Heparin Binding to Protein C Inhibitor*

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Protein C inhibitor is a plasma protein whose ability to inhibit activated protein C, thrombin, and other enzymes is regulated by heparin. The studies were undertaken to further understand how heparin binds to protein C inhibitor and how it accelerates proteinase inhibition. The region of protein C inhibitor from residues 264–283 was identified as the heparin-binding site. This differs from the putative heparin-binding site in the related proteins antithrombin and heparin cofactor. The glycosaminoglycan specificity of protein C inhibitor was relatively broad, including heparin and heparan sulfate, but not dermatan sulfate. Non-sulfated and non-carboxylated polyanions also enhanced proteinase inhibition by protein C inhibitor. Heparin accelerated inhibition of α-thrombin, γ-thrombin, activated protein C, factor Xa, urokinase, and chymotrypsin, but not plasma kallikrein. The ability of glycosaminoglycans to accelerate proteinase inhibition appeared to depend on the formation of a ternary complex of inhibitor, proteinase, and glycosaminoglycan. The optimum heparin concentration for maximal rate stimulation varied from 10 to 100 μg/ml and was related to the apparent affinity of the proteinase for heparin. There was no obvious relationship between heparin affinity and maximum inhibition rate or degree of rate enhancement. The affinity of the resultant protein C inhibitor-proteinase complex was also not related to inhibition rate enhancement, and the results showed that decreased heparin affinity of the complex is not an important part of the catalytic mechanism of heparin. The importance of protein C inhibitor as a regulator of the protein C system may depend on the relatively large increase in heparin-enhanced inhibition rate for activated protein C compared to other proteinases.

Hemostasis requires a balance between procoagulant and anticoagulant forces. Among the anticoagulant mechanisms is the protein C system. Thrombin generated during coagulation binds to thrombomodulin on vessel walls and the thrombin-thrombomodulin complex activates the zymogen protein C. Activated protein C, with its cofactor protein S, proteolytically inactivates coagulant factors V and VI11 (1). The importance of the protein C system is demonstrated by the incidence of thrombosis in individuals who lack protein C (2) or protein S (3). The protein C system is believed to be regulated by a plasma glycoprotein named protein C inhibitor, also known as plasminogen activator inhibitor-3 (4). Three other major plasma proteins, α2-macroglobulin, α2-antiplasmin, and α1-proteinase inhibitor inhibit activated protein C (5) but might be effective only by virtue of their relatively high concentration in plasma. Protein C inhibitor reacts with the active site of defibrinated protein C to form an essentially irreversible complex (6). Interestingly, protein C inhibitor also inhibits thrombin, the final proteinase of the coagulation pathway, as well as other procoagulant enzymes. This broad target proteinase specificity of protein C inhibitor presents a problem in understanding the physiological importance of protein C inhibitor as a regulator of the protein C system. Direct evidence for the involvement of protein C inhibitor is lacking as an inhibitor-deficit, and hitherto unexplained.

Protein C inhibitor is a member of the serine proteinase inhibitor (serpin)1 superfamily of proteins, whose prototype is α1-proteinase inhibitor (7). Protein C inhibitor can be further classified as a heparin-binding serpin, along with the proteinase inhibitors antithrombin (historically known as antithrombin III) and heparin cofactor (also called heparin cofactor II). Heparin and some other glycosaminoglycans act to increase the rate of proteinase inhibition by these three plasma inhibitors, in some cases as much as several thousand-fold (8). The mechanism whereby heparin catalyzes proteinase inhibition is a subject of much study, especially due to the widespread use of heparin as a therapeutic anticoagulant. The ability of heparin to accelerate the inhibition of activated protein C by protein C inhibitor, thereby favoring coagulation, is at odds with the anticoagulant effect of heparin therapy. As a first step toward understanding this apparent contradiction and in order to gather insight into the physiological importance of protein C inhibitor, a series of studies was undertaken. The work presented here describes the heparin-binding site of protein C inhibitor, the polyanion specificity of protein C inhibitor, and the mechanism whereby heparin accelerates proteinase inhibition. Some of these results have appeared previously in abstract form (9). The following report compares protein C inhibitor to antithrombin and heparin cofactor (10).

EXPERIMENTAL PROCEDURES

Materials—Human protein C inhibitor was purified as previously described (11), as were antithrombin and heparin cofactor (12). All proteinases were of human origin, with the exception of bovine chymotrypsin. α-Thrombin was purified as described (13) or converted to γ-thrombin (14). Protein C was prepared as a by-product of the factor X preparation (12), and further purified and activated by incubating it with thrombin, then passing the mixture over immobilized antithrombin to remove thrombin and traces of factor Xa. Urokinase was purchased from Sigma, plasma kallikrein from Calbi-

1 The abbreviations used are: serpin, serine proteinase inhibitor; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PEG, polyethylene glycol; HPLC, high performance liquid chromatography.
ochem, bovine chymotrypsin from Cooper Biomedical, and neutrophil elastase from Elastin Products (Pacific, MD). The following proteinase substrates were used: Chromozym TH (tosyl-Gly-Pro-Arg-p-nitroanilide) for thrombin from Boehringer Mannheim, Spectrozyme PC G (Glu-Arg-p-nitroanilide) for activated protein C, Spectrozyme FXa (MeO-OC-CH-Gly-Gly-p-nitroanilide) for factor Va from American Diagnostica, S-2444 (Glu-Gly-Arg-p-nitroanilide) for urokinase and S-2232 (Pro-Phe-Arg-p-nitroanilide) for kallikrein from KabiVitrum, and Suc-Ala-Ala-Pro-Phe-p-nitroanilide for chymotrypsin from Sigma. The following were purchased from Sigma: bovine serum albumin, polybrene (1,5-dimethyl-1,5-diazaundecamethylene polyacrylamide), bovine heparan sulfate, bovine chondroitin sulfate A, shark chondroitin sulfate C, fucoidan (a sulfated polymer of fucose from a marine alga), phosvitin (a phosphoserine-containing glycoprotein from egg yolk), sulfatides, and tetrapolyphenolic depolymerized heparin from Diosynth (Oss, the Netherlands). Low molecular weight (M, 5500) heparin was from Calbiochem. Dermatan sulfate was purchased from Calbiochem and treated with nitrous acid to remove contaminating heparin (16). DSGC II (a dermatan sulfate proteoglycan from bovine skin) was the gift of Dr. Lawrence Rosenberg, Montefiore Hospital, Bronx, NY. Heparin fractions with low and high affinity for antithrombin were the gift of Dr. Ingemar Bjork, Swedish University of Agricultural Sciences Uppsala, Sweden. Chemically depolymerized heparin (average molecular weight 5000, 5000, and 2500) was the gift of Dr. Chris Griffin, Miami University, OH. Fmoc-amino acids were from Milligen. Heparin-Sepharose was from Pharmacia LKB Biotechnology Inc. and heparin-agarose was prepared as described (12).

**Peptide Synthesis**—Peptides were assembled from Fmoc amino acids using a Milligen pepsynthesizer according to the reported cDNA sequence of protein C inhibitor (17). In peptide 1-16, tyrosine was added to the carboxyl terminus, and in peptide 80-93, Phe-92 was substituted with tyrosine to facilitate spectrophotometric quantitation of the peptides. Purity of the peptides was verified by reverse-phase HPLC, and when necessary, further purification was accomplished by HPLC. Heparin affinity of peptides was measured using a Pharmacia fast protein liquid chromatography system and a 5-ml column of heparin-Sepharose in 20 mM HEPES, 10 mM NaCl, 0.1% PEG, pH 7.4. Samples of protein C inhibitor, proteinase, and protein C inhibitor-proteinase complexes were eluted with a 1 ml/min linear salt gradient from 0 mM to 1.2 M NaCl; 0.25-ml fractions were collected. Peptides were detected by absorbance at 290 nm and by reactivity with a rabbit polyclonal antiserum to protein C inhibitor (to detect protein C inhibitor and protein C inhibitor-proteinase complexes) and by chromogenic substrate hydrolysis (to detect proteinases and protein C inhibitor-proteinase complexes; an extended incubation time was required to detect proteinase activity in complexes). Results were plotted and the salt concentration corresponding to peak elution was determined. The mean and standard deviation were calculated from multiple runs (2-10) of each sample. Protein C inhibitor-proteinase complexes were prepared by incubating proteinase with a slight excess of protein C inhibitor for a time previously determined to allow complete reaction, at least five times the half-life of the reaction. Inactivation of protein C inhibitor by neutrophil elastase was followed by the loss of thrombin inhibition activity.

**RESULTS**

**Identification of the Heparin-binding Site of Protein C Inhibitor**—Because heparin is a negatively charged glycosaminoglycan, it is expected to bind to basic residues of protein C inhibitor. Three regions were identified as potential heparin-binding sites: residues 11-11, 82-90, and 266-278. Residues 266-278 were previously identified as a heparin-binding site by homology to a consensus glycosaminoglycan recognition site (18). Peptide 11 also follows the consensus sequence. Sequences corresponding to the three regions of protein C inhibitor (1-16, 80-93, and 264-283, and the 264-283 random sequence) are shown in Fig. 1, along with helical-wheel projections of these sequences (19). The projections show that each chain was followed by the loss of thrombin inhibition activity. The three peptides were synthesized and their ability to bind heparin was tested in proteinase inhibition assays. The peptides were added to systems containing protein C inhibitor, proteinase, and heparin; with the premise that heparin-binding peptides would compete with protein C inhibitor for heparin, thus decreasing the...
the rate of the proteinase inhibition reaction. The results revealed that of the three peptides, only peptide 264–283 competed with protein C inhibitor for binding to heparin, as shown by the increase in proteinase activity with increasing peptide concentration (Fig. 2). This effect was observed for both thrombin and activated protein C. A peptide comprised of the same residues as peptide 264–283, but in random sequence, did not compete as effectively, suggesting that the three-dimensional distribution of charged residues rather than the number of charged residues was responsible for its heparin binding function. As further confirmation of peptides as models for the heparin binding region in proteins, peptide 264–283 interfered with heparin-catalyzed inhibition of thrombin by two other heparin-binding inhibitors, antithrombin and heparin cofactor, and peptides corresponding to the putative antithrombin heparin-binding site (Ala-124-Leu-140, Ref. 10) competed for heparin binding in the protein C inhibitor assay (results not shown). Furthermore, peptide 264–283 bound to immobilized heparin and was eluted with 800 mM NaCl, indicating even higher affinity than native protein C inhibitor (elution at approximately 600 mM NaCl). The random sequence peptide bound less tightly (elution at 380 mM NaCl, Ref. 10).

**Polyanion Specificity of Protein C Inhibitor**—In addition to heparin, some other glycosaminoglycans have been found to accelerate proteinase inhibition by protein C inhibitor (20, 21). To further assess the specificity of protein C inhibitor, we tested the ability of various polyanions to catalyze protein C inhibitor inhibition of thrombin and activated protein C. Each polyanion was tested at various concentrations in order to determine the maximum degree of rate enhancement and the polyanion concentration at which that occurred. The results are presented in Table I. Among the mammalian glycosaminoglycans, only heparin, heparan sulfate, and chondroitin sulfate A caused acceleration of thrombin and acti-

| Polyanion  | Thrombin Optimum (polyanion) | Rate increase | Activated protein C Optimum (polyanion) | Rate increase |
|------------|------------------------------|--------------|-----------------------------------------|--------------|
| Heparin    | 10 0.4                       | 100 52.2     |                                          |              |
| Heparan sulfate | 100 1.9                   | 300 3.1      |                                          |              |
| Chondroitin sulfate A | >1000 1.7           | >1000 4.1    |                                          |              |
| Chondroitin sulfate C | ND                       | ND           |                                          |              |
| Fucoidan   | 10 9.5                       | 100 10.0     |                                          |              |
| Dermatan sulfate | ND                      | >1000 4.6    |                                          |              |
| DSPG II    | ND                          | ND           |                                          |              |
| Phosvitin  | 30 1.6                       | 100 5.4      |                                          |              |
| AT low affinity heparin | 30 3.0                | 100 10.3     |                                          |              |
| AT high affinity heparin | 30 3.5                | 100 8.7      |                                          |              |
| Low molecular weight heparin | 10 2.6 | 30 12.1 |                                          |              |
| Heparin M, 5000 | 3 1.2                    | 30 10.2      |                                          |              |
| Heparin M, 3500 | 10 1.5                   | 100 12.5     |                                          |              |
| Heparin M, 2500 | ND                       | 300 4.3      |                                          |              |

**Fig. 2. Peptide competition in proteinase inhibition assays.** Synthetic peptides corresponding to potential heparin-binding regions in protein C inhibitor (shown in Fig. 1) were added to thrombin (panel A) or activated protein C (panel B) inhibition assays as described under "Experimental Procedures." The y axes show thrombin and protein C activity relative to the activity in the absence of inhibitor. Greater proteinase activity indicates diminished inhibition due to peptides competing with proteins for heparin binding. ■, peptide 1–16; □, peptide 80–93; ●, peptide 264–283; ○, random peptide 264–283.

**TABLE I**

| Polyanion  | Thrombin Optimum (polyanion) | Rate increase | Activated protein C Optimum (polyanion) | Rate increase |
|------------|------------------------------|--------------|-----------------------------------------|--------------|
| Heparin    | 10 0.4                       | 100 52.2     |                                          |              |
| Heparan sulfate | 100 1.9                   | 300 3.1      |                                          |              |
| Chondroitin sulfate A | >1000 1.7           | >1000 4.1    |                                          |              |
| Chondroitin sulfate C | ND                       | ND           |                                          |              |
| Fucoidan   | 10 9.5                       | 100 10.0     |                                          |              |
| Dermatan sulfate | ND                      | >1000 4.6    |                                          |              |
| DSPG II    | ND                          | ND           |                                          |              |
| Phosvitin  | 30 1.6                       | 100 5.4      |                                          |              |
| AT low affinity heparin | 30 3.0                | 100 10.3     |                                          |              |
| AT high affinity heparin | 30 3.5                | 100 8.7      |                                          |              |
| Low molecular weight heparin | 10 2.6 | 30 12.1 |                                          |              |
| Heparin M, 5000 | 3 1.2                    | 30 10.2      |                                          |              |
| Heparin M, 3500 | 10 1.5                   | 100 12.5     |                                          |              |
| Heparin M, 2500 | ND                       | 300 4.3      |                                          |              |

* The optimum polyanion concentration is the concentration at which the maximum proteinase inhibition rate occurs.

* The rate increase is calculated as the ratio of the maximum rate to the rate in the absence of polyanion.

* ND, no acceleration of inhibition detected at 1 mg/ml polyanion.

The optimum heparin concentration of 1 mg/ml polyanion for maximum inhibition of thrombin by protein C inhibitor, although the effect of chondroitin sulfate A was weak, as shown by the high concentration required to increase the inhibition rate. Dermatan sulfate showed no activity with thrombin and very low activity with activated protein C; a highly purified derman sulfate preparation (DSPG II) showed no activity with either proteinase. Chondroitin sulfate C did not accelerate inhibition. Interestingly, fucoidan (a sulfated polymer of fucose from a marine alga) was relatively effective, and phosvitin (a phosphoserine-containing glycoprotein from egg yolk) was also active. Sulfatides and tetrapolyphosphate did not affect the rate of proteinase inhibition by protein C inhibitor (not shown).

A number of heparin fractions accelerated inhibition of thrombin and activated protein C by protein C inhibitor (Table I). Protein C inhibitor clearly differs from antithrombin in responding equally well to heparin with high or low affinity for antithrombin. Protein C inhibitor exhibited some specificity for the size of the heparin molecule, as greater concentrations of the smaller heparin fractions were required for maximum stimulation of the inhibition reaction.

**Mechanism of Heparin Acceleration of Inhibition**—The mechanism whereby heparin accelerates proteinase inhibition by protein C inhibitor could depend on heparin binding to the inhibitor, to the proteinase, or to both proteins. Examination of the effect of increasing concentrations of heparin on the rate of inhibition of six different proteinases (α-thrombin, γ-thrombin, activated protein C, factor Xa, urokinase, and chymotrypsin, Fig. 3) reveals a bell-shaped curve that is consistent with a ternary complex model for heparin action (22–24). According to this model, heparin binds both inhibitor and proteinase to bring the reactants into closer proximity, and the rate of reaction increases as heparin concentration increases. When heparin concentrations increase beyond a certain point, the proteinase and inhibitor are more likely to bind to separate heparin molecules, thus decreasing the catalytic efficiency of the glycosaminoglycan. If heparin binding to only one of the two reactants was important, then heparin would exhibit a saturation phenomenon in these experiments.
increase in inhibition rate varied among different proteinases. All experiments were performed with identical concentrations of protein C inhibitor and proteinase in order to rule out protein concentration-dependent effects on heparin optimum or maximum rate. Interestingly, the rate of inhibition of factor Xa was diminished at low heparin concentrations, and at optimum heparin the rate was only slightly greater than the rate in the absence of glycosaminoglycan. In addition, increasing concentrations of heparin progressively decreased the rate of inhibition of plasma kallikrein by protein C inhibitor (Fig. 3). These two cases suggest that the catalytic effect of heparin on protein C inhibitor reactivity is not a simple phenomenon, but might involve additional effects of heparin on the particular inhibitor-proteinase pair.

The failure of attempts to demonstrate the importance of simultaneous binding of both inhibitor and proteinase to heparin using a kinetics approach (25, 26) was most likely due to the fact that heparin elicited a relatively mild rate increase. This meant that rate enhancement was not detectable under the required conditions, where proteinase and inhibitor concentrations must saturate a small amount of heparin.

Heparin affinity chromatography was used to further assess the contribution of heparin binding to protein C inhibitor and various proteinases before and after the inhibition reaction. Protein C inhibitor and five proteinases (thrombin, activated protein C, factor Xa, urokinase, and chymotrypsin) bound to immobilized heparin and were eluted by salt concentrations greater than physiological (290–640 mM NaCl, Table II). Therefore, in inhibition assays (containing 150 mM NaCl) heparin binding would occur. Table II demonstrates the inverse relationship between the apparent heparin affinity of a particular proteinase and the heparin concentration required for maximum inhibition rate enhancement; for example, heparin was most efficient at catalyzing the inhibition of thrombin, which had the highest heparin affinity. There is no obvious relationship between heparin affinity and the maximum rate of inhibition or the degree of rate enhancement. Kallikrein, which exhibited no inhibition rate enhancement, did bind to immobilized heparin, eluting at 305 mM NaCl. The salt concentration at which a protein elutes from heparin is an indication of heparin affinity and is useful for comparative purposes, but is not identical to an affinity constant, which requires more rigorous measurement.

Table III describes the apparent heparin affinity of protein C inhibitor following reaction with four proteinases. The reaction between protein C inhibitor and its target proteinases results in a stable bimolecular complex (6). Complexes containing protein C inhibitor and one of four proteinases were applied to immobilized heparin. The protein C inhibitor-thrombin complex eluted at higher salt concentration than protein C inhibitor alone, most likely due to the contribution of thrombin, whose affinity for heparin was even higher. The protein C inhibitor-factor Xa complex eluted at essentially the same salt concentration as protein C inhibitor alone, although the affinity of factor Xa itself was lower than that of protein C inhibitor. The other complexes, protein C inhibitor-activated protein C and protein C inhibitor-urokinase, showed heparin affinities intermediate to those of protein C inhibitor and the individual proteinase. It is obvious from these results that there is no uniform decrease in apparent heparin affinity of protein C inhibitor following reaction with proteinase. Nor does the change in heparin affinity correlate with the degree of inhibition rate enhancement caused by heparin (listed in Table II). Protein C inhibitor inactivated with neutrophil elastase, presumably at or near the reactive site, eluted from heparin-Sepharose at 530 ± 14 mM NaCl.

### Table II

| Proteinase       | Heparin affinityα | Heparin optimumb | Maximum rate   | Rate increasec |
|------------------|-------------------|------------------|----------------|----------------|
|                  | mM NaCl           | µg/ml            | × 10⁻⁶ M⁻¹ min⁻¹ |
| Thrombin         | 640 ± 27          | 10               | 11.2           | 9.4            |
| Factor Xa        | 446 ± 19          | 30               | 0.21           | 1.1            |
| Activated protein C | 357 ± 7    | 100              | 1.54           | 52.2           |
| Urokinase        | 344 ± 7           | 100              | 0.02           | 2.3            |
| Chymotrypsin     | 290 ± 28          | 100              | 203            | 3.0            |

α Heparin affinity is given as the salt concentration required for peak elution from immobilized heparin. This value is an indication of heparin affinity, not a true affinity constant.

β The optimum heparin concentration is the concentration at which the maximum inhibition rate occurs.

γ The rate increase is calculated as the ratio of the maximum rate to the rate in the absence of heparin.

### Table III

| Inhibitor-proteinase complex | Salt conc. at peak elutiond |
|-----------------------------|----------------------------|
| Protein C inhibitor         | 508 ± 11                   |
| Protein C inhibitor-thrombin| 562 ± 15                   |
| Protein C inhibitor-activated protein C | 448 ± 25 |
| Protein C inhibitor-factor Xa | 529 ± 17                  |
| Protein C inhibitor-urokinase| 422 ± 28                   |

α The salt concentration required for peak elution from immobilized heparin.

β Protein C inhibitor affinity was measured in the absence of proteinase.
Heparin Binding to Protein C Inhibitor

DISCUSSION

These studies were undertaken in order to better understand how heparin binds to protein C inhibitor and how this is important for the physiological function of protein C inhibitor. The results focus on three areas: identification of the putative heparin-binding site of protein C inhibitor, the glycosaminoglycan specificity of protein C inhibitor, and the mechanism whereby heparin accelerates proteinase inhibition. A region of the protein C inhibitor molecule, residues 264-283, was identified as the heparin-binding site. This sequence can be represented as an amphipathic helix, with basic and uncharged residues on opposite faces of the helix, which is a property of other heparin-binding peptides. A synthetic peptide corresponding to this region of protein C inhibitor bound to heparin and interfered in heparin-catalyzed proteinase inhibition. This sequence, located in the H helix of protein C inhibitor (7), clearly differs from the heparin-binding sites of the related proteins antithrombin and heparin cofactor, which have been assigned primarily to the D helix on the basis of chemical modification experiments and natural mutations (7). The present results also differ from a recent report implicating the amino terminus of protein C inhibitor (the A+ helix) in heparin binding (27). Failure to detect heparin binding of synthetic peptide 1-16 in the present experiments could be due to the assumption that the protein C inhibitor heparin-binding site is simply a linear sequence of residues and that synthetic peptides would adopt the same structure (and therefore function) as the sequences in the native protein. It is also possible that the monoclonal antibody used in the previous study, which bound to helix A+, might interfere with heparin binding to the H helix; this is certainly a possibility if the A+ and the H helices together form a heparin-binding site, as was suggested (27).

Acceleration of protein C inhibitor proteinase inhibition by a variety of glycosaminoglycans (especially heparin, heparan sulfate, fucoidan, and low molecular weight heparin) and other polyanions (phosvitin) is consistent with a relatively nonspecific heparin-binding site in protein C inhibitor. This is in contrast to antithrombin, which exhibits narrow specificity for heparin and heparan sulfate (28). Protein C inhibitor more closely resembles heparin cofactor, which allows an even wider variety of polyanions to accelerate thrombin inhibition (29-31). Previous studies noted the ability of several sulfated glycosaminoglycans to enhance protein C inhibitor activity, although no effect of heparan sulfate was detected (20, 21). The ability of fucoidan (which contains no carboxyl groups) and phosvitin (which contains no sulfate groups) to enhance proteinase inhibition by protein C inhibitor is further evidence for a relatively nonspecific glycosaminoglycan-binding site in protein C inhibitor. Interestingly, the "permissive" glycosaminoglycan-binding site of protein C inhibitor did not accommodate dermatan sulfate well; this glycosaminoglycan is the most effective at increasing the inhibition rate of heparin cofactor (32). The identity of the glycosaminoglycan that accelerates thrombin or activated protein C inhibitor by protein C inhibitor in vivo remains a mystery, although heparan sulfate, which lines vessel walls and is believed to be the primary antithrombin-activating glycosaminoglycan (28), is a candidate. Protein C inhibitor, however, did not distinguish heparin of high or low affinity for antithrombin. The low activity of chondroitin sulfate A measured in this study could be due to heparan sulfate contamination, as this glycosaminoglycan was not treated with nitrous acid to destroy traces of heparin (16). The present study is at odds with a recent report that dermatan sulfate from various sources accelerated inhibition of urokinase by protein C inhibitor (33). We found that dermatan sulfate that had not been treated with nitrous acid to destroy contaminating heparin did accelerate urokinase inhibition by protein C inhibitor, but acid-treated dermatan sulfate had no effect on the urokinase inhibition rate.
Heparin Binding to Protein C Inhibitor

C by aprotinin is enhanced by heparin (35); this suggests that heparin might directly affect the catalytic properties of activated protein C. Inhibition of activated protein C by protein C inhibitor is accelerated by heparin to a much greater degree than for any other proteinase tested, although no effect of heparin on hydrolysis of a small peptide substrate by activated protein C was detected in the present study.

It has been proposed that part of the ability of heparin to dramatically accelerate thrombin inhibition by antithrombin is due to the decreased heparin affinity of the resultant antithrombin-thrombin inhibitory complex, which allows heparin to efficiently dissociate from the proteins in order to participate in additional rounds of catalysis (36). (Native antithrombin eluted from heparin-Sepharose at 925 mM NaCl, while the antithrombin-thrombin complex and antithrombin inactivated at the reactive site by neutrophil elastase eluted at 430 mM NaCl, in agreement with a previous report (36); results not shown.) This phenomenon is probably less important for protein C inhibitor inhibition of proteinases, as there was no consistent correlation of maximum inhibition rate or rate enhancement with changes in apparent heparin affinity of different protein C inhibitor-proteinase complexes. The absence of a change in heparin affinity of the protein C inhibitor-activated protein C complex has been previously noted (37).

In conclusion, protein C inhibitor clearly belongs to the group of serpins whose reactivity toward certain proteinases is stimulated by heparin. (A comparison of three of these proteins is contained in the following paper, Ref. 10.) The mechanism of heparin acceleration of proteinase inhibition by protein C inhibitor was consistent with a ternary complex model, but there were indications that the exact mechanism is a more complicated function of the particular proteinase involved, as shown by the large rate enhancement for activated protein C inhibition relative to other proteinases. The question remains how protein C inhibitor can regulate the anticoagulant protein C system when it is also an inhibitor of procoagulant enzymes. So far there is little evidence for a protein C system-specific glycosaminoglycan that might preferentially accelerate activated protein C inhibition, since all of the polyanions tested in the present study stimulate inhibition of both thrombin and activated protein C. It is also possible that additional factors, such as the relative concentrations of activated protein C, thrombin, and other enzymes or their localization in vivo, are critical for determining the physiological effectiveness of protein C inhibitor.

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