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

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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 Lys173 and Lys252 are within a region previously shown to contain residues involved in glycosaminoglycan binding. Lys343 and Lys348 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 Lys173 and Lys343 was performed to further dissect the role of these two regions during HC-heparin and HC-dermatan sulfate interactions. The Lys173→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 Lys173→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 Lys173 is involved in the interaction of HC with heparin but not with dermatan sulfate, whereas Lys343 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 Gly35 to Glu50 and Lys107 to Lys136, 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 α2-proteinase inhibitor (α2-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 Gly35 to Glu50 region of AT, but extensive homology to the Lys107 to Lys136 region, which extends from Lys56 to Phe65 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-

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1 The abbreviations used are: HC, heparin cofactor II; serpin, serine proteinase inhibitor; AT, antithrombin III; α2-PI, α2-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, tosylphenylalaninechloromethyl ketone-treated trypsin; Chromozym TH, Nα-p-tosyl-Gly-Pro-Arg-p-nitroanilide.
Critical Lysyl Residue of Heparin Cofactor II

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. Panel D shows detection of all peptides in unprotected HC by absorbance at 325 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 Lys173 and Lys343 in glycosaminoglycan binding.

Experimental Procedures

Results

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. 2A). 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...
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. 2B). 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 α1-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 Lys405 to the Phe495 region of HC known to be involved in glycosaminoglycan binding. This region includes Lys173 and is homologous with the proposed heparin binding region in AT. Lys393 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. Lys345 and Lys348 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 Lys173 and Thr or Asn substitution for Lys343. 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-PC.1. Besides the absence of the signal peptide, the protein product lacked the NH2-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 Lys43 variants 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. 4A). 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 Lys43 → 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, Lys43 → Leu or Gln resulted in protein products that were eluted from heparin-Sepharose at a lower ionic strength (Fig. 4B). The peak of native rHC eluted at ~0.38 M NaCl, whereas rHC (Lys43 → Gln) and rHC (Lys43 → 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 × 107 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 α1-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.](image-url)
by as much as 1000-fold (1, 6, 7). In both cases, binding of heparin and -4-10 pg/ml of dermatan sulfate. The Lys343 variants are shown in Fig. 5. In these experiments, native rHC inhibited 50% of the thrombin with -3-7 pg/ml of heparin for 50% thrombin inhibition. With dermatan sulfate, the Lys343 Thr variant required similar heparin concentrations and only slightly more dermatan sulfate than the native rHC to inhibit thrombin. LYS'~~ is homologous to a residue in AT that remains of AT and the Lys1'j5 to Phe195 region of HC.

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 Lys843 \(\rightarrow\) 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 Lys343 \(\rightarrow\) 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 Lys733 \(\rightarrow\) Leu and Lys733 \(\rightarrow\) 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 Lys843 \(\rightarrow\) 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 Lys843 is probably not critical for glycosaminoglycan-dependent inhibition. However, the Lys843 \(\rightarrow\) 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 glycosaminoglycans (Table II).

In contrast, the rHC molecules containing substitutions of Lys733 required at least a 30-fold higher concentration of heparin for 50% thrombin inhibition. With dermatan sulfate, however, the Lys733 \(\rightarrow\) Leu variant showed normal thrombin inhibition and the Lys733 \(\rightarrow\) Gln variant required only a 2-fold higher concentration for 50% inhibition. These observations suggest that the charge at Lys733 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 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, Gly124 to Glu156 and Lys187 to Lys198, have been designated as forming the heparin binding domain of AT (16-19, 21, 23, 25, 27, 52). HC contains a region, Lys342 to Phe390, that shows extensive homology to the Lys197 to Lys198 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) Lys136 and possibly Arg194 and Arg195 in heparin binding and (ii) Lys105, Arg190, Arg192, Arg193 and possibly Arg194 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 were protected from modification in the presence of glycosaminoglycan.

We then used computer-assisted molecular modeling to interpret the above results. Lys733 was very well protected from chemical modification by heparin and less so by dermatan sulfate. Lys733 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). Lys136 is located just before the start of such an α-helix (D-helix using the nomenclature of α1-PI; Refs. 26 and 29) containing other residues in HC important in glycosaminoglycan binding. These results implicate Lys733 in glycosaminoglycan binding to HC, especially in heparin interactions.

Lys342 was well protected from chemical modification by heparin and fairly well by dermatan sulfate. Lys343 is not involved in heparin binding, but is involved in heparin binding (circles), dermatan sulfate binding (squares), or both (diamond).
within the putative glycosaminoglycan binding site of HC, but from our model it is close to this region in the tertiary structure of the molecule. Lys173, Arg180, Arg192, and possibly Arg214 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 Lys214, that could interact with the negatively charged glycosaminoglycans. Lys214 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 phosphoryliridoxylation.

Lys243 and Lys244 are also in the putative glycosaminoglycan binding site of HC. These residues were moderately well protected by both heparin and dermatan sulfate, with Lys243 being somewhat more protected by each glycosaminoglycan. In our model Lys243 and Lys244 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 Lys173 as the most probable residue involved in glycosaminoglycan binding and we chose to mutate Lys173 in order to investigate the possible involvement of the region around this residue in glycosaminoglycan binding.

The Lys173 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 Lys173. Heparin was better able to protect Lys173 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 LYS173) retained almost no heparin cofactor activity. These results suggest that Lys173 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 Lys173 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 Lys173 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 Lys180 variants. Both of these variants bound to heparin-Sepharose almost identically to native rHC. The heparin cofactor and dermatan sulfate cofactor activities of the Lys180 \(\rightarrow\) Thr variant were very similar to those of the native rHC. The Lys180 \(\rightarrow\) 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 Lys180 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 Lys180 to Phe185 region of HC have been shown to effect both heparin and dermatan sulfate binding and the mutation of Arg214 results in deficient dermatan sulfate binding but normal heparin binding (34, 35, 37). In this study we showed that mutation of Lys173 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.

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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, 635-709
4. Parker, K. A., and Tollefsen, D. M. (1985) J. Biol. Chem. 260, 3501-3508
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 Monoa, 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., Rauh, E., Lindahl, U., and Björk, I. (1986) J. Biol. Chem. 261, 15467-15473
12. Rosenberg, R. D., and Danus, P. S. (1973) J. Biol. Chem. 248, 6490-6505
13. Blackburn, M. N., and Sibiey, C. C. (1980) J. Biol. Chem. 255, 824-828
14. Blackburn, M. N., Smith, R. L., Sibiey, 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 Sibiey, C. C. (1984) J. Biol. Chem. 259, 399-346
17. Koide, T., Odani, S., Takahashi, K., Ono, T., and Sakuragawa, N. (1984) Proc. Natl. Acad. Sci. U. S. A. 81, 280-293
18. Chang, Y.-J., 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. B. (1987) Annu. Rev. Med. 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, 17365-17361
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. (1988) J. Biol. Chem. 264, 11288-11293
29. Loebermann, H., Tukuv, R., Deisenhofer, J., and Huber, R. (1984) J. Mol. Biol. 170, 531-556
30. Church, F. C., and Griffith, M. J. (1984) Biochem. Biophys. Res. Commun. 124, 745-751
31. Gundrill, S., Alach, M., Lane, D., Vidaud, D., Molho-Sabatier, I.
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P. Caso, D. Reo-Moeloese, P. Fieschm, J.J., and Clasen, E. (1990) J. Biol. Chem. 265, 1899-1901.

32. Sun, X.-J., and Chang, J.-Y. (1990) Biochemistry 29, 8957-8962

33. Church, F.C., and Whinna, H.C. (1986) Arch. Biochem. Biophys. 246, 155-178.

36. Church, F.C., Villaneuva, C.B., and Griffith, M.J. (1986) Arch. Biochem. Biophys. 246, 175-184.

39. Ruiz, H., Ullrich, T., and Gerevitz, J.W. (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., Marass, J.C., Reynolds, C.H., Devenn, L.A., and Tollefson, D.M. (1988) J. Biol. Chem. 262, 13773-13779.

40. Nakamaye, K.L., and Eckstein, F. (1986) Nucl. Acids Res. 14, 9679-9698.

41. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. U. S. A. 74, 5463-5467.

42. Wells, J.A., Vasser, M., and Powers, D.B. (1985) Gene (Amst.) 34, 315-323.

43. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, pp. 17-25, 17-24, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

44. Lisewski, M.K., Kumar, V., and Atkinson, J.P. (1989) Bio-Techniques 7, 1079-1081.

45. Li, E., Locke, B., Yang, N.C., Ong, D.E., and Gordon, J.I. (1987) J. Biol. Chem. 262, 13770-13775.

46. Derechin, V., Blinder, M.A., and Tollefson, D.M. (1990) J. Biol. Chem. 265, 5623-5628.

47. Casu, B. (1989) Ann. N.Y. Acad. Sci. 558, 1-17.

48. Church, F.C., Treanor, R.E., Sherrill, G.B., and Whinna, H.C. (1986) Biochem. Biophys. Res. Commun. 148, 362-368.

49. Church, F.C., Pratt, C.W., Treanor, R.E., and Whinna, H.C. (1988) FEBS Lett. 237, 26-30.

50. Pratt, C.W., Whinna, H.C., Meade, J.B., Treanor, R.E., and Church, F.C. (1989) Ann. N.Y. Acad. Sci. 558, 104-114.

51. Blumen, S.O., Ge, F.M., and Jordan, R.E. (1987) FEBS Lett. 219, 43-46.

52. Cardin, A.D., and Weintraub, H.J.R. (1989) Arteriosclerosis, 9, 21-32.

Supplementary Material

THE ROLE OF LYSINE 173 IN HEPARIN BINDING TO HEPARIN COFACTOR II

by

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EXPERIMENTAL PROCEDURES

MATERIALS

1. Thrombin (0.05 M sodium phosphate, pH 6.5; 150mM NaCl; 1mM EDTA) was obtained from American Diagnostica, Inc., New York, USA.

2. Rabbit anti-human HC (total 5623-5628) was purchased from Boehringer-Mannheim.

3. Rabbit anti-human HC antibodies were prepared and labeled with Na-p-tosyl-Gly-Pro-Arg-NH2 (Chromozym TH) by Bierer and cohorts.

4. Rabbit anti-human HC (total 5623-5628) was collected and rechromatographed on the column with sodium borohydride but not PLP. In total volume of 1 ml, thrombin (50 nM) and HC (100 nM) in 0.1 M HEPES, 0.1% polyethylene glycol (Mw=8000), pH 7.4 were incubated at room temperature with increasing amounts of PLP. The reaction mixture was treated with nitrous acid for the removal of contaminating heparin and heparin sulfate (40). Tyrosyl p-phosphates and sodium borohydride were purchased from Sigma. Guanidine hydrochloride (Sequential grade) was purchased from Fisons. Tryptophan/bromophenol blue-assay kit was used with Tris (100mM) and protein samples were purified from Bio-Rad and Research Organizers, respectively. Polyethylene and isocyanate were from Aldrich. The coagulation thrombin substrate N-p-nitro-3,4-diphenyl-l-3-carboxymethyl-threoninyl Pro-Arg-Lys-Thr-Arg-Arg-phenylalnine (Chromozym TH) was purchased from Boehringer-Mannheim.

5. The reduced proteins and buffers were of reagent grade or better.

6. All restriction endonucleases and DNA modifying enzymes were purchased from Amersham or New England Biolabs. DNA sequencing was performed using modified T7 polymerase from United States Biological 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.

7. The synthetic oligonucleotides were provided by the Protein Chemistry Faculty of Washington University and were used without further purification.

METHODS

1. Chemical Modification of HC. HC (20 μM) in 0.05 M triethanolamine acetate, 0.1% polyethylene glycol (Mw=8000), pH 8.5, was added and the reaction mixture was incubated at room temperature with increasing amounts of PLP. The reaction mixture was treated with nitrous acid. Borohydride but not PLP was added to the reaction mixture.

2. Identification of sequence was performed with a Hewlett Packard CAS-2000 DNA sequencer. Oligonucleotides were synthesized as described previously (41). A 3 μl Xho I fragment of the DNA containing the mutation was ligated into the expression vector PMHCII and the final plasmid construct was sequenced by performing the chain termination method of Sanger (42).

3. Mutations of HC. DNA-DNA, Single nucleotide mutation of the l343 codon was confirmed in the M13mp18 containing the corresponding strand of HC by the method of Nakamaye and Herbert as described previously (41). A 2 μl Xho I fragment of the DNA containing the mutation was ligated into the expression vector PMHCII and the final plasmid construct was sequenced by performing the chain termination method of Sanger (42).

4. Mutations of HC. DNA-DNA, Single nucleotide mutation of the l343 codon was confirmed in the M13mp18 containing the corresponding strand of HC by the method of Nakamaye and Herbert as described previously (41). A 2 μl Xho I fragment of the DNA containing the mutation was ligated into the expression vector PMHCII and the final plasmid construct was sequenced by performing the chain termination method of Sanger (42).

5. Mutations of HC. DNA-DNA, Single nucleotide mutation of the l343 codon was confirmed in the M13mp18 containing the corresponding strand of HC by the method of Nakamaye and Herbert as described previously (41). A 2 μl Xho I fragment of the DNA containing the mutation was ligated into the expression vector PMHCII and the final plasmid construct was sequenced by performing the chain termination method of Sanger (42).

6. Mutations of HC. DNA-DNA, Single nucleotide mutation of the l343 codon was confirmed in the M13mp18 containing the corresponding strand of HC by the method of Nakamaye and Herbert as described previously (41). A 2 μl Xho I fragment of the DNA containing the mutation was ligated into the expression vector PMHCII and the final plasmid construct was sequenced by performing the chain termination method of Sanger (42).
Critical Lysyl Residue of Heparin Cofactor II

Expression of Recombinant HC and HC variants. For each recombinant HC preparation, transformed E. coli JM101 were grown to an optical density of ~1.2 at 550 nm in 500 ml of medium and expression was induced with arabinose and IPTG. 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 immunoassay as described previously (85). There was no apparent difference in the recovery of the expressed rHC compared to the variant HC 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 (85). 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 HEPES buffer at room temperature. At specific times, 100 μl aliquots were added to 600 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 | Amino Acid Sequence |
|-------------|---------------------|
| 1           | Leu245-Met-Ser-Cys-Met-Lys |
| 2,3,4       | Glu236-Val-Val-Lys339-Val-Ser-Met-Met-Glu-Thr-Lys |
| 5           | Leu245-Thr-Lys339-Cys-Leu-Bel-Lys |
| 7           | Met344-Ser-Cys-Met-Lys351-Thr-Leu-Glu-Ala-Glu-Leu-Thr-Thr |
| 8           | Arg245-Thr-Leu-Ala-Ser-Lys473-Thr-Glu-Val-Thr-Gly-Cys-Thr-His-Ala-Leu-Thr-Arg |

*Positions are from Fig. 1, panel A. Bold residues are those lysines modified by PLP.