N-Glycans and the N Terminus of Protein C Inhibitor Affect the Cofactor-enhanced Rates of Thrombin Inhibition*

Wei Sun, Simon Parry, Maria Panico, Howard R. Morris, Margareta Kjellberg, Åke Engström, Anne Dell, and Sophia Schedin-Weiss

From the Department of Medical Biochemistry and Microbiology, Uppsala University, Box 582, Uppsala SE-751 23, Sweden, the Division of Molecular Biosciences, Imperial College London, London SW7 2AZ, United Kingdom, and the Department of Clinical Chemistry, Lund University, University Hospital, Malmö SE-205 02, Sweden

Protein C inhibitor (PCI) is a serine protease inhibitor, displaying broad protease specificity, found in blood and other tissues. In blood, it is capable of inhibiting both procoagulant and antiocoagulant proteases. Mechanisms that provide specificity to PCI remain largely unrevealed. In this study we have for the first time provided a full explanation for the marked size heterogeneity of blood-derived PCI and identified functional differences between naturally occurring PCI variants. The heterogeneity was caused by differences in N-glycan structures, N-glycosylation occupancy, and the presence of a Δ6-N-cleaved form. Bi-, tri-, and tetra-antennary complex N-glycans were identified. Fucose residues were identified both on the core GlcNAc and as parts of sialyl-Leα epitopes. Moreover, a glycan with a composition that implied a di-sialyl antenna was observed. PCI was N-glycosylated at all three potential N-glycosylation sites, Asn-230, Asn-243, and Asn-319, but a small fraction of PCI lacked the N-glycan at Asn-243. The overall removal of N-glycans affected the maximal heparin- and thrombomodulin-enhanced rates of thrombin inhibition differently in different solution conditions. In contrast, the Δ6-N-region increased both the heparin- and the thrombomodulin-enhanced rates of thrombin inhibition at all conditions examined. These results thus demonstrate that the N-linked glycans and the N-terminal region of blood-derived PCI in different ways affect the cofactor-enhanced rates of thrombin inhibition and provide information on the mechanisms by which this may be achieved. The findings are medically important, in view of the documented association of PCI with atherosclerotic plaques and the promising effect of PCI on reducing hypercoagulability states.

Protein C inhibitor (PCI) is a serine protease inhibitor that belongs to the serpin superfamily of proteins (1, 2). Like other inhibitory serpins, PCI utilizes a unique suicide mechanism for protease inactivation. In this mechanism the inhibitor exposes its reactive site in a loop on the protein surface, called the reactive center loop, RCL. The reactive site is recognized and cleaved by the protease. Instead of being released as with a normal substrate, the protease remains covalently bound to the inhibitor. The N-terminal part of the RCL is subsequently inserted as a sixth strand in β-sheet A, while the protease is dragged along the surface to the opposite pole of the serpin. The protease-inhibitor complex is inactive and rapidly cleared from the circulation (3). Protease-serpin specificity is determined by the reactive site and by exosite interactions. PCI is capable of inhibiting diverse proteases, including thrombin, thrombin bound to thrombomodulin, active protein C, factor Xa, factor Xla, plasma and tissue kallikreins, urokinase, and tissue plasminogens (4–7).

The identification of factors that restrict PCI to performing a single task instead of the several tasks it is biochemically capable of carrying out, remain elusive. Some factors have, however, been found to affect the ability of PCI to inactivate proteases. For instance, heparin and other glycosaminoglycans and polyanions activate the inhibitory potential of PCI toward most target proteases (8, 9) but can also lower the inhibitory potential, as exemplified by a decreased activity of PCI for the inhibition of tissue kallikrein (10). Glycosaminoglycans can thus determine the activity as well as the specificity of PCI for proteases. Thrombomodulin is another compound found to affect the protease-inhibitory function of PCI by enhancing the inhibitory effect toward thrombin (11). Because thrombin bound to thrombomodulin functions as an activator of protein C, rather than a fibrinogen-degrading enzyme, the presence of thrombomodulin favors the procoagulant effect of PCI. Although active protein C was identified as the first PCI target protease (12), PCI has been reported to have considerably higher inhibitory activity toward thrombin, both in the absence and the presence of the cofactors (9, 13). In the present study, we have used MALDI-TOF MS for peptide mapping and glycan structure analyses, to establish that the size heterogeneity of human blood-derived PCI is explained by differences in N-glycan structures, N-glycosylation occupancy, as well as in the length of the N terminus. Additionally we showed that PCI is O-glycosylated, but this does not contribute significantly to the size heterogeneity, because >95% of the O-glycans are either mono- or di-sialylated core type 1 structures. Furthermore, the functional effects of the N-glycans and
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the N terminus on protease inhibition were explored using a discontinuous assay of thrombin inhibition in the absence or presence of various concentrations of heparin or saturating concentrations of thrombomodulin. The results demonstrate that the N-glycans and the Δ6-N terminus of PCI can fine-tune the rates of cofactor-enhanced inhibition of thrombin and that solution conditions can differentially affect these inhibition rates by the PCI variants.

EXPERIMENTAL PROCEDURES

Proteins and Saccharides—PCI was isolated from fresh-frozen human blood plasma that originated from several healthy blood donors, as described previously (14, 15). Briefly, the plasma was purified with three consecutive affinity chromatography steps. In the first step, a column with immobilized monoclonal M11-5 anti-PCI antibody was employed. The bound protein fraction, eluted with 50 mm triethylamine, 0.5 m NaCl (pH 11.5), was collected. This fraction was further applied to a 5-ml HiTrap heparin-Sepharose column (GE Healthcare, Uppsala, Sweden) and eluted with a linear gradient from 0.1–1 m NaCl. The protein fraction that bound to the column and appeared as one broad peak upon elution was collected. In the last chromatography step, an antibody specific for RCL-cleaved PCI, M36 (16), immobilized on a HiTrap column, was used to separate the native inhibitor from the RCL-cleaved form. The native form did not bind to the column, whereas the cleaved form bound and was eluted with 0.1 m glycine, 0.5 m NaCl, pH 2.7. For the N-glycan structure determination PCI was further applied to a Superdex 75 column (GE Healthcare) coupled to an Äkta explorer 10 purifier system (Pharmacia Biotech, Uppsala, Sweden), eluted with 20 mm Tris-HCl, 0.15 m NaCl, pH 7.4, at a flow rate of 2.5 ml/min.

The concentration of the purified native protein fraction was determined by amino acid analysis. The homogeneity of the purified protein was investigated by 10–12% SDS-PAGE under reducing conditions with the Laemmli system. The gels were stained with Coomassie Brilliant Blue or colloidal Coomassie. The identity of the resulting bands was determined with peptide mapping using MALDI-TOF MS. Quantitative analysis of gel bands was performed using the gel image analyzing software Quantity One V4.5 (Bio-Rad).

Human α-thrombin was purchased from Enzyme Research Laboratories (South Bend, IN). The active concentration, determined by titrations with 100% active human α-thrombin (17), was used throughout this study. Rabbit thrombomodulin was purchased from Hematologic Technologies Inc. (Essex Junction, VT). Size-fractionated heparin with an average mass of ~11 kDa, derived from pig intestinal mucosa, was a gift from Prof. Ingemar Björk, Swedish University of Agricultural Sciences, Uppsala, Sweden.

Peptide Mapping—Bands were excised from the Coomassie-stained SDS-PAGE gels, washed, reduced with dithiothreitol, and alkylated with iodoacetamide. The proteins in the bands were cleaved by modified porcine trypsin (Promega, Madison, WI). The digests were analyzed by MALDI-TOF using an Ultraflex ToF/ToF instrument (Bruker). An α-cyano-4-hydroxycinnamic acid matrix was used. Mass spectra were used for searches in NCBIINr data base using Mascot search engine (Matrixsciences.com). Determined peptide masses were also compared with theoretical tryptic fragments of alkylated PCI.

PNGase F Treatment for the Determination of N-Glycan Occupancy—To determine the occupation of potential N-glycosylation sites, N-glycosidically linked carbohydrate chains were removed by incubating 45 μg of PCI with PNGase F (Sigma-Aldrich) under denaturing conditions according to the standard protocol, except that the concentration of PNGase F was lower than recommended (0.96 International Union of Biochemistry milliunit). Aliquots were collected at 5 s, 1 min, 5 min, 15 min, 30 min, 60 min, 3 h, and 24 h of incubation and snap frozen in ethanol with dry ice. The products obtained after the various time points were analyzed by SDS-PAGE followed by peptide mapping. In addition to Coomassie, these gels were stained with PRO-Q Emerald 300 glycoprotein gel stain and SYPRO Ruby protein gel stain (both from Invitrogen).

Digestion of PCI with Trypsin Prior to N-Glycan Determination—Purified PCI was reduced in 600 mm Tris-HCl buffer, pH 8.4, containing 2 mg/ml dithiothreitol (37 °C for 45 min), and carboxymethylated by addition of 12 mg/ml iodoacetic acid (room temperature for 1 h). Carboxymethylation was terminated by dialysis against 50 mm NH4HCO3, pH 8.5, at 4 °C for 48 h, followed by lyophilization. PCI was incubated with trypsin (Sigma) at a 50:1 ratio (w/w) in 50 mm NH4HCO3, pH 8.4, for 16 h at 37 °C. The digestion was terminated by heating at 100 °C for 3 min, followed by C18 Sep-Pak chromatography (Waters Corp.). Bound peptides were eluted with either 20% (v/v) or 40% (v/v) propanol in 5% aqueous acetic acid, pooled, and lyophilized.

Preparation of N-Glycans—PNGase F (Roche Applied Science) digestion was carried out in 50 mm ammonium bicarbonate, pH 8.5, for 16 h at 37 °C with 3 Roche units of enzyme. The released N-glycans were separated from peptides by Sep-Pak C18 (Waters Corp.) as described (18). Permethylation of glycans was performed using the sodium hydroxide procedure (18).

Preparation of O-Glycans—O-Linked oligosaccharides were liberated from glycopeptides by reductive elimination (400 μl of 1 m NaBH4 in 0.05 m NaOH at 45 °C for 16 h) and desalted through a Dowex 50W-X8 (H+) column. Excess borates were removed by co-evaporation with 10% (v/v) acetic acid in methanol under a stream of nitrogen. Desalted eluate was passed through a C18 Sep-Pak and the glycans collected in the unbound fraction.

MS for Glycan Determination—MALDI-TOF data were acquired on a Voyager-DE sSTR mass spectrometer (PerSeptive Biosystems, Framingham, MA) in the reflectron mode with delayed extraction.Permethylated samples were dissolved in 10 μl of 80% (v/v) methanol in water, and 1 μl of dissolved sample was pre-mixed with 1 μl of matrix (10 mg/ml 2,5-dihydroxybenzoic acid in 80% (v/v) aqueous methanol) before loading onto a metal plate. The MALDI-TOF/TOF experiments were performed on a 4800 Proteomics Analyzer (Applied Biosystems, Framingham, MA) operated in reflectron positive ion mode. Both 2,5-dihydroxybenzoic acid and α-cyano-4-hydroxycinnamic acid matrices (10 mg/ml in 50% (v/v) acetonitrile in 0.1% (v/v) aqueous trifluoroacetic acid) were used in conjunction with setting the potential difference between the
source acceleration voltage and the collision cell at 1 kV to obtain different degrees and patterns of fragmentation.

**PNGase F Treatment for Functional Studies**—For functional studies PNGase F treatments were conducted on the native protein. In this case, 300 μg of PCI was deglycosylated with 580 International Union of Biochemistry milliunits of PNGase F (New England Biolabs), essentially as described in the standard protocol, but in the absence of heat, SDS, and β-mercaptoethanol, to maintain the native protein structure. The treated protein was purified on a 1-ml HiTrap Heparin HP column (GE Healthcare). Protein fractions were collected, and the buffer was exchanged into 20 mM Tris-HCl, pH 7.4, by filter centrifugation with Amicon Ultra-4 centrifugal filter devices (Millipore Corp., Bedford, MA).

**Isolation of PCI Variants with Heparin-Sepharose Chromatography**—To further isolate natural PCI variants a second heparin-Sepharose chromatography was employed, using a more extended NaCl gradient than in the initial purification of PCI. Running buffers were 0 and 2 mM NaCl in 20 mM sodium phosphate, 0.1 mM EDTA, pH 7.4, in buffers A and B, respectively. A 1-ml HiTrap Heparin HP column was used. A pH gradient was additionally introduced in some experiments with pH 6.0 and 7.4 in the A and B buffers, respectively. The gradient was segmented as follows: 0 min, 0 mM NaCl; 60 min, 1.2 mM NaCl; 65 min, 1.6 mM NaCl; 66 min, 0.0 mM NaCl; and 70 min, 0 mM NaCl. The flow rate was 0.5 ml/min. The column was coupled to a fast-protein liquid chromatography system, monitoring the eluant by the absorbance at 280 nm with a UV-detector. The collected protein fractions were concentrated, and the buffer was exchanged into 20 mM Tris-HCl, pH 7.4, with Amicon Ultra-4 centrifugal filter devices (Millipore Corp.). The protein concentration was determined with the Bradford reagent (Bio-Rad, Hercules, CA). Protein fractions were collected, and the buffer of such plots to a single exponential decay function (17). The linear dependence of $k_{obs}$ versus [PCI] was verified for the initial PCI preparation, giving the second order rate constant, $k_2$. For the isolated PCI variants, second order rate constants were therefore calculated by dividing $k_{obs}$ by the active PCI concentration.

**Effects of Cofactors on the Rates of Thrombin Inhibition**—The rates of thrombin inhibition by the PCI variants in the presence of heparin were measured under the same conditions as described above except that 9 nM to 90 nM heparin was added in the incubation mixtures. The dependence of $k_{obs}$ on the heparin concentration was computer-fitted by the following equation, described previously for the antithrombin-thrombin-heparin ternary complex (19), with KaleidaGraph software,

$$k_{obs} = k \left[ [PCI]_i + k_{CL}[PCI-T] \times \frac{K_{f,PCI}}{[H]_f} + K_{CL} + [H]_f \right] \times \frac{[H]_f}{K_{f,PCI} + [H]_f}$$

(Eq. 1)

where

$$[PCI-H] = \frac{[PCI]_i + [H]_i + K_{f,CL} - \sqrt{([PCI]_i + [H]_i + K_{f,CL})^2 - 4[PCI]_i[H]_i}}{2}$$

(Eq. 2)

$$[PCI]_i = [PCI]_0 - [PCI-H]$$

(Eq. 3)

$$[H]_i = [H]_0 - [PCI \cdot H]$$

(Eq. 4)

[PCI]_0 and [H]_0 are the total PCI and heparin concentrations, $K_{f,PCI}$ and $K_{f,CL}$ are dissociation equilibrium constants for the thrombin-heparin and PCI-heparin interactions, $k_1$, $k_{CL,1}$, and $k_{CL,2}$ are second order rate constants for the reactions of thrombin with PCI, PCI-heparin binary complex with thrombin, and the thrombin-heparin binary complex with the PCI-heparin binary complex, respectively. Measured $k_q$ values, determined by the method described above (i.e. the $k_q$ values measured in the absence of cofactor), were used. Published values for $K_{f,CL}$ were used, because the $K_{f,CL}$ for low and high affinity heparins binding to thrombin are indistinguishable, i.e. 0.7 ± 0.1 and 0.9 ± 0.1 μM, respectively (20).

The thrombomodulin-catalyzed rates of thrombin inhibition by the PCI variants were measured using close to saturating concentrations of thrombomodulin (50–200 nM), based on the previously described affinity of thrombin for thrombomodulin (21). Thrombomodulin enhances the reactivity of thrombin with PCI by providing both a binding site for the serpin and allosterically modulating the activity of thrombin (22). It was
recently shown that the dependence of thrombomodulin on this inhibition rate follows a bell-shaped curve (23). We therefore reported the results determined at 50 nm thrombomodulin, because the values determined at 100–200 nm were somewhat lower, to ensure that we were measuring the maximal effects. Ca$^{2+}$ and albumin were found not to affect these inhibition rates and, therefore, were not included in the incubation assays.

RESULTS

Purification and Homogeneity of PCI—The isolation of PCI from human blood plasma was based on three consecutive affinity chromatography steps, two of which employ monoclonal antibodies specific for PCI. Additionally, gel chromatography on S-75 Superdex, was included prior to the N-glycosopeomics experiments. SDS-PAGE analysis of the isolated PCI fraction revealed several bands, in agreement with earlier reports (15), demonstrating a substantial size heterogeneity of the PCI fraction. We therefore ran a number of SDS-PAGE gels to achieve a better separation between these bands, resulting in the identification of at least six apparent bands (see the first lane of Fig. 1A). The same band pattern was observed in two different PCI preparations, each derived from several blood donors.

To identify these bands, PCI was subjected to SDS-PAGE in a large (18 × 16 cm) gel, and the resulting bands were analyzed by peptide mapping. Mascot search results gave probability based Mowse scores between 110 and 200 for all bands, verifying that all the bands were PCI. No other proteins could be detected. Bands 1–6 constituted ~16, 23, 41, 15, 3, and 3%, respectively.

N-Glycosylation Site Occupancy—To determine the N-glycosylation site occupancy, PCI was treated with PNGase F under denaturing conditions. This enzyme cleaves the linkage between the asparagine on the core protein and the attached oligosaccharide for all types of mammalian N-glycans. The released sugar chain remains intact, whereas the asparagine residue, to which the sugar had been attached, becomes deaminated to aspartic acid (24). Peptides containing N-linked glycan sites, from which the sugar had been removed, could thus be identified by a one mass unit higher mass than predicted from the amino acid sequence. The SDS-PAGE band pattern after treatment with PNGase F at different time points is shown in Fig. 1. The deglycosylation process started immediately, because the first aliquot that was removed (5 s in Fig. 1A) already showed a shift in the gel band pattern toward lower molecular weights. Bands 1–6 from the untreated PCI sample and bands 7–13 from the deglycosylated samples were analyzed by peptide mapping. Bands 7–12 were composed of mixtures of products, being partially deglycosylated by the enzyme. The presence or absence of signals of peptides from bands 1–6 and 13, containing the N-glycosylation sites and the N terminus, are shown in Fig. 2. The absence of a signal corresponding to the potential N-glycosylation site peptide in the untreated sample combined with appearance of the corresponding signal with one mass unit increment after PNGase F treatment, verifies that the N-glycosylation site in that sequence was originally occupied (Fig. 2). For bands 1–4, none of the peptides containing N-glycosylation sites were detected. For band 5 the peptide containing the Asn-243 glycosylation site was detected with a small signal in one experiment but was absent in another experiment. For band 6, the peptide containing the Asn-243-glycosylation site was detected in both experiments. We therefore assumed that the detection of the Asn-243-containing peptide in band 5 in one of the experiments was due to contamination from band 6, which is a reasonable assumption considering the proximity of the bands in the gel. Band 13 contained all three predicted N-glycosylated peptides with a one mass unit shift. The results thus show that all N-glycans were efficiently removed in band 13 of the treated sample and that the proteins in bands 1–5 from the untreated sample are glycosylated at all three N-glycosylation sites, whereas band 6 lacks the N-glycan attached to Asn-243. Although more than two bands can be visualized at the 24-h time point in Fig. 1A, most repeated experiments gave only two final products. Interestingly, staining with PRO-Q Emerald 300 glycoprotein gel of Fig. 1

FIGURE 1. SDS-PAGE image of human blood-derived untreated and PNGase F-treated PCI. A, the sequential removal of carbohydrate chains after PNGase F treatment under denaturing conditions for various time points is shown. SDS-PAGE was conducted under reducing conditions and stained with Coomassie. Untreated PCI (lane 1) and samples taken after treatment for 5 s, 1 min, 5 min, 15 min, 30 min, 1 h, 3 h, and 24 h of incubation, as described under “Experimental Procedures,” are shown. B, SDS-PAGE of PCI treated with PNGase F for 3 h, stained with SYPRO Ruby stain (A and B) and with PRO-Q Emerald 300 glycoprotein stain (C and D). Lanes A and C, untreated PCI; lanes B and D, PNGase F-treated PCI. Candycane glycoprotein molecular weight standard was used as a marker in the first and the third lanes.
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FIGURE 2. Peptides obtained by in-gel digestion with trypsin of blood-derived PCI (bands 1–6 in the SDS-PAGE in Fig. 1A) and PNGase F-treated PCI (band 13 in the SDS-PAGE in Fig. 1A) and schematic drawing of the major constituents of the SDS-PAGE bands. PCI was reduced and alkylated prior to digestion with trypsin. Molecular ions [M+H]+ were determined by MALDI-TOF. Tryptic peptides containing the N-terminal sequence and the potential N-glycosylation sites (the latter marked with gray shadows) are shown. Presence or absence of a signal is marked with + or −, respectively. The presence of a signal corresponding to the mass expected from the amino acid sequence of the peptides containing potential N-glycosylation sites (649.3, 2160.2, and 3617.8) shows that a natural non-glycosylated form exists. In bands 1–3, peptides containing N-glycosylated sites had very strong signals of the deaminated peptide products with one mass unit increase compared with that expected from the amino acid sequence. Therefore, the latter were not determined (nd) in band 13. The major components of the six bands are schematically drawn to the right. Horizontal lines represent the protein backbone, and "y" symbols represent covalently bound N-glycans. Potential N-glycosylation sites are Asn-230, Asn-243, and Asn-319, from the left to right. * and # indicate that the size differences are caused by differences in N-glycan structures.

| SDS-PAGE band | HHPR (3-6) | NR-SCR (230-234) | VVGYPYQGEO (235-255) | VLPNSLGVFTSHSEMVHK (298-331) | Schematic drawing of the major constituents of the SDS-PAGE bands |
|---------------|------------|-----------------|----------------------|-----------------------------|---------------------------------------------------------------|
| 1-2           | +          | −               | −                    | −                           | NIL. Y Y Y COOH *                                             |
| 3             | +          | −               | −                    | −                           | NIL. Y Y Y COOH                                               |
| 4             | −          | −               | −                    | −                           | NIL. Y Y Y COOH                                               |
| 5             | −          | −               | −                    | −                           | NIL. Y Y Y COOH                                               |
| 6             | +          | nd              | nd                   | nd                          | NIL. Y Y Y COOH                                               |
| 13            | +          | nd              | nd                   | nd                          | NIL. Y Y Y COOH                                               |

FIGURE 3. MALDI-TOF mass spectrum of permethylated PCI N-glycans from human blood plasma. N-Glycans are derived from the 50% (v/v) acetonitrile fraction from a C$_{18}$ Sep-Pak. All molecular ions are [M+Na]+, and nominal masses of the 13C isotope are shown. Putative structures based on composition, tandem mass spectrometry analysis, and knowledge of biosynthetic pathways are shown. Signals at m/z 1763, 1967, 2212, and 2416 were likely to be formed as a result of fragmentation of the major ion at m/z 2791. Peaks marked with a cross are metastable fragment ions. ○, galactose; gray circle, mannose; ■, GicNAc; gray triangle, fucose; and ●, NeuAc. stain gave a signal in band 13, although migration behavior and peptide mapping showed that all the N-linked glycans were removed in this band (Fig. 1B). These findings indicate that PCI contains O-linked glycans, in addition to the N-linked glycans. This was confirmed by mass spectrometric analysis of glycans released by alkaline elimination (see later).

Detection of N-terminally Cleaved PCI—Another explanation for the size heterogeneity of PCI was the partial cleavage of the N terminus. For bands 1–3 and 6 the ion at m/z 546.3 corresponding to the peptide HHPR was detected, whereas the peptide maps of the bands 4 and 5 lacked this signal (Fig. 2). These results show that an N-terminal fragment is lacking in a fraction of the isolated PCI and are in agreement with a previous study (15), which showed that a portion of PCI isolated from fresh human plasma is cleaved at the N terminus, lacking the six most N-terminal amino acid residues, HRHHP. Further size heterogeneity, as observed for instance among bands 1–3, is likely to be caused by differences in the N-glycan structures. This was confirmed by structure analysis experiments described in the following section.

MALDI-MS Analysis of N-Glycans Released from PCI—Purified PCI from human blood plasma was reduced/carboxymethylated and tryptically digested to facilitate deglycosylation with PNGase F. The released glycans were separated from peptides and were analyzed by MALDI-MS after permethylation and Sep-Pak purification (Fig. 3). The spectrum indicates that PCI N-glycans have compositions consistent with complex type bi-, tri-, and tetra-antennary glycans with (NeuAc$_{1-4}$ Fuc$_{0-2}$Hex$_{5-7}$HexNAc$_{4-6}$). The most abundant ion (m/z 2791) has a composition (NeuAc$_{3}$Hex$_{3}$HexNAc$_{4}$), consistent with a non-fucosylated bi-antennary glycan with both antennae capped with sialic acid (Fig. 3). Thus blood-derived PCI appears to be dif-
ferently glycosylated to urinary PCI, which is reported to carry core fucose on 60% of its glycans (25). Moreover a portion of the urinary glycans have antennae composed of lacdNAc(GalNAc1–4GlcNAc), a sequence that is absent in blood-derived PCI.

Characterization of PCI N-Glycan Structures by MALDI-TOF/TOF—Where possible, components observed in the MALDI-MS profile (Fig. 3) were subjected to MALDI-TOF/TOF mass spectrometry to assist sequence assignment. MALDI-TOF/TOF of the ion at m/z 2791 (NeuAc2Hex5HexNAc4) produced strong signals at m/z 2416 and 1967 corresponding to the loss of NeuAc and NeuAcHexHexNAc from the parent ion, respectively (data not shown). A strong b ion at m/z 847 (NeuAcHexHexNAc) further supported the presence of this antenna (data not shown). The fucosylated counterpart (m/z 2965) of the component at m/z 2791 was also subjected to MALDI-TOF/TOF analysis to gain information on whether the fucose residue was present on an antenna or on the core (Fig. 4). The presence of fragment ions at m/z 1021 indicates that the fucose can be on either one, and when it is on the antenna it forms part of a sialyl-Le^a/x epitope (see annotations in Fig. 3). Tri- and tetra-antennary glycans (e.g. m/z 3775, 3949, 4585, and 4759) are also predicted to carry sialyl-Le^a/x structures. In addition, a glycan mass consistent with di-sialyl antennae was observed at m/z 4136. The abundance of this component was too low to be rigorously characterized, but it is likely that it carries the additional NeuAc as part of a NeuAc(α2–3)Gal(β1–3)[NeuAc(α2–6)]GlcNAc(β1–2)Man epitope (26).

MALDI-MS Analysis of O-Glycans Derived from PCI—O-Glycans were released from tryptic (O-glyco)peptides by reductive elimination, derivatized, and then analyzed by MALDI-MS (supplemental Fig. S1). The most intense ions are at m/z 895 and 1256, which correspond to glycans with the compositions NeuAcHexHexNAc and NeuAc2HexHexNAc, respectively. These core 1 structures were confirmed by CAD-ESI-MS/MS (data not shown). Accompanying peaks at m/z 1344 and 1705 correspond to N-acetyllactosamine extensions of these glycans (supplemental Fig. S1).

Separation of Native and Deglycosylated PCI Variants by Heparin Chromatography—PCI is a glycosaminoglycan-binding serpin (reviewed in Ref. 5). Upon heparin-Sepharose chromatography conducted with an extended NaCl gradient at pH 7.4, untreated PCI eluted as two peaks (not shown), demonstrating that natural PCI variants with different heparin binding properties exist. Adding a pH gradient to the same NaCl gradi-
ent resulted in improved separation between the two peaks and
the appearance of a small third peak between the two major
peaks (Fig. 5A). The SDS-PAGE image of the proteins in these
three peaks is shown in Fig. 5C. The migration behavior in SDS-
PAGE of PCI, which had been treated with PNGase F under
denaturing conditions, showed that the protein lacked all
N-glycans (data not shown). This protein fraction was sub-
jected to heparin-Sepharose chromatography in the same man-
ner as the non-treated protein. The resulting chromatogram is
shown in Fig. 5B. PNGase F was found to bind to heparin (the
first peak in Fig. 5B). The two major deglycosylated PCI peaks
appeared at similar positions as the two major non-treated PCI
peaks (Fig. 5, A and B). SDS-PAGE analysis of the contents in
the two peaks derived from the deglycosylated sample revealed
bands that were >90% homogeneous (Fig. 5D). Peptide map-
ing of the bands in Fig. 5D demonstrated that the only differ-
ence between the two peaks was in the N terminus. The peak
eluting at 0.34–0.46 M NaCl, pH 6.2–6.3, was lacking the
N-terminal tryptic peptide, whereas the peak eluting at 0.58–
0.72 M NaCl, pH 6.4–6.5, contained the full-length protein. The
PCI variant lacking the N-terminal tryptic peptide in peptide
mapping experiments thus represents the N-terminally cleaved
form that lacks the six most N-terminal residues HRHHHPR
reported previously (15). These results demonstrate that the
overall removal of N-glycans does not seem to affect heparin
affinity much, whereas PCI lacking the six most N-terminal
residues, here denoted the Δ6-N-cleaved form, has consider-
ably lower affinity for heparin than full-length PCI.

Effects of PCI Variants on Thrombin Inhibition—The effects of
N-glycan removal and the Δ6-N-region of PCI on protease
inhibition were studied using thrombin as the protease at 25 °C
in the presence of physiological Na+. Thrombin inhibition by
untreated and PNGase F-treated PCI fitted well to a monopha-
sic single-exponential decay function. Similarly, deglycosylated
PCI with and without the N terminus also fitted this type of
function. Second order rate constants for thrombin inhibition
were (11 ± 1) × 10³ and (11 ± 2) × 10³ M⁻¹ s⁻¹ for the untreated
and PNGase F-treated PCI, respectively
(Table 1), demonstrating that the
rate of thrombin inhibition in the
absence of cofactors was not
affected by the overall removal of the
N-glycans. Moreover, second order rate constants for the deglyco-
sylated full-length and the Δ6-N-
cleaved form were (9 ± 0.9) × 10³ and (7 ± 0.9) × 10³ M⁻¹ s⁻¹, respec-
tively (Table 1), demonstrating that
there were no major differences
between the rates of thrombin inhibi-
tion by the full-length and the
Δ6-N-cleaved forms in the absence of
cofactors.

Effects of PCI Variants on Cofactor-enhanced Thrombin Inhibition—The effects of N-glycan removal and the Δ6-N-region of
PCI on thrombin inhibition were also studied in the presence of varying
concentrations of heparin or

**TABLE 1**

Bimolecular association rate constants of thrombin inhibition by full-length and Δ6-N-cleaved forms of untreated and PNGase F-treated PCI

| PCI form                        | No cofactor | Optimal heparin* | -fold increase | Thrombomodulin* | -fold increase |
|---------------------------------|-------------|------------------|----------------|-----------------|---------------|
| Untreated full-length           | 0.11 ± 0.01*| 3.7              | 34             | 2.3 ± 0.4       | 21            |
| Untreated Δ6-N-cleaved          | 0.11 ± 0.01*| 1.2              | 11             | 1.1 ± 0.1       | 10            |
| Deglycosylated full-length      | 0.09 ± 0.009| 6.9              | 77             | 2.3 ± 0.2       | 26            |
| Deglycosylated Δ6-N-cleaved     | 0.07 ± 0.009| 3.1              | 44             | 0.95 ± 0.2      | 14            |

* Second-order rate constants ± S.E. were calculated from the observed pseudo-first order rate constants obtained by nonlinear regression fitting to a monophasic exponential decay function as described under “Experimental Procedures.”

* The highest heparin-enhanced thrombin inhibition rates were obtained from the computer-fitted curves of the dependence of k_{on} on the heparin concentration shown in Fig. 6.

* The thrombomodulin concentrations used were close to saturating with respect to thrombin binding.

* Measured in blood-derived PCI, which contains the full-length as well as the Δ6-N-cleaved forms. Similarly, deglycosylated blood-derived PCI containing the full-length and the Δ6-N-cleaved form gave a k_{2} value of (0.11 ± 0.02) × 10³ M⁻¹ s⁻¹.

**FIGURE 5.** Binding of PCI variants to heparin. A shallow gradient with increasing pH and NaCl concentration was used on a 5-ml HiTrap heparin-Sepharose column, as described under “Experimental Procedures.” A, elution pattern of the original PCI fraction. C, SDS-PAGE gel of the eluant from the PNGase F-treated PCI sample shown in A. B and D, elution pattern of the non-treated PCI fraction.
close to saturating conditions of thrombomodulin at 25 °C in the presence of physiological [Na\(^+\)]. The dependence of the pseudo-first order rate constants on the heparin concentration followed a bell-shaped curve for the inhibition of thrombin by all four PCI forms, which fitted well to Equation 1 (Fig. 6). The maximal heparin-enhanced second order rate constants \(k_2\) are shown in Table 1. These values were derived by computer-fitting to Equation 1 followed by dividing the maximal \(k_{\text{obs}}\) values by the PCI concentration. There was a 6-fold difference between the most and the least active variant. The maximal \(k_2\) values obtained for untreated full-length and \(\Delta 6\)-N-cleaved forms were \((3.7 \pm 0.2) \times 10^5\) and \((1.2 \pm 0.1) \times 10^5\) s\(^{-1}\), respectively, and the maximal \(k_2\) values for the deglycosylated full-chain and \(\Delta 6\)-N-cleaved forms were \((6.9 \pm 0.4) \times 10^5\) and \((3.1 \pm 0.3) \times 10^5\) s\(^{-1}\), respectively. These results show that the \(\Delta 6\)-N-region enhances the maximal second order rate constant 3-fold, whereas the presence of \(N\)-linked glycans reduces the maximal second order rate constants 2-fold. In contrast the thrombomodulin-enhanced rate of thrombin inhibition was 2-fold enhanced by the \(\Delta 6\)-N-region, but was unaffected by the removal of \(N\)-glycans (Table 1). At 25 °C in the presence of physiological [Na\(^+\)], the overall effect of the \(N\)-glycans of PCI was thus a lowered maximal rate of heparin-enhanced, but not thrombomodulin-enhanced, thrombin inhibition, whereas the \(\Delta 6\)-N-terminal region enhanced the maximal rates of both the heparin- and the thrombomodulin-enhanced thrombin inhibition.

Effects of Solution Conditions on Thrombin Inhibition by the PCI Variants—The inhibition of thrombin by the PCI variants was also determined at 37 °C. The uncatalyzed rates of thrombin inhibition were 1.4- to 1.9-fold higher at 37 °C than 25 °C for all PCI variants. The cofactor-enhanced rates were, however, somewhat differently affected by the PCI variants at higher temperature. In the presence of 150 nM heparin, these rate constants were \((11 \pm 2, 3.6 \pm 0.1, 12 \pm 2,\) and \(1.3 \pm 0.05) \times 10^5\) M\(^{-1}\) s\(^{-1}\) for the full-length untreated, \(\Delta 6\)-N-cleaved untreated, full-length PNGase F-treated and \(\Delta 6\)-N-cleaved PNGase F-treated forms, respectively. The increased temperature thus enhanced the heparin-enhanced inhibitory rates for all variants except the one lacking both the \(N\)-glycans and the N-terminal peptide, for which a decrease by the increased temperature was observed. The temperature effect on the thrombomodulin-enhanced rates were also investigated and was found to increase the inhibition rate constants for all PCI forms, which were \((4.1 \pm 0.3, 1.6 \pm 0.1, 8.7 \pm 1.8,\) and \(1.2 \pm 0.1) \times 10^5\) M\(^{-1}\) s\(^{-1}\) for the full-length untreated, \(\Delta 6\)-N-cleaved untreated, full-length PNGase F-treated and \(\Delta 6\)-N-cleaved PNGase F-treated forms, respectively. The enhancing effect of the N-terminal peptide on the thrombomodulin-enhanced rates was thus clear also at this increased temperature.

Another solution condition that was explored was the effect of using K\(^+\) instead of Na\(^+\). Thrombin activities are affected by Na\(^+\) by an allosteric mechanism, which affects the catalytic site and thus favors several prothrombotic and procoagulant properties of this protease (28). Here we explored the effects of Na\(^+\) on the inhibition by the PCI variants by using a buffer containing K\(^+\) instead of Na\(^+\). This change enhanced the uncatalyzed rates 1.3- to 2.4-fold for the PCI variants. The heparin-enhanced rates were, however, differentially altered for the PCI variants. These rate constants were \((6.4 \pm 0.9, 1.0 \pm 0.1, 5.1 \pm 0.4,\) and \(0.7 \pm 0.1) \times 10^5\) M\(^{-1}\) s\(^{-1}\) for the full-length untreated, \(\Delta 6\)-N-cleaved untreated, full-length PNGase F-treated, and \(\Delta 6\)-N-cleaved PNGase F-treated forms, respectively. These findings interestingly show that the full-length variant reached the highest heparin-enhanced rate in the presence of Na\(^+\), whereas the \(\Delta 6\)-N-cleaved form reached the highest rate in the presence of K\(^+\) and may, thus, reflect different regulatory mechanisms for these two variants. The thrombomodulin-enhanced inhibition rates increased 1.4- to 2.1-fold for the PCI variants by replacing Na\(^+\) by K\(^+\).

**DISCUSSION**

This study was conducted to pinpoint the reasons for the large size heterogeneity of human blood-derived PCI observed in this and earlier work (15, 29) and to explore whether natural blood-derived PCI variants have different functional properties. We hypothesized that the size heterogeneity is due to differences in post-translational modifications. Such information may be valuable for multifunctional proteins in general, because post-translational modifications are known to add complexity to higher organisms. This study provided evidence that the size heterogeneity of blood-derived PCI was due to differences in N-glycan structures, N-glycosylation occupancy as well as in the length of the N terminus. The finding that PCI was homogeneous in SDS-PAGE after the removal of N-glycans by PNGase F treatment followed by separation between the \(\Delta 6\)-N-cleaved and the full-length form, demonstrated that no other factors contributed to the size heterogeneity. The results further demonstrated O-glycosylation. Such glycosylation did not, however, contribute to the size heterogeneity, because only N-linked glycans are removed by PNGase F. The differences
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between the major constituents of PCI resolved by SDS-PAGE could thus be schematically pictured (Fig. 2).

The presence of bi-, tri-, and tetra-antennary complex N-glycans in blood-derived PCI clearly demonstrated the contribution of the N-glycan structures to the different sizes. The smallest underivatized N-glycan mass would be ~1900 Da, whereas the largest would be ~3800 Da. Because most of the PCI molecules contain three N-glycans, the theoretically largest mass difference due to heterogenous N-glycan structures would be 5.7 kDa, which is substantial. The presence of sialyl-Le<sup>α</sup>/epitopes on bi-, tri-, and as well as tetra-antennary N-glycans of PCI is highly interesting. These epitopes are known to bind to selectins that are expressed on, e.g. endothelial cells and platelets in response to inflammation (30). PCI has been found associated with atherosclerotic plaques, co-localized with annexin V (31), and, according to a recent report, PCI is involved in wound healing and tissue repair by inhibiting hepatocyte growth factor activator (32). Furthermore, it has been documented that PCI has improving effects on hypercoagulability states, when thrombomodulin concentrations are low, such as disseminated intravascular coagulation (33) and pulmonary hypertension (34). All these conditions are accompanied by inflammatory responses. The function of sialyl-Le<sup>α</sup>/epitopes of PCI remains to be established, but it is possible that these epitopes direct PCI to inflammatory sites in blood, such as atherosclerotic plaques, where it could be enriched for the efficient local inhibition of proteases.

It is known that N-X-T consensus sequences are usually more efficiently glycosylated than N-X-S consensus sequences (35, 36). Therefore the finding that the Asn-243 (N-X-T sequence) site was not fully occupied in the PCI sample, whereas Asn-230 and Asn-319 (both N-X-S sequences) were fully carbohydrates, was somewhat surprising. It appears therefore that residues in the vicinity of the consensus sequences may contribute to the efficiency of N-glycosylation. A previous study on recombinant N-glycosylated PCI mutants expressed in a Baculovirus system (37) showed that the removal of Asn-243 reduced the rates of inhibition of thrombin and plasma kallikrein, measured in the absence and presence of heparin. In contrast, the removal of Asn-230 enhanced these rates, and the removal of Asn-319 had no apparent effect. Therefore our results suggest that the presence of a natural non-glycosylated form of Asn-243 may be of functional significance.

The bell-shaped curves for the rates of thrombin inhibition by the PCI forms at various heparin concentrations are in agreement with previous reports, which suggested that the enhancing effect of heparin is due to a bridging effect, bringing the protease and the inhibitor into a ternary complex (8, 38, 39). The mechanism by which this has occurred has not been fully clarified. A crystal structure of the Michaelis complex of PCI in complex with heparin and thrombin was recently published (23). This structure suggested that the cofactor effect of heparin involved a bridging mechanism and substrate-induced exosite contacts. The PCI used was expressed in *Escherichia coli* and lacked both the N-glycans and the N-terminal region (23). Our study indicates that heparin may contribute with structural effects, because the overall presence of N-linked glycans of PCI reduced the maximal inhibitory rate of thrombin inhibition in the presence of heparin, but not in the absence of it, although the affinity for heparin, based on heparin chromatography, did not seem to be much changed after N-glycan removal. It is possible that heparin alters the PCI-thrombin complex structure and that this structure is also affected by the N-linked glycans. This seems like a plausible explanation, considering the proximity between the Asn-243 and Asn-230 position by heparin chromatography not necessarily reflects the affinity for heparin, although this is usually the case. The lack of effect of the N-glycans of PCI on the thrombomodulin-enhanced rate of thrombin inhibition probably relates to the fact that one effect of thrombomodulin is to induce a conformational change of thrombin to better accommodate the reactive site of PCI (11, 22).

Although the H-helix is considered to be the major heparin-binding site of PCI (Fig. 7) (5, 40), the N-terminal region, also called the A + helix region, has been proposed to contribute to heparin binding in some studies. For instance, based on modeling studies, positively charged amino acids in the N-terminal...
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region have been implicated in heparin binding (41). A synthetic peptide corresponding to the N-terminal region was found to compete with PCI for heparin binding in one study (13) but was found not to compete in another (9). According to two studies where recombinant PCI expressed in E. coli were used, the N-terminal region did not contribute significantly to heparin affinity (39, 40). However, recombinant PCI expressed in baby hamster kidney cells demonstrated that the lack of the 11 N-terminal residues resulted in decreased affinity for heparin and lowered maximal heparin-enhanced inhibition of thrombin and active protein C (13). Our results suggest that the differences between the previous reports are due to differences in post-translational modifications that arose because different expression systems were used. Our results also show that PCI carries a limited repertoire of short O-glycans. Therefore, the factors that can differ between the expression systems are several and include the occupation and structures of N-linked and other glycans as well as the length of the N terminus. Only N-linked glycans are removed by PNGase F, which ensures that, in this study, the effect of glycans was restricted to the N-linked glycans.

This is the first report on the isolation of the Δ6-N-cleaved form, lacking the N-terminal residues HRHHPR (15), from the full-length variant found in human blood. The separation between the two forms allowed us to show that this region in blood-derived PCI affected both the heparin- and the thrombomodulin-enhanced rates of thrombin inhibition. Thrombomodulin binds to exosite 1 of thrombin, whereas heparin binds to exosite 2 (21, 42). In contrast, thrombomodulin and heparin have been suggested to bind to the same or similar sites of PCI (22, 23). The mechanism by which the Δ6-N-terminal region contributes to the heparin- and thrombomodulin-enhanced rates of thrombin inhibition could be an interaction with the cofactors or induction of conformational changes at the reactive site or exosites of PCI. Although the full-length form was a more potent thrombin inhibitor than the Δ6-N-cleaved form in the presence of heparin at all conditions studied, these two forms were, interestingly, differently affected by Na⁺. The full-length form reached the highest enhancing effect in the presence of K⁺, whereas the Δ6-N-cleaved form reached the highest effect in the presence of Na⁺. The Δ6-N-terminal region contains three histidines and two arginines and therefore has a high positive charge density, particularly at low pH, which enhances the affinity of PCI for negatively charged ligands. A potential pH dependence may be important for the role of PCI at inflammatory sites, if PCI is targeted to sites of inflammation, as indicated by the presence of sialyl-Leₐ/β epitopes and by the functions of PCI in e.g. atherosclerosis and wound healing (31, 32). Future investigations are required to investigate whether these findings reflect different functions of the two forms in vivo.

In conclusion, this study demonstrates that the observed heterogeneity of human blood-derived PCI is fully explained by differences in N-glycan structures, N-glycosylation occupancy, as well as the length of the N terminus. The interesting carbohydrate epitopes found on the N-linked glycans, including sialyl putative Leₐ/β epitopes and di-sialyl antennae, suggest that PCI is directed to specific sites on the endothelial surface. The study also provides novel information regarding the purification of human blood-derived PCI, by showing that a Δ6-N-cleaved form can be readily isolated from the full-length form by heparin chromatography. The full-length forms of PCI reach 2- to 7-fold higher rates of thrombomodulin-enhanced thrombin inhibition and 2- to 9-fold higher rates of heparin-enhanced thrombin inhibition, depending on the solution conditions, than the Δ6-N-cleaved forms. The heparin- and thrombomodulin-enhanced inhibitory rates of the PCI variants lacking the N-linked glycans were differently affected by the solution conditions. These findings suggest that not only the form of PCI but also the solution conditions, i.e. the temperature and Na⁺, contribute to bringing specificity to this serpin. The results are also medically important in consideration of clinical studies on PCI treatments of patients with hypercoagulability conditions, for which PCI variants with enhanced anticoagulant efficiency should be able to lower the doses required.

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