Contribution of Basic Residues of the 70−80-Loop to Heparin Binding and Anticoagulant Function of Activated Protein C†

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ABSTRACT: The role of basic residues of the 70−80-loop, Arg^{74}, Arg^{75}, and Lys^{78} (chymotrypsin numbering) in the catalytic function of activated protein C (APC) was investigated by expressing mutants of protein C in which these residues were replaced with Ala in three separate constructs. Following purification to homogeneity and activation by thrombin, the catalytic properties of the mutants were characterized with respect to their ability to cleave the chromogenic substrate Spectrozyme PCa, react with protein C inhibitor (PCI), and inactivate factor Va. Relative to wild-type APC, the mutants cleaved Spectrozyme PCa with identical or improved catalytic efficiencies. Similarly, PCI inhibited mutants with identical or improved second-order rate constants (k_2) in the absence of heparin. However, the heparin-catalyzed inhibition of mutants by PCI was impaired ∼10-fold. Analysis of k_2 values by a ternary complex model revealed that the affinities of mutants for heparin were impaired to a similar extent. Moreover, analysis of the NaCl gradient elution profiles of APC derivatives from Heparin-Sepharose supported this conclusion. An oligosaccharide containing 14 residues efficiently catalyzed the PCI inhibition of APC by a template mechanism. Further studies revealed that the ability of Arg^{74} and Arg^{75} mutants to inactivate factor Va was markedly impaired. We conclude that basic residues of the 70−80-loop are critical for the catalytic function of APC.

Protein C is a multidomain vitamin K-dependent serine protease zymogen in plasma that, upon activation by the complex of thrombin and thrombomodulin (TM),[1] down-regulates the coagulation cascade by degrading factors Va and VIIIa by limited proteolysis (*1*−*3*). Activated protein C (APC) circulates in plasma as a light- and heavy-chain molecule held together by a single disulfide bond (*4*). Similar to other vitamin K-dependent coagulation proteases, the N-terminal light chain of APC contains the noncatalytic γ-carboxyglutamic (Gla) domain and two epidermal growth factor (EGF)-like domains (*5*). The catalytic domain of APC with a trypsin-like primary specificity pocket is located on the C-terminal heavy chain of the molecule. APC is a highly specific enzyme with factors Va and VIIIa being the only known substrates for the protease in plasma (*1*). APC also reacts very slowly with natural protein inhibitors; thus, it has a long circulating plasma half-life of ∼27 min (*6*). The proteolytic activity of APC is believed to be regulated by the serine protease inhibitors (serpins), protein C inhibitor (PCI) (*7*, *8*), plasminogen activator inhibitor-1 (PAI-1) (*9*), and α_1-antitrypsin (*10*). Among the three serpins, the reactivity of APC with α_1-antitrypsin is the slowest (∼10 M^{-1} s^{-1}). However, due to its high concentration (∼40 μM), α_1-antitrypsin is also believed to contribute to regulation of APC activity in plasma (*10*). The reactivity of APC with other two serpins is also relatively slow (10^2−10^3 M^{-1} s^{-1}). However, cofactors heparin and vitronectin markedly accelerate the reactivity of APC with PCI and PAI-1, respectively (*8*, *9*).

It is not known exactly how vitronectin promotes the PAI inhibition of APC, but it is well established that the rate accelerating effect of heparin in the PCI inhibition of APC is mediated through a template mechanism in which heparin binding to both the serpin and protease facilitates the encounter between the two proteins (*8*, *11*). Previous mutagenesis and molecular modeling studies have indicated that heparin binds to a cluster of basic residues present on the H-helix of the serpin (*8*, *11*−*13*). The X-ray crystal structure of Gla-domainless APC suggests that several positively charged residues are clustered at a region on the right side of the active site pocket of the protease that constitutes the fibrinogen binding site, also termed exosite 1, in the homologous region of thrombin (*14*). The basic residues of this region are clustered on three conserved surface loops known as 39-loop (Lys^{37}-Lys^{39}), 60-loop (Lys^{62}, Lys^{63}), and 70−80-loop (Arg^{74}, Arg^{75}, and Lys^{78}). Results of several recent structure−function studies suggest that,

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[1] Abbreviations: APC, activated protein C; APC R74A, R75A, and K78A, APC mutants in which Arg^{74}, Arg^{75}, and Lys^{78} (in the chymotrypsin numbering system of Bode et al. (*43*)) have been replaced with Ala by the recombinant DNA methods; Gla, γ-carboxyglutamic acid; GDPC, Gla-domainless protein C; GDPC R74E/R75E/K78E, GDPC in which the indicated residues in the chymotrypsin numbering system (*43*) have been replaced with Glu; TM, thrombomodulin; aPTT, activated partial thromboplastin time; PCI, protein C inhibitor; PAI-1, plasminogen activator inhibitor-1; serpin, serine protease inhibitor; PEG, poly(ethylene glycol).

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similar to thrombin, the basic residues of these loops play critical roles in the catalytic function of APC. The Lys residues of the 39-loop have been demonstrated to be critical for the catalytic function of APC with respect to degradation of factor Va (*15*), binding to heparin (*11*, *16*), and the specificity of its interaction with serpins (*9*). In addition, these residues on protein C are crucial for thrombin to efficiently activate the zymogen in the presence of thrombomodulin (TM) (*17*). This is consistent with recent structural and molecular modeling data, which suggest that certain basic residues of this loop may interact with the acidic residues of TM4 in the thrombin−TM complex (*18*). The basic residues of the 60-loop appear to be dispensable for the anticoagulant function of APC; they are, however, required for effective binding of the protease to heparin (*16*). Similar to trypsin and other vitamin K-dependent coagulation proteases (*19*), the 70−80-loop of APC constitutes a functionally important Ca^{2+}-binding site on the protease (*20*). Our previous data revealed that the binding of the metal ion on this loop allosterically modulates the structure and catalytic function of APC in the anticoagulant pathway (*21*). However, the contribution of the basic residues of the 70−80-loop to heparin binding and anticoagulant function of APC has not been studied.

In this study, the basic residues of the 70−80-loop of protein C, Arg^{74}, Arg^{75}, and Lys^{78}, were substituted with Ala in three separate constructs. These mutants, together with PCI, were expressed in mammalian cells and purified to homogeneity. Following activation by thrombin, the catalytic activities of the APC mutants were characterized with respect to their ability to hydrolyze chromogenic substrates, react with PCI, and inactivate factor Va. The inhibitory activity of recombinant PCI in reaction with APC was identical to that observed with the plasma-derived PCI. The amidolytic activity of all APC mutants toward cleavage of Spectrozyme PCa was normal or improved. The reactivity of mutants with PCI was also normal or improved ~2-fold in the absence of heparin. However, the heparin-catalyzed PCI inhibition of mutants was impaired ~10-fold. The affinity of mutants for binding heparin was impaired to a similar extent. This conclusion was supported by direct binding studies using a Heparin−Sepharose column. Inhibition studies in the presence of heparin fragments of varying size suggested that an oligosaccharide containing 14 residues is capable of bridging the protease and serpin in a ternary complex. Further studies revealed that the anticoagulant activity of both Arg^{74} and Arg^{75} mutants was also impaired. We conclude that the basic residues of the 70−80-loop are critical for heparin binding and the anticoagulant function of APC.

MATERIALS AND METHODS

Construction and Expression of Recombinant Proteins. Wild-type and protein C mutants in which the basic residues Arg^{74}, Arg^{75}, and Lys^{78} were individually substituted with Ala (R74A, R75A, and K78A, respectively) were prepared by PCR methods, expressed in HEK293 cells using pRC/RSV (Invitrogen, San Diego, CA), and purified to homogeneity as described (*22*). Gla-domainless protein C (GDPC) and a triple mutant of GDPC in which Arg^{74}, Arg^{75}, and Lys^{78} were replaced with Glu (R74E/R75E/K78E) were constructed by PCR methods and expressed in HEK cells using the RSV-PL4 mammalian expression/purification vector system as described (*22*). RSV-PL4 was also used to express human protein C inhibitor (PCI) in the same cell line. This vector contains the sequence of the transferrin signal peptide for secretion, the sequence of a 12-residue epitope for a Ca^{2+}-dependent monoclonal antibody, HPC4, for purification and a neomycin gene for G418 selection in mammalian cells (*22*). Following transfer of the vector into mammalian cells, G418 resistant clones were selected and examined for PCI expression by a sandwich enzyme-linked immunosorbent assay using the HPC4 monoclonal (*23*) and a polyclonal antibody against human PCI (Enzyme Research laboratories, South Bend, IN). A high-expressing clone was identified and expanded, and 20 L of cell culture supernatant was collected, concentrated, and purified by immunoaffinity chromatography using the HPC4 antibody linked to Affi-gel 10 (Bio-Rad) as described (*22*). The PCI concentration was determined from the absorbance at 280 nm assuming a molecular weight of 57 000 and an extinction coefficient ($E^{1\%}_{1cm}$) of 14.1 (*24*) and by stoichiometric titration of the serpin with known concentrations of APC.

Human plasma proteins APC, protein S, factor Xa, factor Va, and prothrombin were purchased from Haematologic Technologies, Inc. (Essex Junction, VT). Human plasma PCI as a control was the courtesy of Dr. Frank Church from the University of North Carolina at Chapel Hill. The oligosaccharides, ranging in size from 6 to 18 saccharides, were generous gifts from Dr. Ingemar Björk (Swedish University of Agricultural Sciences, Uppsala, Sweden), and high-affinity heparin fragments of 22−64 saccharides were generous gifts from Dr. Steven Olson (University of Illinois−Chicago). Heparin−Sepharose (CL-6B) was purchased from Amersham Pharmacia (Piscataway, NJ). Unfractionated heparin from porcine intestinal mucosa (sodium salt), Polybrene, and the activated partial prothrombin time (aPTT) reagent, Alexin, were purchased Sigma (St. Louis, MO). The normal pooled plasma was purchased from George King Bio-Medical, Inc. (Overland Park, KS). Phospholipid vesicles containing 80% phosphatidylcholine and 20% phosphatidylserine (PC/PS) were prepared as described (*25*). The chromogenic substrate, Spectrozyme PCa (SpPCa), was purchased from American Diagnostica (Greenwich, CT), and S2238 was purchased from Kabi Pharmacia/Chromogenix (Franklin, OH).

Protein C Activation. Following purification on the HPC4 immunoaffinity column, 1 mg of each protein C derivative was incubated with thrombin (100 μg) in 0.1 M NaCl, 0.02 M Tris−HCl, pH 7.4 (TBS buffer) containing 5 mM EDTA for 2 h at 37 °C. Activated protein C derivatives were separated from thrombin by an FPLC Mono Q column developed with a 40 ml linear gradient from 0.1 to 1.0 M NaCl, 0.02 M Tris−HCl, pH 7.4. Partially and fully γ-carboxylated APC derivatives were eluted from the Mono Q column as two distinct peaks at ~0.3 and ~0.4 M NaCl, respectively, as described (*9*). The APC concentrations were determined from the absorbance at 280 nm assuming a molecular weight of 56 200 and an extinction coefficient ($E^{1\%}_{1cm}$) of 14.5 (*26*) and by stoichiometric titration of enzymes with known concentrations of PCI.

Cleavage of Chromogenic Substrates. The steady-state kinetics of hydrolysis of SpPCa (15−2000 μM) by the APC derivatives (2 nM) was studied in 0.1 M NaCl, 0.02 M Tris−HCl (TBS, pH 7.5) containing 2.5 mM Ca^{2+}, 0.1 mg/mL BSA, and 0.1% poly(ethylene glycol) (PEG 8000). The rate

of hydrolysis was measured at 405 nm at room temperature in a V_{max} kinetic microplate reader (Molecular Devices, Menlo Park, CA) as previously described (*27*). The apparent K_m and k_{cat} values for substrate hydrolysis were calculated from the Michaelis−Menten equation, and the catalytic efficiencies were expressed as ratios of k_{cat}/K_m.

Inhibition Kinetic Methods. The PCI inhibition rates of APC derivatives were measured under pseudo-first-order rate conditions by a discontinuous assay method in both the absence and presence of heparin as described (*28*, *29*). In the absence of heparin, 2 nM APC was incubated at room temperature with 50−200 nM PCI in 50 μL reactions in TBS buffer containing 2.5 mM Ca^{2+}, 0.1 mg/mL BSA, and 0.1% PEG 8000. At the end of the incubation time (15−30 min), 50 μL SpPCa was added to a final concentration of 0.5 mM. The remaining activities of APC derivatives were measured from the rate of chromogenic substrate hydrolysis at 405 nm using a V_{max} Kinetics Microplate Reader (Molecular Devices, Menlo Park, CA). The pseudo-first-order inactivation rate constants (k') were calculated by fitting the time-dependent change of the APC activity to a first-order rate equation as described (*28*, *29*). The uncatalyzed second-order inactivation rate constants ($k_{2,uncat}$) were calculated from the slope of the linear plot of the k' values vs PCI concentrations. In all assays, less than 10% chromogenic substrate was utilized.

For the inactivation studies in the presence of heparin, the heparin concentration dependence of reactions was determined by incubating 1 nM of wild-type or mutant APC with 10−50 nM PCI and 0−50 μM heparin in 50 μL reactions in the same TBS buffer system containing 2.5 mM Ca^{2+} or 100 μM EDTA. Following 1−20 min of incubation at room temperature (~25 °C), 50 μL of SpPCa in TBS containing 1 mg/mL Polybrene (to block heparin action immediately) was added to a final concentration of 0.5 mM. The catalyzed k_{obs} values at each heparin concentration were determined from a first-order rate equation, and the k_2 values were calculated by dividing k_{obs} values by the concentration of the serpin. The bell-shaped dependence of k_2 on heparin concentrations was then computer fit to the following equation as described (*30*).

$$k_2 = ([PCI]_o + [H]_o + K_{PCI,H} - \{([PCI]_o + [H]_o + K_{PCI,H})^2 - (4[PCI]_o \times [H]_o)\}^{1/2})/(2 \times [PCI]_o) \times (k_{2,max} \times K_{APC,H})/(K_{APC,H} + [H]_o) \quad (1)$$

In the equation above, $[PCI]_o$ is the total PCI concentration, $[H]_o$ is the total heparin concentration, $K_{PCI,H}$ is the dissociation constant for heparin−PCI interaction, $k_{2,max}$ is the maximum second-order inhibition rate constant, and $K_{APC,H}$ is the dissociation constant for the APC−heparin interaction. The heparin concentration represents the independent variable in the equation. This equation neglects the uncatalyzed rate constant since its contribution was found to be negligible within experimental error.

Determination of Inhibition Stoichiometry (*SI*). The SI values for the PCI inactivation of the APC derivatives in the presence of heparin were determined by titration of 50 nM APC with increasing concentrations of PCI corresponding to PCI/APC molar ratios of 0−2 plus a fixed 2-fold molar excess of heparin over the highest concentration of the serpin. The residual amidolytic activities of the wild-type and mutant enzymes were monitored for up to 2 h at room temperature by the hydrolysis of SpPCa as described above. After completion of the inhibition reactions, the PCI/APC ratios were plotted versus the residual activities of enzymes and the SI values were determined from the *x*-intercept of the linear regression fit of the inhibition data as described (*31*).

Binding to Heparin−Sepharose. APC derivatives (0.5 mL, 200 nM) in 0.02 M Tris−HCl (pH 7.5), 0.05 M NaCl containing 5 mM Ca^{2+} were applied on a 0.5 mL Heparin−Sepharose column preequilibrated with the same buffer as described previously (*28*). The column was washed with 2 mL of the same buffer followed by elution with a step gradient of 0.05−0.3 M NaCl using increments of 10 mM salt. At each step, 0.5 mL of buffer was used to elute proteins. Aliquots (25 μL) from each fraction were transferred to a 96 well plate, and the amidolytic activities of the APC derivatives toward cleavage of SpPCa were determined as described above.

Measurement of Anticoagulant Activity. Anticoagulant activities of APC derivatives were evaluated in both purified and plasma-based assay systems. The time course of factor Va inactivation by the APC mutants was measured by a two-stage assay in the purified system as described (*32*). Briefly, in the first stage, factor Va (2.5 nM) was incubated with wild-type or mutant APC (0.5 nM) on 25 μM PC/PS vesicles in TBS containing 2.5 mM Ca^{2+}, 0.1 mg/mL BSA, and 0.1% PEG 8000. In the second stage, at different time intervals (0−20 min), the remaining factor Va activity was determined in a prothrombinase assay from the factor Va-catalyzed rate of prothrombin activation by factor Xa as described (*32*). The prothrombinase assay was carried out for 30 s with excess prothrombin (1 μM) and saturating factor Xa (10 nM) at room temperature. The remaining activity of factor Va was determined from the decrease of the rate of thrombin generation as monitored by an amidolytic activity assay using 100 μM S2238. An aPTT assay was employed to evaluate the anticoagulant activities of the APC mutants in plasma using a STart 4 fibrinometer (Diagnostica/Stago, Asnieres, France). Briefly, 0.050 mL of TBS lacking or containing 1.25−20 nM final concentrations of wild-type or mutant APC was incubated with a mixture of 0.05 mL of normal pooled plasma plus 0.05 mL of aPTT reagent (Alexin) for 5 min before the initiation of clotting by the addition of 0.05 mL of 35 mM $CaCl_2$ at 37 °C. Clotting times were plotted as a function of APC concentrations.

Data Analysis. The ENZFITTER (R. J. Leatherbarrow, Elsevier, Biosoft, London) computer program was used to fit data to appropriate equations. All values are the average of at least three independent measurements ± standard error.

RESULTS

Expression and Purification of Recombinant Proteins. Wild-type and mutant protein C derivatives were expressed in HEK293 cells and purified to homogeneity on the HPC4 immunoaffinity column as described (*22*). SDS−PAGE analysis of zymogens prior to and following activation by thrombin indicated that, similar to plasma protein C, recombinant protein C derivatives were expressed as two closely migrating subforms that are glycosylation variants observed previously with this protein (data not shown) (*27*, *33*). Human PCI was expressed in the same cell line using

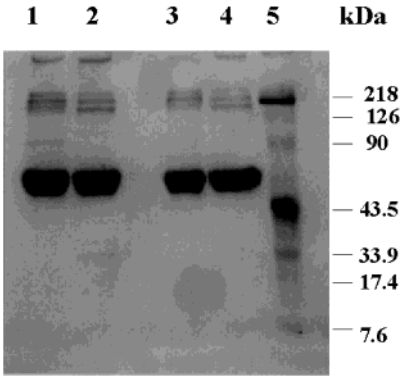

FIGURE 1: SDS−PAGE analysis of plasma and recombinant PCI. Under nonreducing conditions: lane 1, human plasma PCI; lane 2, recombinant PCI. Under reducing conditions: lane 3, plasma PCI; lane 4, recombinant PCI; lane 5, molecular mass standards.

the universal RSV-PL4 expression/purification vector system as described (*22*). PCI expressed in this system contains a 12 residue epitope for the Ca^{2+}-dependent monoclonal antibody HPC4 at its N-terminal domain for easy purification. Following passage of PCI supernatants through the HPC4 column, the substitution of Ca^{2+} with EDTA in the wash buffer was sufficient to elute protein from the antibody column (*22*). SDS−PAGE analysis suggested that the serpin had been purified to homogeneity in this single-step immunoaffinity chromatography (Figure 1). As expected from the purity on the gel, plasma and recombinant PCI exhibited indistinguishable reactivities with APC as determined in both the absence and presence of heparin. Thus, only recombinant PCI was utilized in kinetic reactions throughout the study.

Protein C derivatives were activated to APC by thrombin in the presence of EDTA and purified on an FPLC mono Q column as described under in Materials and Methods. Relative to wild-type plasma or recombinant protein C, all three Ala substitution mutants of protein C were activated by thrombin with ~1.5−2-fold improvement of rate constants in the absence of thrombomodulin (TM). However, the activation of the mutants by thrombin in complex with TM was impaired. The impairment in the rate of activation ranged from ~2−3-fold for K78A and was greater than 50-fold for both the R74A and R75A mutants of protein C (data not shown). Consistent with structural and other mutagenesis data (*18*, *34*), these results suggest that the basic residues of the 70−80-loop of protein C are cofactor-dependent recognition sites for the thrombin−TM complex. An improved activation rate in the absence of cofactor further suggested that the mutagenesis had not adversely affected the folding of the mutant zymogens. As described below, this is also consistent with the observation that the cofactor-independent catalytic function of the mutants toward cleavage of tripeptidyl chromogenic substrates and reaction with PCI are normal or improved.

Amidolytic Activity. Kinetic parameters for the hydrolysis of the chromogenic substrate SpPCa by the APC derivatives are presented in Table 1. Relative to cleavage by wild-type APC, a similar or slightly improved amidolytic activity was observed with all mutants, supporting the contention that the mutagenesis of these basic residues did not adversely affect the folding and the catalytic pocket of APC. Results of previous studies have indicated that occupancy of the 70−80-loop of APC with Ca^{2+} leads to ~30−50% enhancement

Table 1. Kinetic Constants for Cleavages of Spectrozyme PCa by APC Derivatives[a]

	K_m (μM)	k_{cat} (s^{-1})	k_{cat}/K_m ($\mu M^{-1}s^{-1}$)
Wt (plasma)	177 ± 1	44 ± 4	0.25
Wt (recombinant)	164 ± 2	47 ± 7	0.29
R74A	131 ± 10	47 ± 2	0.36
R75A	146 ± 7	48 ± 1	0.33
K78A	133 ± 9	48 ± 5	0.36

[a] Steady-state hydrolysis of Spectrozyme PCa (15−2000 μM) by the APC derivatives (2 nM) was measured in TBS containing 2.5 mM Ca^{2+}, 0.1 mg/mL BSA, and 0.1% PEG 8000 as described in Materials and Methods.

in the amidolytic activity of APC toward cleavage of tripeptidyl chromogenic substrates (*20*, *35*, *36*). A similar Ca^{2+}-mediated enhancement with an apparent dissociation constant of 40−60 μM was observed for the amidolytic activities of all APC derivatives, suggesting that the mutants interact normally with the metal ion.

Inactivation by PCI. Similar to cleavage of Spectrozyme PCa, the catalytic activity of APC mutants toward reaction with PCI was slightly improved (Table 2). However, the heparin-catalyzed inhibition rate constants were impaired with all mutants. Similar to the thrombin−antithrombin reaction (*37*), high molecular weight heparin is known to accelerate the PCI inhibition of APC by a template mechanism (*8*). A bell-shaped dependence on the heparin concentration is characteristic for this mechanism of heparin action in the inhibition reaction. Recently, we demonstrated that the template function of heparin in the antithrombin inhibition of factor Xa is masked unless the Gla-domain is stabilized by Ca^{2+} ions (*28*, *29*). Since, APC is also a vitamin K-dependent protease with an N-terminal Gla-domain, we initially evaluated the heparin-catalyzed PCI inhibition of APC in both the absence and presence of physiological levels of Ca^{2+}. Interestingly, we discovered that Ca^{2+} was also required for heparin to effectively bridge the enzyme−inhibitor in a ternary complex (Figure 2A). In contrast to a similar k_2 of 1.2×10^3 M^{-1} s^{-1} for the uncatalyzed PCI inhibition of APC in both the absence (100 μM EDTA) and presence of 2.5 mM Ca^{2+}, the heparin-catalyzed inhibition rate constant at an optimal concentration of the polysaccharide (0.1−1 μM) was higher by at least an order of magnitude in the presence of Ca^{2+} (Figure 2A). On the other hand, an efficient heparin-catalyzed PCI inhibition of the Gla-domainless APC was independent of the metal ion ($1.4 \pm 0.1 \times 10^6$ M^{-1} s^{-1} in Ca^{2+} and $1.5 \pm 0.1 \times 10^6$ M^{-1} s^{-1} in EDTA). These results support the proposal that for an effective interaction with heparin, negative charges of the Gla-domain of APC must be neutralized by Ca^{2+} ions. Analysis of k_2 values according to eq 1 yielded a $k_{2,max}$ value of $1.2 \pm 0.1 \times 10^5$ M^{-1} s^{-1} for the heparin-catalyzed PCI inhibition of wild-type APC and an apparent dissociation constant ($K_{APC,H}$) of 7.8 μM for the APC−heparin interaction in the presence of 100 μM EDTA. These values are at least an order of magnitude lower than the corresponding values in the presence of Ca^{2+} (Table 2).

To evaluate the contribution of the basic residues of the 70−80 loop to the APC−heparin interaction, the heparin concentration dependence of PCI inhibition of wild-type and mutant APC derivatives were compared in the presence of 2.5 mM Ca^{2+} (Figure 2B). Relative to wild-type APC, the

Table 2. Kinetic Constants for Uncatalyzed and Heparin-Catalyzed PCI Inhibition of APC Derivatives

	$k_{2,uncat}$ 10^3 (M^{-1} s^{-1})[a]	$k_{2,max}$ 10^5 (M^{-1} s^{-1})	$K_{PCI,H}$ (nM)	$K_{APC,H}$ (μM)	$k_{2,max}/k_{2,uncat}$ fold enhancement
Wt	1.2 ± 0.2	27.8 ± 1.3	150 ± 16	0.7 ± 0.1	2317
R74A	2.4 ± 0.1	2.3 ± 0.1	97 ± 17	5.1 ± 0.7	96
R75A	2.3 ± 0.1	3.7 ± 0.1	138 ± 17	5.0 ± 0.6	161
K78A	1.9 ± 0.1	3.0 ± 0.1	152 ± 34	2.8 ± 0.4	158
R74E/R75E/K78E[b]	1.2 ± 0.2	0.1 ± 0.1	206 ± 40	ND[c]	8

[a] Uncatalyzed second-order inhibition rate constants ($k_{2,uncat}$) were determined by incubating 2 nM APC with 50−200 nM PCI in TBS containing 2.5 mM Ca^{2+}, 0.1 mg/mL BSA, and 0.1% PEG 8000 as described in Experimental Procedure. Kinetic constants in the presence of unfractionated heparin are derived from a computer fit of kinetic data shown in Figure 2B to eq 1. [b] Triple mutant is a Gla-domainless form of APC. [c] Heparin-catalyzed PCI inhibition of the triple mutant did not exhibit a bell-shaped dependence on polysaccharide concentrations; thus, the $K_{APC,H}$ value could not be determined (ND).

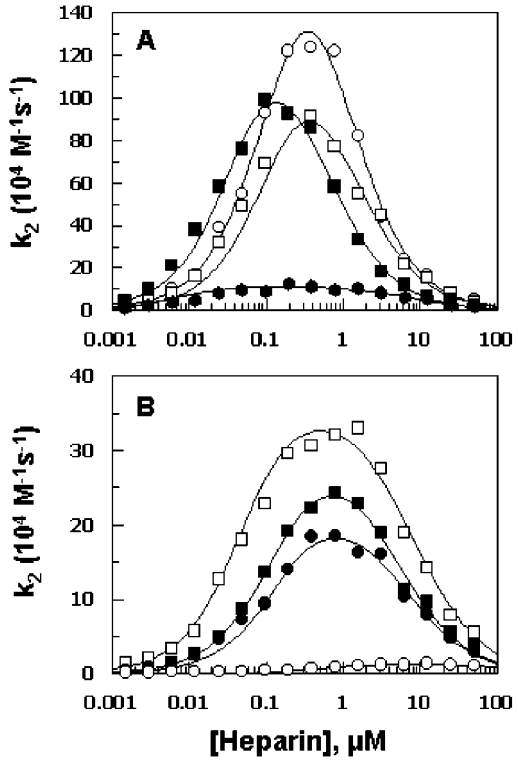

FIGURE 2: Heparin concentration dependence of wild-type mutant APC inactivation by PCI in the absence and presence of Ca^{2+}. (A) Second-order rate constants were determined by incubating 1 nM of either wild-type (○, ●) or Gla-domainless APC (□, ■) with PCI (10−50 nM) at indicated concentrations of unfractionated heparin in TBS containing 0.1 mg/mL BSA, 0.1% PEG 8000, and either 100 μM EDTA (closed symbols) or 2.5 mM Ca^{2+} (open symbols) and computer fit to eq 1 as described in Materials and Methods. (B) The same as A, except that the second-order rate constants for the APC mutants, R74A (●), R75A (□), K78A (■), or Gla-domainless R74E/R75E/K78E (○), were determined in the presence of 2.5 mM Ca^{2+}.

heparin-catalyzed second-order rate constants were impaired by approximately an order of magnitude with each mutant of APC (Table 2). Ascending and descending limbs of the bell-shaped curves represent the apparent affinity of heparin for interaction with PCI ($K_{PCI,H}$) and APC ($K_{APC,H}$), respectively. The computer fit of the k_2 values to eq 1 describing a ternary complex-bridging model (*30*) estimated $K_{PCI,H}$ values of 100−150 nM for the PCI−heparin interaction with all APC derivatives (Table 2). However, similar to the impairment in the $k_{2,max}$ values, the mutants exhibited approximately 4−7-fold higher $K_{APC,H}$ values, suggesting that their ability to interact with heparin is impaired (Table 2). It should be noted that qualitatively similar results were obtained if the reactions were carried out in the absence of Ca^{2+}. Thus, $k_{2,max}$ values with mutants were impaired by the same extent in the absence of Ca^{2+} (data not shown). A similar inhibition stoichiometry of 1:1 was observed in reaction with all APC derivatives in the presence of heparin, suggesting that the lower k_2 values with the mutants are not due to their enhanced reactivity with PCI in the substrate pathway of the reaction. These results suggest that the basic residues of the 70−80-loop are part of the heparin binding site of APC. Further support for this proposal was provided by the observation that the heparin-catalyzed inhibition of a triple mutant of Gla-domainless APC in which all three residues, Arg^{74}, Arg^{75}, and Lys^{78}, were substituted with Glu was severely impaired (Figure 2B, open circles). This mutant was activated normally by thrombin in EDTA, exhibited normal amidolytic activity, and was inhibited by PCI with a k_2 of 1.2×10^3 M^{-1} s^{-1} in the absence of heparin and k_2 of 1.1×10^4 M^{-1} s^{-1} in the presence of a saturating concentration of heparin. Thus, the catalytic effect of heparin in the PCI inhibition of this mutant was impaired by 2 orders of magnitude. Moreover, unlike a bell-shaped curve, a saturable dependence on heparin concentration was observed for the rate accelerating effect of heparin in the PCI inhibition of the triple mutant at up to 50 μM heparin, suggesting that the triple mutant does not detectably interact with heparin (Figure 2B). Fitting the saturable dependence of the rate accelerating effect of heparin to a hyperbolic equation yielded an apparent dissociation constant of ~200 nM for the binary heparin−PCI interaction, which agrees relatively well with the $K_{PCI,H}$ values determined by eq 1 (Table 2).

To confirm results of the kinetic data, the ability of APC derivatives to interact with heparin was also evaluated by a direct binding assay using Heparin−Sepharose. The NaCl gradient elution profiles of enzymes from the Heparin−Sepharose column are presented in Figure 3. The peak activity of wild-type APC was eluted at a NaCl concentration of 150−170 mM. On the other hand, lower concentrations of salt (110 mM for R74A, 120−130 mM for R75A, and 130−140 mM for K78A) were required to elute the mutants from the column. The triple mutant of the Gla-domainless APC did not bind to the Heparin−Sepharose column under the experimental conditions described in the legend of Figure 3. Taken together, these results strongly suggest that the basic residues of the 70−80-loop are part of the heparin binding exosite of APC.

To determine the minimum chain-length requirement for the template effect of heparin in the reaction, the inhibition studies were carried out with oligosaccharides containing 6,

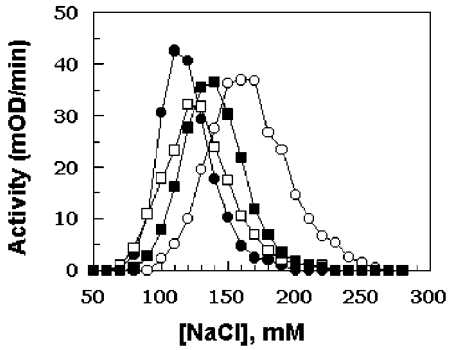

FIGURE 3: NaCl gradient elution profiles of APC derivative from Heparin−Sepharose. APC derivatives (0.5 mL, 200 nM) in 50 mM NaCl, 20 mM Tris−HCl (pH = 7.5) containing 5 mM Ca^{2+} were applied on a 0.5 mL Heparin−Sepharose column followed by elution with a step gradient of 50−300 mM NaCl using increments of 10 mM salt as described in Materials and Methods. The symbols are APC (○), R74A (●), R75A (□), and K78A (■). The peak activities were eluted at NaCl concentrations of 150−170 mM for APC, 110 mM for R74A, 120−130 mM for R75A, and 130−140 mM for K78A.

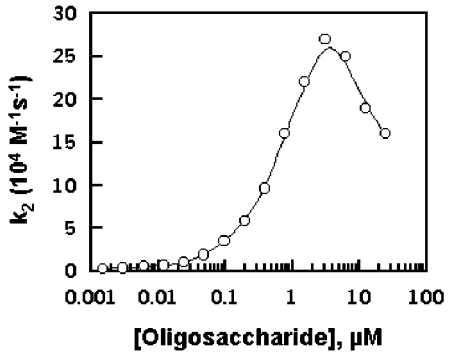

FIGURE 4: Oligosaccharide (14-mer) concentration dependence of wild-type APC inactivation by PCI in the presence of Ca^{2+}. Conditions were the same as those in Figure 2, except that a 14-mer oligosaccharide was used to measure the PCI inhibition of wild-type APC in the presence of 2.5 mM Ca^{2+}. The computer fit of data to eq 1 yielded the following kinetic constants with the oligosaccharide: $k_{2,max} = (3.6 \pm 0.3) \times 10^5$ M^{-1} s^{-1}, $K_{PCI,H} = 853 \pm 21$ nM, and $K_{APC,H} = 19.3 \pm 0.6$ μM.

10, 14, or 18 saccharides or high-affinity heparin fragments containing 22, 26, 35, 50, and 64 saccharides as described (*39*). Interestingly, the 14-mer oligosaccharide efficiently accelerated the PCI inhibition of APC with a catalytic effect that was only 5-fold lower than that observed with the 64-saccharide or unfractionated heparin (Figure 4). These results suggested that unlike the heparin-catalyzed antithrombin inhibition of thrombin (*40*, *41*) and factor Xa (*28*), which require heparin chains of longer than 18 saccharides for inhibition by a template mechanism, the PCI inhibition of APC can be accelerated by heparin chains as short as 14 saccharides (Figure 4).

Anticoagulant Activity of APC Mutants. The anticoagulant activities of APC mutants were studied in both purified and plasma-based assay systems. Results of both assays suggested that the basic residues Arg^{74} and Arg^{75} are important for the anticoagulant function of APC (Figure 5). In contrast to a significant decrease in the anticlotting activities of both Arg^{74} and Arg^{75} mutants, the anticlotting activity of the Lys^{78} mutant did not appear to be affected in the factor Va degradation assay performed in the purified system (Figure 5B). However, relative to APC, the anticoagulant activity

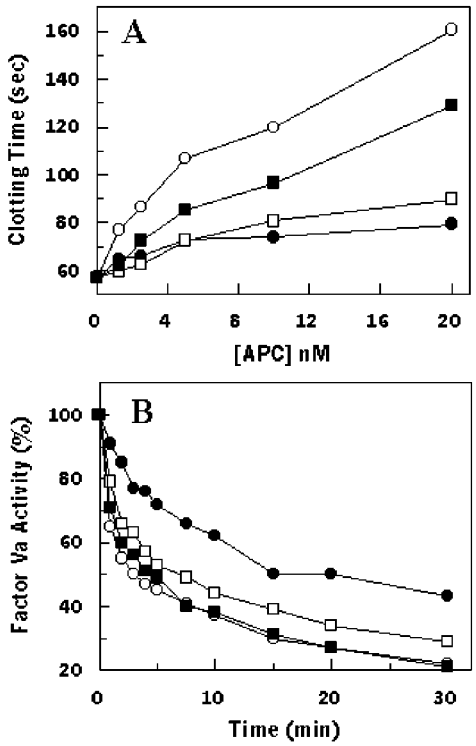

FIGURE 5: Anticoagulant activities of wild-type and mutant APC. (A) Clotting time of normal human plasma in the presence of 0−20 nM wild-type (○), R74A (●), R75A (□), or K78A (■) APC was determined at 37 °C in an aPTT assay. In all measurements, 0.05 mL of APC was incubated with 0.05 mL of plasma and 0.05 mL of aPTT reagent (Alexin) for 5 min followed by the initiation of clotting by addition of 0.05 mL of 35 mM $CaCl_2$ as described in Materials and Methods. (B) Human factor Va (2.5 nM) was incubated with wild-type (○), R74A (●), R75A (□), or K78A (■) APC (0.5 nM each) on 25 μM PC/PS vesicles in TBS containing 0.1 mg/mL BSA, 0.1% PEG 8000, and 2.5 mM Ca^{2+}. At the indicated time intervals, the remaining factor Va activity was determined from the rate of thrombin generation in a prothrombinase assay as described in Materials and Methods.

of the Lys^{78} mutant was impaired, though to a lesser extent, in the plasma-based assay system (Figure 5A). The reason for the discrepancy between the results of two assay systems for the Lys^{78} mutant is not known. One possibility is that, unlike the inactivation of factor Va in the purified system, the aPTT assay may be reporting the inactivation of both factors Va and VIIIa in the plasma-based assay system. To determine whether the decreases in the anticlotting activities of the Arg^{74} and Arg^{75} mutants are due to an impairment in their interaction with protein S or if direct interactions with the procoagulant cofactor Va account for the catalytic defect, the factor Va-degradation assay in the purified system was carried out in both the absence and presence of protein S (220 nM). The results suggested that the cofactor function of protein S is not affected with either mutant since similar defects in the anticoagulant activities of both mutants were observed in both the absence and presence of protein S (Figure 5B, data shown in the absence of protein S only). These results suggest that the basic residues of the 70−80-loop are not recognition sites for protein S on APC.

DISCUSSION

Previous studies have indicated that, similar to factor Xa and thrombin, APC has a binding site for heparin on its catalytic domain (*11*, *16*, *39*, *42*, *43*). However, heparin does

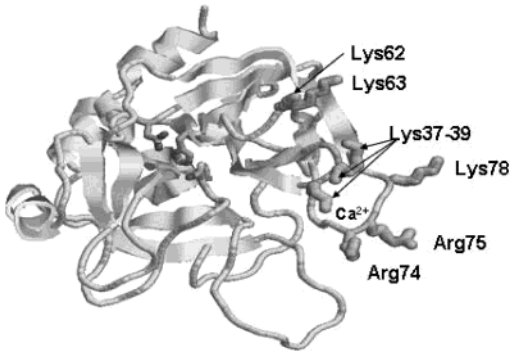

FIGURE 6: RasMol plot of the catalytic domain of the Gla-domainless APC−PPACK complex. Relative three-dimensional locations of side chains of the basic residues of three surface loops 39, 60, and 70−80 are shown. The catalytic triad residues Asp^{102}, His^{57}, and Ser^{195} (from top to bottom) are shown in the middle. Coordinates (Protein Data Bank entry 1AUT) of the Gla-domainless APC were used to prepare the figure (*14*).

not bind to the same site on APC, since the basic residues of this site are not conserved at the same three-dimensional locations (*14*, *42*, *43*). Instead, heparin binds to clusters of basic residues on a site of APC that constitutes exosite 1 in the homologous region of thrombin (*14*, *43*). These basic residues, located on the right side of the active site pocket of APC, are constituents of the solvent-exposed surface loops 33−40 (39-loop), 59−64 (60-loop), and 70−80 (Ca^{2+}-loop) (Figure 6). The relative contribution of basic residues of the 39-loop (Lys^{37}-Lys^{39}) and 60-loop (Lys^{62} and Lys^{63}) for the binding of APC to heparin has been investigated in the past (*16*). The results of the current study suggest that in addition to basic residues of the 39- and 60-loop, the basic residues of the 70−80-loop of APC are also part of the heparin binding exosite of the protease. Our results further suggest that the basic residues of this loop are critical for the anticoagulant function of APC as evidenced by the poor anticoagulant response of the mutants (particularly Arg^{74} and Arg^{75} mutants) in both purified and plasma-based assay systems. Impairments of the anticoagulant activities of both Arg^{74} and Arg^{75} mutants in both the aPTT and the purified assay systems suggested that these residues may constitute a direct binding site for factor Va and most likely for factor VIIIa on the catalytic domain of the protease. Previous studies have indicated that the three Lys residues of the 39-loop also play a crucial role in the anticoagulant function of APC (*15*). It appears, therefore, that similar to exosite 1 of thrombin, the cluster of basic residues of APC, conserved at the same three-dimensional locations, are key recognition sites for interaction with macromolecular substrates and cofactors. Consistent with these results, recent molecular modeling data have predicted that electrostatic interactions between negatively charged residues of factor Va with positively charged residues of APC are critical for the specificity of reaction (*44*). In this context, it is interesting to note that the binding of both cofactors V and VIII on exosite 1 of thrombin is also required for their rapid activation by thrombin (*45*−*47*). It is not known whether the same sites of procofactors, which interact with exosite 1 of thrombin, are also involved in interaction of activated cofactors with the homologous exosite of APC.

Results of inhibition kinetic studies indicated that the heparin-catalyzed rate of inactivation of APC by PCI was an order of magnitude higher if the inactivation reaction was carried out in the presence of physiological levels of Ca^{2+}. Analysis of data revealed that the affinity of heparin for binding APC in the absence of Ca^{2+} was markedly impaired. Previously, we made a similar observation for the catalytic effect of high molecular weight heparins in inhibition of factor Xa by antithrombin (*28*, *39*). It appears, therefore, that the γ-carboxyglutamic acid residues of Gla-domains in vitamin K-dependent proteases, if not stabilized by the Ca^{2+} ions, interfere with efficient binding of heparin to coagulation proteases. Such findings may explain the underlying reason for the enormous variability (50−800-fold) in the reported values of the heparin catalytic effect in the PCI inhibition of APC in previous studies (*8*, *16*). It is worth noting that we previously demonstrated that heparin and other polyanionic compounds also accelerate protein C activation by both thrombin and factor Xa by a template mechanism (*39*). Similarly, the Gla-domain of protein C had to be stabilized by Ca^{2+} for an optimal template effect of heparin in the activation reaction (*39*).

Heparin is known to promote the inhibition of coagulation proteases by their target serpins primarily by a template mechanism (*48*). The template effect of heparin exhibits a characteristic bell-shaped dependence on the polysaccharide concentration, which is indicative of heparin possessing binding sites for both protease and serpin. It follows, therefore, that heparin has a minimum chain-length requirement for catalysis of protease inhibition by serpins through a template mechanism. In the case of thrombin, heparin fragments containing at least 18 saccharides are required to catalyze the antithrombin inhibition of thrombin by a template mechanism (*40*, *41*). The minimum chain-length requirement for observing the same effect in the heparin-catalyzed antithrombin inhibition of factor Xa is even longer (>26 saccharides) (*28*, *49*). On the other hand, the results of this study suggest that an oligosaccharide containing 14 residues is long enough to efficiently accelerate the PCI inhibition of APC by a template mechanism. A shorter heparin chain requirement for the APC−PCI reaction may be due to observation that the heparin binding sites of APC and PCI are not conserved at the same three- dimensional locations as in thrombin and antithrombin. Heparin binds to the D-helix of all heparin-activatable serpins with the exception of PCI (*12*, *50*). In PCI, the basic residues of the H-helix constitute the heparin binding site of the serpin (*12*). Moreover, unlike thrombin and factor Xa, in which the heparin binding site is extended through the C-terminal of both molecules (*29*, *51*, *52*), the heparin binding site of APC is located closer to the active site pocket of the protease (*14*). Adding to this, previous molecular modeling of the APC−PCI interaction suggests that the H-helix of PCI is spatially closer than the D-helix to the catalytic domain of protease in the binary enzyme−inhibitor complex (*11*). Assuming an average length of ~7.5 Å for a disaccharide unit of heparin (*53*) and a minimum heparin chain length of nine disaccharides, which is required to catalyze the antithrombin inhibition of thrombin by a template mechanism (*40*, *41*), it is estimated that the heparin binding sites of two proteins would be ~67.5 Å apart from each other. This mirrors the distance between the two heparin binding sites in the molecular model of the thrombin−antithrombin complex (*11*) where Lys^{240}, the last residue on the C-terminal helix of thrombin, is

~70 Å apart from the α-carbon atom of Lys^{114}, which is the farthest critical residue for heparin binding on antithrombin (*54*). On the basis of the result that a 14-mer oligosaccharide was sufficient to observe the template effect of heparin in the APC−PCI reaction, we estimate that the distance between the heparin binding site of APC and the H-helix of PCI would be less than 52.5 Å. This also mirrors the molecular model of the APC−PCI interaction where Lys^{78}, the farthest heparin binding residue in APC, is located ~50 Å away from Arg^{269}, which is the farthest heparin binding residue on the H-helix of PCI (*11*). Thus, relative to thrombin and antithrombin, the heparin binding exosites of APC and PCI are spatially closer to each other for the bridging function of heparin.

Finally, the observation that the activation of protein C mutants by thrombin was severely impaired in the presence of TM but improved in its absence suggests that in addition to the important role of these basic residues in the catalytic function of APC, they are also cofactor-dependent recognition sites for protein C activation by thrombin. The molecular basis for this observation may be explained on the basis of recent structural and molecular modeling data, which suggest that binding of the TM5 domain to exosite 1 of thrombin may position the TM4 domain of the cofactor in such a way that a negatively charged region on this domain juxtaposes positively charged regions of protein C, including the basic residues of the 70−80-loop (*18*). On the basis of this model, electrostatic interactions between the basic residues of the 70−80-loop of protein C and acidic residues of TM4 are required for rapid zymogen activation (*18*). It is also likely that the basic residues of the 70−80-loop make repulsive interactions with basic residues of exosite 1 of thrombin in the absence of TM, explaining the inability of thrombin to efficiently activate protein C in the absence of the cofactor. These results are consistent with other previous protein C mutagenesis data, which suggest that the basic residues of the 39-loop are also required for thrombin to activate protein C in the presence of TM (*17*). Taken together, these results suggest that, as in exosite 1 of thrombin, the basic residues of 39-loop and 70−80-loop constitute a functionally important exosite in protein C/APC that is indispensable in the regulation of the protein C anticoagulant pathway.

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