–224
20. Rosenfeld, L., and Danishefsky, I. (1986) *Biochem. J.* **237,** 639–646
21. Liu, C.-S., and Chang, J.-Y. (1987) *J. Biol. Chem.* **262,** 17356–17361
22. Liu, C.-S., and Chang, J.-Y. (1987) *Eur. J. Biochem.* **167,** 247–252
23. Peterson, C. B., Noyes, C. M., Pecon, J. M., Church, F. C., and Blackburn, M. N. (1987) *J. Biol. Chem.* **262,** 8061–8065
24. Smith, J. W., and Knauer, D. J. (1987) *J. Biol. Chem.* **262,** 11964–11972
25. Owen, M. C., Borg, J. Y., Soria, C., Soria, J., Caen, J., and Carrell, R. W. (1987) *Blood* **69,** 1275–1279
26. Borg, J. Y., Owen, M. C., Soria, C., Soria, J., Caen, J., and Carrell, R. W. (1988) *J. Clin. Invest.* **81,** 1292–1296
27. Chang, J.-Y., (1989) *J. Biol. Chem.* **264,** 3111–3115
28. Sun, X.-J., and Chang, J.-Y. (1989) *J. Biol. Chem.* **264,** 11288–11293
29. Loebermann, H., Tukuok, R., Deisenhofer, J., and Huber, R. (1984) *J. Mol. Biol.* **107,** 531–556
30. Church, F. C., and Griffith, M. J. (1984) *Biochem. Biophys. Res. Commun.* **124,** 745–751
31. Gandrille, S., Aiach, M., Lane, D., Vidaud, D., Molho-Sabatier,

P., Caso, R., de Moerloose, P., Fiessinger, J.-N., and Clauser, E. (1990) *J. Biol. Chem.* **265,** 18997–19001
32. Sun, X.-J., and Chang, J.-Y. (1990) *Biochemistry* **29,** 8957–8962
33. Church, F. C., Meade, J. B., Treanor, R. E., and Whinna, H. C. (1989) *J. Biol. Chem.* **264,** 3618–3623
34. Blinder, M. A., Andersson, T. R., Abildgaard, U., and Tollefsen, D. M. (1989) *J. Biol. Chem.* **264,** 5128–5133
35. Blinder, M. A., and Tollefsen, D. M. (1990) *J. Biol. Chem.* **265,** 286–291
36. Church, F. C., Villaneuva, C. B., and Griffith, M. J. (1986) *Arch. Biochem. Biophys.* **246,** 175–184
37. Ragg, H., Ulshöfer, T., and Gerewitz, J. (1990) *J. Biol. Chem.* **265,** 5211–5218
38. Church, F. C., and Whinna, H. C. (1986) *Anal. Biochem.* **157,** 77–83
39. Blinder, M. A., Marasa, J. C., Reynolds, C. H., Deaven, L. L., and Tollefsen, D. M. (1988) *Biochemistry* **27,** 752–759
40. Teien, A. N., Abildgaard, U., and Höök, M. (1976) *Thromb. Res.* **8,** 859–867
41. Nakamaye, K. L., and Eckstein, F. (1986) *Nucleic Acids Res.* **14,** 9679–9698
42. Sanger, F., Nicklen, S., and Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U. S. A.* **74,** 5463–5467
43. Wells, J. A., Vasser, M., and Powers, D. B. (1985) *Gene (Amst.)* **34,** 315–323
44. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual,* pp. 17.23–17.24, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
45. Liszewski, M. K., Kumar, V., and Atkinson, J. P. (1989) *BioTechniques* **7,** 1079–1081
46. Li, E., Locke, B., Yang, N. C., Ong, D. E., and Gordon, J. I. (1987) *J. Biol. Chem.* **262,** 13773–13779
47. Derechin, V., Blinder, M. A., and Tollefsen, D. M. (1990) *J. Biol. Chem.* **265,** 5623–5628
48. Casu, B. (1989) *Ann. N. Y. Acad. Sci.* **556,** 1–17
49. Church, F. C., Treanor, R. E., Sherrill, G. B., and Whinna, H. C. (1987) *Biochem. Biophys. Res. Commun.* **148,** 362–368
50. Church, F. C., Pratt, C. W., Treanor, R. E., and Whinna, H. C. (1988) *FEBS Lett.* **237,** 26–30
51. Pratt, C. W., Whinna, H. C., Meade, J. B., Treanor, R. E., and Church, F. C. (1989) *Ann. N. Y. Acad. Sci.* **556,** 104–114
52. Brennan, S. O., George, P. M., and Jordan, R. E. (1987) *FEBS Lett.* **219,** 431–436
53. Cardin, A. D., and Weintraub, H. J. R. (1989) *Arteriosclerosis,* **9,** 21–32

Supplementary Material
to
THE ROLE OF LYSINE 173 IN HEPARIN BINDING TO HEPARIN COFACTOR II
by
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EXPERIMENTAL PROCEDURES

MATERIALS

HC and α-thrombin were purified as described previously (1, 34, 38). Affinity purified rabbit anti-HC antibodies were prepared and labeled with $Na^{125}I$ as described previously (39). Heparin was obtained courtesy of Dr. G. van Dedem of Diosynth BV, Oss, The Netherlands or from Sigma. Dermatan sulfate was from Calbiochem or Sigma, and was treated with nitrous acid for the removal of contaminating heparin and heparan sulfate (40). Pyridoxal 5'-phosphate and sodium borohydride were purchased from Sigma. Guanidine hydrochloride (Sequanal grade) was purchased from Pierce. Tosylphenylalanylchloromethyl ketone-treated trypsin (TPCK-trypsin) and dithiothreitol were obtained from Cooper-Biomedical and Research Organics, respectively. Polybrene and iodoacetic acid were from Aldrich. The chromogenic thrombin substrate N^{α}-p-tosyl-Gly-Pro-Arg-p-nitroanilide (Chromozym TH) was purchased from Boehringer-Mannheim. All other reagents and buffers were of reagent grade or better.

All restriction endonucleases and DNA modifying enzymes were products of Amersham or New England Biolabs. DNA sequencing was performed using modified T7 polymerase from United States Biochemical Corp. The reagents for oligonucleotide-directed mutagenesis were purchased from Amersham. Taq DNA polymerase and other reagents used in the polymerase chain reaction were products of Perkin Elmer Cetus. The synthetic oligonucleotides were provided by the Protein Chemistry Facility of Washington University and were used without further purification.

METHODS

Chemical Modification of HC- HC (20 μM) in 0.05 M triethanolamine acetate, 0.1 % polyethylene glycol (M_r= 8,000), pH 8.0, was modified for 15 minutes with a 92-fold excess of PLP in the absence and presence of 1 mg/ml heparin or dermatan sulfate, at 25 °C and in the dark as much as possible. The samples were treated with 0.1 M sodium borohydride (dissolved in ice-cold water) for another 15 minutes at 0 °C. The reduced samples were then run over a Sephadex G-25 column (1 X 40 cm) equilibrated in 0.05 M triethanolamine acetate, 0.1 % polyethylene glycol (M_r= 8,000), pH 8.0 containing 0.25 M NaCl in order to remove the majority of unreacted PLP and sodium borohydride. Fractions containing protein (determined by absorbance at 280 nm using an extinction coefficient of 0.593 ml mg^{-1} cm^{-1} for HC) were pooled and dialyzed against 2 X 2 liters of 0.05 M triethanolamine acetate, 0.1 % polyethylene glycol (M_r= 8,000), pH 8.0 with 0.25 M NaCl in order to remove any remaining reagents. Concentration of PLP bound to HC was determined by absorbance at 325 nm using an extinction coefficient of 9710 M^{-1} cm^{-1} (30).

Identification of Modified Residues in HC- Tryptic digests of the modified proteins were prepared essentially as described (23). The PLPHC species were denatured by dialysis overnight against 6 M guanidine HCl, 0.5 M TRIS, 0.002 M EDTA, pH 8.1. The proteins were reduced and S-carboxymethylated by addition of 1 mg of dithiothreitol, incubated at room temperature for 3 hours, followed by the addition of 200 μL of 120 mg/ml iodoacetic acid in 1 N NaOH (prepared in the dark) and reacted for 15 minutes in dim light. The samples were then dialyzed against 2 X 2 liters of 0.1 M ammonium bicarbonate overnight. After dialysis 50 μL of 0.5 mg/ml TPCK-trypsin in 1 mM HCl was added and the samples digested for 4 hours at 37 °C. At that time another 50 μL of TPCK-trypsin was added and the samples digested for an additional 27 hours at 37 °C.

Tryptic maps were produced on a Phenomenex, W-Porex C-18 column (4.6 mm inner diameter X 250 mm, with 5 μm particles) at 45 °C and a flow rate of 1 ml/min using a Beckman Model 332 gradient liquid chromatograph with a Hewlett-Packard 1040A diode array detector. Solvent A was 0.05 M sodium phosphate, pH 6.5; solvent B was acetonitrile. Solvent strength was increased linearly from 1 to 28% solvent B in 100 minutes and then to 62% solvent B in 55 minutes. The elution was monitored at 210, 280, and 325 nm. Peptides absorbing at 325 nm were collected and rechromatographed on the same column with solvent A being 0.1% (v/v) trifluoroacetic acid in water and solvent B being 0.085% trifluoroacetic acid in acetonitrile. The gradient was 1% solvent B to 62% solvent B in 61 minutes. Amino acid sequences were determined on an Applied Biosystems 470A gas phase sequencer with an online Waters HPLC. Phosphopyridoxylated lysine was identified by the absence of the usual lysine peak (23).

Functional Analysis of Modified HC- The effect of glycosaminoglycan concentration on the inhibition of thrombin by the various modified species was determined in the following manner. Modified HC species were compared to a "reduced control" HC treated with sodium borohydride but not PLP. In a total volume of 0.1 ml, thrombin (5 nM) and HC (50 nM) in 0.02 M HEPES, 0.15 M NaCl, 0.1% polyethylene glycol (M_r= 8,000), pH 7.4 were incubated at room temperature with increasing amounts of glycosaminoglycan (heparin 0.1-120 μg/ml; dermatan sulfate 5-1000 μg/ml). After 15 seconds, 0.8 ml of 150 μM Chromozym TH in 0.02 M HEPES, 0.15 M NaCl, 0.1% polyethylene glycol (M_r= 8,000), pH 7.4 with enough polybrene to complex the glycosaminoglycan (1 mg/ml for heparin; 10 mg/ml for dermatan sulfate) was added to the reaction mixture. The remaining active thrombin was allowed to react with Chromozym TH for 10 minutes at which time the reaction was stopped by the addition of 0.1 ml of glacial acetic acid. The samples were centrifuged to precipitate any glycosaminoglycan/polybrene complex and the amount of Chromozym TH hydrolyzed, measured at 404 nm, was taken to be proportional to remaining thrombin activity. Controls, with thrombin and glycosaminoglycan only, showed no effect of glycosaminoglycan concentration on the synthetic substrate activity of thrombin.

Computer-Aided Molecular Modeling - The amino acid sequences of HC and α_1-PI were obtained from the literature. The three-dimensional structure of α_1-PI was obtained from the Brookhaven Protein Data Bank based on the work of Loebermann *et al* (29). The amino acid sequence of α_1-PI was changed to that of HC using the SYBYL/MENDYL software package from TRIPOS ASSOCIATES, INC., St. Louis, MO. Regions lacking coordinates, such as the amino-terminus, insertions, or deletions, were ignored in the simulation. The mutated protein was minimized using the force-fields in SYBYL/MENDYL. The drawings were made using the Tripos NITRO program on a Macintosh II.

Mutagenesis of HC cDNA- Single nucleotide mutagenesis of the Lys^{343} codon was performed in the M13mp18 containing the noncoding strand of HC by the method of Nakamaye and Eckstein as described previously (41). A *Bsu* 36 I- *Xho* I fragment of the DNA containing the mutation was ligated into the expression vector pMON-HCII and the final plasmid construct was confirmed by sequencing using the chain termination method of Sanger *et al* (42).

Random mutations were generated in the Lys^{173} codon by the procedure of Wells *et al* (43). In brief, sequential site-directed mutagenesis was performed in M13mp18 containing the HC cDNA to create a *Sac* I site at nucleotides 595-600 followed by the formation of an *Rsr* II site in nucleotides 683-689 (numbered according to Blinder *et al* Fig. 2; 39). The predicted amino acid sequence was not altered by the nucleotide substitutions. A 660 bp *Bsu* 36 I- *Xho* I fragment of the cDNA which contained both new restriction sites was isolated from the replicative form and ligated into similarly digested pMON-HCII that had been digested with mung bean nuclease to eliminate a *Sac* I site in the 3' polylinker region of the HC insert (44). Proper ligation was verified by digestion with *Rsr* II and *Sac* I and the final plasmid construct was confirmed by dideoxy sequencing of both strands. Approximately 5 μg of the pMON-HCII plasmid was digested with both restriction enzymes and the vector product was isolated from the small *Sac* I-*Rsr* II fragment by agarose gel electrophoresis and purified from the gel using "glass milk" beads (Gene Clean, Bio101). Five oligonucleotides were synthesized to span the *Sac* I-*Rsr* II domain. Two of the oligonucleotides, a 44mer and a 41mer, formed the coding strand and three complementary oligonucleotides (34, 28, and 30 nucleotides in length) comprised the non-coding strand. The sequence of the oligonucleotides varied from the HC cDNA by replacement of all three positions in the codon for Lys^{173} with any nucleotide, allowing for generation of random changes at those positions. In addition, the codon for Arg^{184} was changed from CGT to CGG to create a *Bsp* EI restriction site that was used for screening. Oligonucleotides were pooled at a final concentration of 10 μM in H_2O and 2.5 μl of the pool was phosphorylated with T4 polynucleotide kinase. Approximately 25 fmol of the phosphorylated oligonucleotide were mixed with ~12 fmol of the digested DNA in a buffer containing 25 mM Tris-HCl, 10 mM $MgCl_2$, 10 mM DTT, 4 mM ATP, pH 7.5 and annealed by heating to 68 °C for 5 min and cooled to room temperature over 2 hours. T4 DNA ligase was added (1 Weiss unit) and the reaction was incubated at 14 °C for 16 hours. *Escherichia coli* JM109 were transformed with a portion of the ligation mix, 24 colonies were isolated and amplified, and the plasmid was purified (45). A 448 bp DNA fragment spanning the oligonucleotide insert was amplified using the Taq polymerase chain reaction as described by the manufacturer and the fragments were digested with *Bsp* EI to screen for the inserted oligonucleotides. Seven plasmids containing the *Bsp* EI site were sequenced between the *Rsr* II-*Sac* I sites in both orientations and of those, two clones were detected that contained only mutations in the codon for Lys^{173}.

Expression of Recombinant HC and HC variants- For each recombinant HC preparation, transformed *Escherichia coli* JM101 were grown to an optical density of ~1.2 at 550 nm in 500 ml of medium, and expression was induced with nalidixic acid according to Li *et al* (46). The cells were washed, resuspended in phosphate-buffered saline, and lysed by sonication. The product was subjected to ammonium sulfate precipitation and the concentration of the rHC was determined by a quantitative immunoblot as described previously (35). There was no apparent difference in the recovery of the expressed rHC compared to the variant rHC preparations.

Functional Analysis of Recombinant HC and HC variants- Binding of rHC to heparin was determined using a 2-ml heparin-Sepharose column as described previously (35). Thrombin inhibition was determined in the absence of glycosaminoglycans by incubating 900 µl of 40-168 nM rHC, 50 µl of 280 nM thrombin and 50 µl of H_2O at room temperature. At specific times 100 µl aliquots were added to 400 µl of 100 µM Chromozym TH and the rate of change of absorbance at 405 nm was monitored continuously for 100 seconds. The rate of change of absorbance was proportional to the concentration of active thrombin remaining in the incubation. The effect of glycosaminoglycans was determined using thrombin and rHC at the concentrations used above and adding various concentrations of heparin or dermatan sulfate to a final volume of 100 µl. Absorbance at 405 nm was determined after the addition of Chromozym TH as described above.

TABLE I

Amino Acid Sequences of PLP Modified Peptides in Heparin Cofactor II.

Peak Number[a]	Amino Acid Sequence[b]
1	Lys^{343}-Met-Ser-Gly-Met-Lys
2,3,4,6	Glu^{300}-Val-Val-**Lys^{303}**-Val-Ser-Met-Met-Gln-Thr-Lys
2,3,4	Glu^{43}-Asn-Thr-Val-Thr-Asn-Asp-Trp-Ile-Pro-Glu-Gly-Glu-Glu-Asp-Asp-Asp-Tyr-Leu-Asp-Leu-Glu-**Lys^{65}**-Ile-Phe-Ser-Glu-Asp-.....Phe-His-Gly-Lys
5	Leu^{250}-Thr-**Lys^{252}**-Gly-Leu-Ile-Lys
7	Met^{344}-Ser-Gly-Met-**Lys^{348}**-Thr-Leu-Glu-Ala-Gln-Leu-Thr-Pro-Arg
8	Asp^{166}-Phe-Val-Asn-Ala-Ser-Ser-**Lys^{173}**-Tyr-Glu-Ile-Thr-Thr-Ile-His-Asn-Leu-Phe-Arg

[a]Peak numbers are from Fig. 1, Panel A.

[b]**Bold** residues are those lysines modified by PLP.

TABLE II

Modification of Lysines in the Presence of Heparin and Dermatan Sulfate

Modified Residue	Percent Modification[a] with 1 mg/ml Heparin present	Percent Modification with 1 mg/ml Dermatan Sulfate present
Lys65	117%	100%
Lys^{173}	21%	81%
Lys^{252}	19%	57%
Lys303	95%	91%
Lys^{343}	31%	52%
Lys^{348}	57%	74%

[a]Percentages are relative to amount of modification of each residue in unprotected PLPHC.

TABLE III

Antithrombin Activity of Recombinant Heparin Cofactor II Variants

Variant	Final HC Concentration	Second Order Rate Constant
	nM	M^{-1} min^{-1}
rHC (native)	168	1.6×10^5
rHC (Lys^{343}→ Thr)	155	2.2×10^5
rHC (Lys^{343}→ Asn)	168	2.3×10^4
rHC (native)	40	1.1×10^5
rHC (Lys^{173}→ Leu)	48	9.2×10^4
rHC (Lys^{173}→ Gln)	40	1.3×10^5