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The functional significance of the autolysis loop in protein C and activated protein C

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Summary

The autolysis loop of activated protein C (APC) is five residues longer than the autolysis loop of other vitamin K-dependent coagulation proteases. To investigate the role of this loop in the zymogenic and anticoagulant properties of the molecule, a protein C mutant was constructed in which the autolysis loop of the protein was replaced with the corresponding loop of factor X. The protein C mutant was activated by thrombin with ~5-fold higher rate in the presence of Ca^{2+} . Both kinetics and direct binding studies revealed that the Ca^{2+} affinity of the mutant has been impaired ~3-fold. The result of a factor Va degradation assay revealed that the anticoagulant function of the mutant has been improved 4–5-fold in the absence but not in the presence of protein S. The improvement was due to a better recognition of both the P1-Arg⁵⁰⁶ and P1-Arg³⁰⁶ cleavage sites by the mutant protease. However, the plasma half-life of the mutant was markedly shortened due to faster inactivation by plasma serpins. These results suggest that the autolysis loop of protein C is critical for the Ca^{2+} -dependence of activation by thrombin. Moreover, a longer autolysis loop in APC is not optimal for interaction with factor Va in the absence of protein S, but it contributes to the lack of serpin reactivity and longer half-life of the protease in plasma.

Keywords

Activated protein C; thrombin; thrombomodulin; factor Va; antithrombin

Introduction

Protein C is a vitamin K-dependent plasma serine protease zymogen that upon conversion to activated protein C (APC)¹ by thrombin in complex with thrombomodulin (TM) down-regulates the coagulation cascade by degrading factors Va (fVa) and VIIIa (fVIIIa) by limited proteolysis (1–4). Similar to other vitamin K-dependent coagulation zymogens, with the exception of prothrombin, the domain organization of protein C includes a γ -carboxyglutamic acid (Gla) domain at the N-terminus followed by two epidermal growth factor (EGF)-like domains, an activation peptide and a trypsin-like catalytic domain at the C-terminus end of the molecule (5,6). The thrombin-TM complex removes a 12-residue peptide from the activation peptide of protein C to convert the zymogen to an active protease (7). The activation of protein C by the thrombin-TM complex is a Ca^{2+} dependent reaction that is also stimulated by the complex formation of the zymogen with the endothelial cell protein C receptor on the surface

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Abbreviations - APC, activated protein C; Gla, γ -carboxyglutamic acid; GDPC, Gla-domainless protein C; fX, factor X; fVa, factor Va; fVIIIa, factor VIIIa; TM, thrombomodulin; aPTT, activated partial thromboplastin time; PEG, polyethylene glycol

of endothelium (7–9). Previous studies have demonstrated that the Ca^{2+} binding site important for the zymogen activation by the thrombin-TM complex is located in the protease domain of protein C on a loop called 70–80 loop in the chymotrypsinogen numbering system (10,11). This loop is also known to interact with Ca^{2+} in other vitamin K-dependent coagulation proteases (12–14) as well as in trypsin (15). In addition to this site, protein C has a high affinity Ca^{2+} binding site in the first EGF-like domain and several low affinity sites in Gla domain (6,16). However, the binding of Ca^{2+} to either one of these sites is not required for protein C activation by thrombin in solution since the deletion derivatives of the zymogen lacking either the Gla domain (Gla-domainless protein C, GDPC) or both the Gla and first EGF domains exhibit similar kinetics and Ca^{2+} dependence of protein C activation by thrombin in both the absence and presence of TM (16,17).

Ca^{2+} plays a complex role in the zymogenic properties of protein C because it not only stimulates the zymogen activation by thrombin in the presence of TM, but it also potently inhibits the activation by thrombin in the absence of TM (1,8). The mechanism of the dual function of Ca^{2+} in protein C activation by thrombin in the absence and presence of TM is not well understood. The consensus developed from several studies is that the binding of Ca^{2+} to the 70–80 loop of protein C is associated with a conformational change in the activation peptide of the zymogen that is optimal for interaction with thrombin in the presence of TM, but inhibitory for interaction in the absence of the cofactor (1,18,19). It is believed that the Ca^{2+} -stabilized conformer of protein C makes inhibitory interactions with the extended binding pocket residues of thrombin, thus impeding its optimal docking into the catalytic pocket of the protease (20,21). We recently provided direct support for this hypothesis by showing that the activation peptide of protein C is energetically coupled to the Ca^{2+} -binding loop of the protease domain (19). Further support for the Ca^{2+} -induced conformational change in the activation peptide of protein C has been provided by the observation that the mutagenesis of the P3 and/or P3' residues (nomenclature of Schechter and Berger (22)) of the thrombin recognition site in the activation peptide of protein C impairs the affinity of Ca^{2+} for its binding site in the protease domain, leading to an improvement in the ability of thrombin to recognize the mutant substrates (17,23). Although the x-ray crystal structure of the zymogen protein C has not yet been determined, nevertheless, the autolysis loop (residues 143–154 in the chymotrypsinogen numbering (11)) of protein C has been found in the proximity of the Ca^{2+} loop in the crystal structure of the activated GDPC (24). Moreover, previous molecular modeling of the zymogen protein C, based on the structures of activated GDPC and the homologous zymogens chymotrypsinogen and prethrombin-2 have indicated that the acidic residues of the activation peptide can interact with basic residues of the autolysis loop (25,26). Taken together, these previous results support the hypothesis that a network of non-covalent interactions among the residues of the activation peptide, the 70–80-loop and the autolysis loop of protein C may be responsible for trapping the zymogen in a conformation that is inhibitory for recognition by thrombin in the absence of TM.

The autolysis loop of protein C from residues 143–154 is five residues longer than the corresponding loop of the other vitamin K-dependent family of coagulation proteases (24, 27). To investigate the role of this loop in the zymogenic and enzymatic properties of protein C and APC, we prepared two mutant constructs in the full-length and Gla-domainless (GDPC) forms in which the autolysis loop was substituted with the corresponding loop of factor X (fX). Both constructs were expressed in mammalian cells and the mutant proteins were purified to homogeneity. The characterization of these mutants in protein C activation studies by thrombin in both the absence and presence of TM and Ca^{2+} suggested that the affinity of Ca^{2+} for the mutant zymogens has been weakened 3-fold. Moreover, the inhibitory effect of Ca^{2+} has been partially eliminated so that the mutants are activated by thrombin 5-fold faster than the wild-type counterparts in the presence of Ca^{2+} . Direct binding studies with the GDPC mutant supported the weaker affinity of Ca^{2+} for the protease domain of protein C. Interestingly,

further studies revealed that a longer autolysis loop of APC may also contribute to the regulation of the anticoagulant properties of the protease since the mutant inactivated fVa with 4–5-fold higher rates in the absence, but not in the presence of protein S. However, the APC mutant did not exhibit a superior anticoagulant effect in plasma because it reacted faster with the plasma serpins, suggesting that this loop also contributes to lack of the reactivity of APC with the inhibitors and the longer half-life of the protease in plasma.

Materials and Methods

Construction, Expression and Purification of Recombinant Proteins

The construction, expression and purification of wild-type protein C and the mutant in which the autolysis loop (¹⁴³Tyr-His-Ser-Ser-Arg-Glu-Lys-Glu-Ala-Lys-Arg-Asn-Arg-Thr-Phe-Val¹⁵⁴) of the zymogen was replaced with the corresponding sequence of fX (¹⁴³Arg-Thr-His-Glu-Lys-Gly-Arg-Gln-Ser-Thr-Arg¹⁵⁴) (PC-fX^{143–154}) has been described previously (28). The construction and expression of a deletion derivative of protein C lacking the Gla-domain (GDPC) has been described previously (17). A GDPC homologue of PC-fX^{143–154} was also constructed and expressed in the same expression/purification vector system as described (17). Following confirmation of the accuracy of the mutagenesis, the mutant constructs were expressed in HEK293 cells as described (17). Both wild-type and mutant zymogens were purified to homogeneity by immunoaffinity chromatography using the Ca²⁺-dependent monoclonal antibody HPC4 as described (17,28). The TM fragment containing epidermal growth factor-like domains 456 (TM456) (17), protein C inhibitor (29), and antithrombin (AT) (30) were expressed in mammalian cells as described. The prothrombin derivative lacking both the Gla and kringle-1 domains (prethrombin-1) (31) and recombinant fX (32) were expressed in mammalian cells and purified to homogeneity as described. All recombinant proteins were tested for homogeneity by sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Human plasma proteins including prothrombin, protein S, APC and factors Va, IXa and Xa were purchased from Haematologic Technologies Inc. (Essex Junction, VT). Human recombinant fVIIIa was a generous gift from Dr. Philip Fay (University of Rochester, Rochester, NY). Factor Va Leiden, purified from the plasma of a homozygous patient (33) was a generous gift from Dr. Charles Esmon (Oklahoma Medical Research Foundation, Oklahoma City, OK). Phospholipid vesicles containing 80% phosphatidylcholine and 20% phosphatidylserine (PC/PS) were prepared as described (34). The normal pooled plasma was purchased from George King Bio-Medical, Inc. (Overland Park, KS) and the activated partial prothrombin time (aPTT) reagent, Alexin, was purchased from Sigma (St. Louis, MO). α_1 -antitrypsin was obtained from Athens Research and Technology, Inc. (Athens, GA). The chromogenic substrates, Spectrozyme PCa (SpPCa) and Spectrozyme FXa (SpFXa) were purchased from American Diagnostica (Greenwich, CT); S2238 was purchased from Kabi Pharmacia/Chromogenix (Franklin, OH).

Zymogen Activation

Two milligrams of protein C derivatives in both full-length and Gla-domainless forms were incubated with thrombin (25 μ g) 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. The APC 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 as described (28). The concentrations of proteases were determined from the absorbance at 280 nm and extinction coefficient ($E^{1\%}_{1\text{cm}}$) of 14.5 (assuming molecular masses of 56 kDa and 51 kDa for APC and activated GDPC, respectively), by an amidolytic activity assay using SpPCa, and by stoichiometric titration of enzymes with known concentrations of protein C inhibitor as described (28).

The initial rate of protein C or GDPC activation by thrombin was measured in both the absence and presence of Ca^{2+} , EDTA and TM456 as described (17). In the absence of TM456, the time course of protein C or GDPC (1 μM) activation by thrombin (5–50 nM) was studied at room temperature in 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.4 (TBS) containing 1 mg/mL bovine serum albumin (BSA), 0.1% polyethylene glycol (PEG) 8000 in the presence of either 2.5 mM Ca^{2+} or 1 mM EDTA in 96-well assay plates. At different time intervals, thrombin activity was quenched by 1 μM AT and the rate of protein C activation was measured from the cleavage rate of SpPCa (200 μM) at 405 nm by a V_{max} Kinetic Microplate Reader (Molecular Devices, Menlo Park, CA) as described (17). The experimental conditions in the presence of TM456 were the same with the exception that the activation by thrombin (1.0 nM) was carried out in the presence of a saturating concentration of TM456 (200 nM). The concentration of APC in reaction mixtures was determined by reference to a standard curve which was prepared by the total activation of protein C derivatives with the excess thrombin at the time of the experiments. The inhibitory effect of Ca^{2+} on protein C activation by thrombin alone and the stimulatory effect of Ca^{2+} on the zymogen activation by the thrombin-TM456 complex was studied as a function of increasing concentrations of Ca^{2+} as described above.

Measurement of the Anticoagulant Activity

Anticoagulant activities of APC derivatives were evaluated in both the purified and plasma based assay systems. Time course of both wild-type and Leiden fVa inactivation by APC was measured by a three-stage assay in the purified system as described (29). Briefly, in the first stage, fVa (5 nM) was incubated with wild-type or mutant APC (1 nM) on 25 μM PC/PS vesicles in TBS containing 2.5 mM Ca^{2+} , 0.5 mg/ml BSA and 0.1% PEG 8000. In the second stage, at different time intervals (0–40 min), the remaining fVa activity was determined in a prothrombinase assay from the fVa-catalyzed prothrombin activation by fXa as described (29). The prothrombinase assay was carried out for 30 sec with excess prothrombin (1 μM) and a saturating fXa (10 nM) at room temperature. The remaining activity of fVa was determined from the decrease of the rate of thrombin generation as monitored by an amidolytic activity assay in the third stage using 100 μM S2238. The same procedures were used to monitor the rate of fVa inactivation by the activated GDPC derivatives in solution. In this case, however, the concentration of activated GDPC was increased to 2.5 nM, and prethrombin-1 was used as a substrate for fXa to measure the remaining cofactor activity of fVa. A similar assay was utilized to monitor fVIIIa degradation by the APC derivatives as described (35). In this case, however, the remaining activity of fVIIIa was determined by an intrinsic Xase assay using factor IXa (15 nM) as the activator and recombinant fX (500 nM) as the substrate. The rate of fXa generation was measured by an amidolytic activity assay using 150 μM SpFXa as described (35). The APC concentration dependence of fVa inactivation was carried out in both the absence and presence of protein S. In this case, the experimental conditions were the same with the exception that the inactivation of fVa (5 nM) by APC was monitored in either the absence (10 min) or presence of 220 nM protein S (1 min) and different concentrations of APC (0–5 nM). The time course analysis of fVa degradation was carried out with 1 nM APC in the absence and 0.5 nM APC in the presence of protein S.

The anticoagulant activities in plasma were evaluated in an aPTT assay using a STart 4 fibrinometer (Diagnostica/Stago, Asnieres, France). Briefly, 0.050 ml TBS lacking or containing 1–20 nM final concentrations of wild-type or mutant APC was incubated with a mixture of 0.05 ml normal pooled plasma plus 0.05 ml 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.

Inhibition by Plasma Inhibitors

The PCI and α_1 -antitrypsin inhibition rates of APC derivatives were measured under pseudo-first order rate conditions by a discontinuous assay method as described (29). Briefly, 3 nM

APC was incubated at room temperature with 50–200 nM PCI or 20 μ M α_1 -antitrypsin in 50 μ l reactions in TBS buffer containing 2.5 mM Ca^{2+} , 1 mg/ml BSA and 0.1 % PEG 8000. At the end of incubation time (15–60 min) 50 μ l SpPCa was added to a final concentration of 0.2 mM. The remaining activities of APC derivatives were measured from the rate of chromogenic substrate hydrolysis at 405 nm as described above. The second-order inactivation rate constants were calculated as described (29). The reactivity of the APC derivatives with plasma inhibitors was also assessed by incubating 5–10 nM APC with normal plasma and monitoring the chromogenic substrate activity of the protease for 0–45 min at room temperature. The half-life of each protease was calculated based on the time required to observe 50% decline in the amidolytic activity.

Fluorescence Measurements

Direct binding of Ca^{2+} to both wild-type and mutant GDPC was evaluated from the changes in the intrinsic protein fluorescence associated with the binding of the metal ion to the zymogen as described (18,19). The fluorescence titrations were carried out with a Spex FluoroMax-3 spectrophotometer (Jobin-Yvon, Edison, NJ) at 25 °C. The excitation and emission wavelengths were 295 and 345 nm, respectively. The titration experiments were carried out using Chelex-treated GDPC (1 μ M) in 0.1 M NaCl, 20 mM Tris-HCl, pH 7.4 containing 0.1% PEG 8000. The equilibrium dissociation constants were determined from nonlinear regression analysis of the decreases in the intrinsic protein fluorescence upon binding Ca^{2+} as described (19).

Results

Protein C Activation

The initial rate of protein C activation by thrombin was studied in both the absence and presence of TM456 and Ca^{2+} . Protein C contains a single Ca^{2+} -binding site in the protease domain which has been localized to a loop consisting of residues Glu⁷⁰-Glu⁸⁰ (10). The binding of Ca^{2+} to this loop of protein C is inhibitory for activation by thrombin in the absence of TM (10). However, the metal ion is also an obligatory cofactor for activation by thrombin in the presence of TM (1). Since the Gla-domain of protein C has several low affinity Ca^{2+} -binding sites, to make the interpretations easier, we decided to delete the Gla-domain and carry out the direct binding studies with this mutant. Previously, we have also demonstrated that both full-length protein C and GDPC function similarly as a substrate for thrombin in both the absence and presence of TM and both exhibit the same kinetics and Ca^{2+} -dependence of activation (17). The initial rate of wild-type and mutant protein C activation by thrombin in both the absence and presence of TM456 is presented in Figure 1. In the absence of TM, the activation of protein C mutant by thrombin was improved 5-fold in the presence of Ca^{2+} (Figure 1A). The initial rate of activation of mutants by thrombin in the presence of TM456 was also improved, though to a lesser extent of ~2.5-fold (Figure 1B). The marked improvement in the substrate properties of the protein C mutant was specific for the presence of Ca^{2+} , since only an ~1.5-fold enhancement in the reactivity of thrombin with the mutant was observed in the presence of EDTA (data not shown). It should be noted that the autolysis loop of protein C harbors a known glycosylation site (Asn¹⁵⁰, chymotrypsinogen numbering (11)). A previous mutagenesis study, which eliminated the glycosylation of Asn¹⁵⁰ by its substitution with a Gln, also reported an ~2-fold improvement in the $K_{m(\text{app})}$ of protein C activation by the thrombin-TM complex (36). Thus, it appears that the glycosylation of the zymogen at this site also impairs the affinity of the zymogen for the activation complex.

To evaluate the affinity of the mutant for binding Ca^{2+} , the initial rate of GDPC activation by thrombin was studied as a function of varying Ca^{2+} concentrations. To simplify the comparisons of Ca^{2+} -dependence of the activations, the data were normalized to maximal

inhibition or fold stimulation in the absence and presence of TM, respectively. As shown in Figure 2, similar to wild-type, Ca^{2+} inhibited protein C activation by thrombin in the absence of TM and stimulated its activation in the presence of the cofactor. However, in both cases the $K_{d(\text{app})}$ (the half-maximal effect) for interaction of Ca^{2+} with the mutant was impaired 3-fold. Thus, in contrast to a half-maximal value of $30 \pm 5 \mu\text{M}$ Ca^{2+} in the absence of TM for the wild-type GDPC, the corresponding value was elevated to $90 \pm 10 \mu\text{M}$ ($n = 3 \pm \text{SD}$) for the mutant protein (Figure 2A). Similarly, in contrast to a half-maximal value of $12 \pm 5 \mu\text{M}$ Ca^{2+} in the presence of TM456 for wild-type, the corresponding value was elevated to $36 \pm 5 \mu\text{M}$ ($n = 3 \pm \text{SD}$) for the mutant protein (Figure 2B). These results suggest that the autolysis loop influences the affinity of protein C for interaction with Ca^{2+} .

Fluorescence Measurements

Binding of Ca^{2+} to GDPC caused an ~6% decrease in the intrinsic fluorescence, as previously reported (18), with a K_d of $20 \pm 6 \mu\text{M}$ (Figure 3). On the other hand, binding of Ca^{2+} to the mutant GDPC decreased the intrinsic fluorescence ~8%, with a K_d of $40 \pm 11 \mu\text{M}$ ($n = 3 \pm \text{SD}$). The direct binding study supports the kinetic data that the affinity of Ca^{2+} for protein C has been weakened in the mutant protein. Kinetics and direct binding studies have indicated that Ca^{2+} binding to protease domain of protein C induces a conformational change in the activation peptide which results in the trapping of the zymogen in an inhibitory conformation for recognition by thrombin in the absence of TM (17–19). Thus, the improvement in the substrate property of the mutant together with its weaker Ca^{2+} affinity indicate that the autolysis loop of protein C also interacts directly or indirectly with the activation peptide and/or the Ca^{2+} -binding loop of the zymogen.

The interaction of Ca^{2+} with activated GDPC is also associated with ~20–50% improvement in the amidolytic activity of the protease with a $K_{d(\text{app})}$ of ~50 μM Ca^{2+} (10). This property of Ca^{2+} was utilized to evaluate the affinity of the mutant protease for the metal ion. Unlike the zymogen GDPC mutant, the activated GDPC mutant exhibited slightly improved affinity for Ca^{2+} ($77 \pm 5 \mu\text{M}$ for wild-type and $47 \pm 9 \mu\text{M}$ for the mutant), though the extent of the stimulation of the amidolytic activity of the mutant by the metal ion was diminished ~2-fold (Figure 4). These results suggest that the autolysis loop and/or the Ca^{2+} -binding 70–80 loop undergo a conformational change upon activation by thrombin.

Anticoagulant Activity

The anticoagulant activity of the full-length APC mutant was evaluated in both the purified and plasma based clotting assays. In the purified system, both the time course and concentration dependence of fVa degradation suggested that the anticoagulant activity of the mutant has been improved 4–5-fold (Figure 5A and B, time course and concentration-dependence, respectively). Interestingly, however, the same studies in the presence of protein S revealed that both wild-type and mutant APC have essentially identical activities (Figure 5C and D, time course and concentration-dependence, respectively). Comparisons of the time course and APC concentration dependence of fVa degradation revealed that protein S improves the activity of wild-type APC ~20–25-fold. On the other hand, the extent of the cofactor activity of protein S with the APC mutant was decreased to ~5-fold (compare Fig. 5A in the absence of protein S for the time course analysis with 1 nM APC derivatives, with Fig. 5C for the time course analysis with 0.5 nM APC derivatives in the presence of protein S). These results suggest that the alleviation of possible inhibitory interactions of a longer autolysis loop of wild-type APC with fVa by protein S may partially account for the cofactor effect of protein S in enhancing the anticoagulant function of the protease. APC must sequentially cleave at least two bonds P1-Arg⁵⁰⁶ and P1-Arg³⁰⁶ to completely inactivate the cofactor (37). It is known that the cleavage at the Arg³⁰⁶ site is membrane dependent and is also slower than the cleavage at the Arg⁵⁰⁶ site which is independent of the membrane (38,39). Thus, activated GDPC in solution

primarily recognizes the Arg⁵⁰⁶ cleavage site (38,39). To determine the basis for the improved fVa degradation by the APC mutant, two additional experiments were carried out. First, the inactivation of fVa by the activated GDPC derivatives was compared. As shown in Figure 6A, similar to the APC mutant, the activated GDPC mutant exhibited a similar improvement in the fVa degradation assay suggesting that the ability of the mutant to recognize the Arg⁵⁰⁶ cleavage site has been improved. In the second experiment, the ability of the full-length APC derivatives to inactivate fVa Leiden was examined. In this natural fVa variant, the Arg⁵⁰⁶ is mutated to a Gln so the resulting Gln⁵⁰⁶ is resistant to cleavage by APC (40,41). As shown in Figure 6B, the activity of the APC mutant toward fVa Leiden was also improved to a similar extent. These results suggest that the catalytic efficiency of the APC mutant toward both cleavage sites of fVa has been improved. A similar fVIIIa degradation assay indicated that the ability of the APC mutant to inactivate fVIIIa has also been improved (data not shown). However, the effect of protein S on fVIIIa degradation by APC derivatives was not studied.

Unlike the improved anticoagulant activity of the APC mutant in the purified system, the plasma clotting activity of the mutant was slightly impaired (Figure 7). To understand the basis for this paradoxical effect in the activity of the mutant in these two systems, the extent of the reactivity of APC derivatives with plasma inhibitors was studied. The time course of the incubation of each APC with normal plasma followed by monitoring the amidolytic activity yielded a half-life of ~30 min for wild-type APC at room temperature, which is consistent with the literature (42). Interestingly, the half-life of the APC mutant in plasma was decreased to ~5 min, suggesting that the APC mutant has become susceptible to inhibition by the plasma inhibitors. Thus, certain structural features in the autolysis loop of APC appear to be responsible, at least partially, for the slow reactivity of the protease with plasma inhibitors. We previously showed that the reactivity of the APC mutant with AT has been dramatically improved in the presence of heparin or the pentasaccharide fragment of heparin suggesting that the mutant recognizes the activated conformation of the serpin (28). Although the reactivity of the APC mutant with the native conformation of AT was also improved, nevertheless, a second-order rate constant of $3 \text{ M}^{-1}\text{s}^{-1}$ for the APC mutant-AT interaction (28) cannot solely account for the dramatic decrease in the half-life of the APC mutant in plasma. Thus, it is likely that the reactivity of the APC mutant with other serpins has also been improved. In support of this hypothesis, inhibition studies with protein C inhibitor (PCI) revealed that relative to wild-type APC ($k_2 = 1.7 \pm 0.2 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$) the reactivity of the mutant with the serpin has been increased ~3-fold ($k_2 = 4.6 \pm 0.1 \times 10^3 \text{ M}^{-1}\text{s}^{-1}$). A slight improvement in reaction with α_1 -antitrypsin ($k_2 = 5.9 \pm 0.7 \text{ M}^{-1}\text{s}^{-1}$ for wild-type and $8.1 \pm 0.6 \text{ M}^{-1}\text{s}^{-1}$ for the mutant) was also observed. Whether the APC mutant reacts faster with other plasma serpins was not further evaluated.

Discussion

Protein C activation by thrombin requires the cofactor function of both Ca^{2+} and TM (1). Surprisingly, however, Ca^{2+} also potently inhibits protein C activation by thrombin when TM is absent (1). The mechanism of the inhibitory effect of Ca^{2+} on protein C activation by thrombin is not well understood. Previous studies have indicated that the conformation of the activation peptide of protein C and the Ca^{2+} binding loop of the zymogen is allosterically linked (17,19). This has been evidenced by direct binding studies (19) and the observation that the mutagenesis of the residues surrounding the scissile bond weakens the affinity of Ca^{2+} for its binding site in the protease domain (10,17). Interestingly, the weakening of the interaction of the metal ion by mutagenesis of either P3-Asp and/or P3'-Asp residues has been shown to be associated with a significant improvement in the activation rates of the mutant zymogens by thrombin in the presence of Ca^{2+} (17). Based on these and other similar studies, it has been hypothesized that the binding of Ca^{2+} to the protease domain induces structural changes in the activation peptide of protein C that lead to trapping of the zymogen in an inhibitory

conformation for recognition by thrombin in the absence of TM (1). Recent molecular modeling studies supported this hypothesis and further predicted that the acidic residues of the activation peptide can also directly interact with residues of both the Ca^{2+} -binding 70–80 loop and the autolysis loop of the protein (25,26). Thus, a complex network of electrostatic interactions between the charged residues of the activation peptide, the Ca^{2+} -binding loop and the autolysis loop of protein C may make the activation peptide of protein C inaccessible for interaction with the catalytic pocket of thrombin. It is likely therefore that the autolysis loop of the chimeric protein C mutant is unable to participate in such interactions, leading to its weaker affinity for Ca^{2+} and a lower inhibitory effect for the metal ion during its activation by thrombin. The crystal structure of the zymogen protein C needs to be determined to validate this hypothesis.

Previous mutagenesis studies have indicated that the autolysis loop of APC also contributes to the anticoagulant properties of the protease (43,44). Thus, in one previous study, the longer autolysis of human APC was replaced with the corresponding shorter sequence of the bovine APC (43). The resulting APC mutant exhibited a 2–3-fold higher anticoagulant activity as determined by both fVa degradation and plasma based clotting assays. The results of this study are in agreement with the hypothesis that the shortening of the autolysis loop of APC yields a mutant with a higher anticoagulant activity. The current study further indicates that a cofactor function for protein S in enhancing the anticoagulant function of APC may be mediated through the ability of the cofactor to eliminate the potential inhibitory interactions of the longer autolysis loop with fVa. This is derived from the observation that the anticoagulant activity of the APC mutant was improved 4–5-fold in the fVa degradation assay in the absence of protein S, but its activity was identical to the wild-type protease in the presence of the cofactor. The observation that the APC mutant cleaved both Arg⁵⁰⁶ and Arg³⁰⁶ sites with similar improved efficiency in the absence of protein S suggests that the cofactor can promote the catalytic efficiency of APC with both cleavage sites. This is consistent with a recent mutagenesis study in the literature (45).

In a second study, Ala-scanning of the charged residues of the autolysis loop of APC suggested that these residues constitute a binding site for fVa (44). Based on the observation that the Ala substitution mutants of APC exhibited a dramatic impairment in the cleavage rate of the Arg⁵⁰⁶ site and a lesser effect on the cleavage rate of the Arg³⁰⁶ site, this previous study concluded that the preferential recognition of the Arg⁵⁰⁶ site by the autolysis loop helps to explain the differences in the cleavage rates of the two sites in fVa (44). The observation here that the APC mutant containing the autolysis loop of fXa inactivated both fVa and fVIIIa with a markedly improved activity compared to wild-type APC may seem somewhat surprising. But the current results also do not contradict the previous results because, similar to APC, the autolysis loop of fXa has four basic residues which might functionally substitute for the basic residues of the APC autolysis loop in the mutant protease.

Finally, the results of this study suggest that, in addition to a critical role in interaction with the substrate fVa, a longer autolysis loop may have been evolved in APC to prevent the interaction and subsequent neutralization of the anticoagulant protease by the major plasma serpin, AT, in complex with the glycosaminoglycans of the endothelium. Indeed, in support of this hypothesis, previous results have indicated that the chimeric APC mutant, unlike wild-type APC, reacts efficiently with the heparin-activated conformation of AT (28), suggesting that structural features within this loop contribute to the complete lack of reactivity of the anticoagulant APC with this serpin. Similarly, the mutant also reacted with protein C inhibitor with an ~3-fold enhanced second-order rate constants. Thus, the enhanced reactivity of the APC mutant with the plasma serpins accounts for the marked decline in the half-life and the plasma anticoagulant activity of the mutant protease.

In summary, we showed that the autolysis loop of the zymogen protein C contributes to stabilization of the activation peptide of the protein in the characteristic Ca^{2+} -dependent inhibitory conformation that is suboptimal for recognition by thrombin in the absence of TM. The autolysis loop also plays a critical role in the anticoagulant function of APC and may partially be responsible for the lack of reactivity of the protease to inactivation by the plasma serpins. Moreover, it appears that another role for the cofactor function of protein S in the regulation of the anticoagulant function of APC is the optimization of the interaction of the longer autolysis loop of APC with the target procoagulant substrate fVa and perhaps also fVIIIa.

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Figure 1.

Time course of the initial rate of protein C activation by thrombin in the absence and presence of TM. A, either wild-type (○) or mutant GDPC (●) (1 μ M each) was incubated with thrombin (10–50 nM) at room temperature in TBS containing 1 mg/mL BSA, 0.1% PEG 8000 and 2.5 mM Ca^{2+} . At indicated time points, small aliquots of the activation reactions were transferred to a 96-well assay plate, the thrombin activity was neutralized by AT, and the rate of activated GDPC generation was determined by an amidolytic activity assay using SpPCa as described under “Materials and methods”. B, the same as A, except that the time course of activation by thrombin (1 nM) was monitored in the presence of TM456 (200 nM). The plots are representative experiments out of 3 independent and reproducible measurements.

Figure 2.

The Ca^{2+} -dependence of protein C activation by thrombin in the absence and presence of TM. A, the inhibitory effect of Ca^{2+} on the initial rate of wild-type (○) and mutant GDPC (●) (1 μM each) activation by thrombin (10 nM) was monitored at room temperature in TBS containing 1 mg/mL BSA, 0.1% PEG 8000 and different concentrations of Ca^{2+} . The % inhibitory effect of Ca^{2+} was calculated as described under "Materials and methods". B, the same as A, except that the fold stimulatory effect of Ca^{2+} on wild-type (○) and mutant GDPC (●) activation by thrombin (1 nM) was calculated in the presence of TM456 (200 nM). The solid lines are nonlinear regression fits of kinetic data to a hyperbolic equation. The plots are representative experiments out of 3 independent and reproducible measurements.

Figure 3.

Ca²⁺ binding to wild-type and mutant GDPC. The binding of Ca²⁺ to either wild-type (○) or mutant GDPC (●) (1 μM each) was monitored from a decrease in the intrinsic fluorescence upon interaction with the metal ion as described under “Materials and methods”. The plot is a representative experiment out of 3 independent and reproducible measurements.

Figure 4.

The Ca^{2+} -dependence of stimulation of the amidolytic activity of activated GDPC derivatives. The amidolytic activity of wild-type (○) and mutant activated GDPC (●) (3 nM each) was monitored using SpPCa (100 μM) at room temperature in TBS containing 1 mg/mL BSA, 0.1% PEG 8000 and 5 mM Ca^{2+} . The solid lines are nonlinear regression fits of kinetic data to a hyperbolic equation. The plot is a representative experiment out of 2 independent and reproducible measurements.

Figure 5.

Factor Va degradation by wild-type and mutant APC in the absence and presence of protein S. A, the time course of fVa (5 nM) degradation by wild-type (○) and mutant APC (●) (1 nM each) was carried out on PC/PS vesicles (25 μM) at room temperature in TBS containing 0.5 mg/mL BSA, 0.1% PEG 8000 and 5 mM Ca²⁺. At each time point, small aliquots of the inactivation reaction was transferred to a 96-well assay plate and the remaining cofactor activity of fVa was determined by a prothrombinase assay as described under “Materials and methods”. B, the same as A, except that the APC concentration dependence of fVa degradation was carried out for 10 min. C, the same as A, except that the time course of fVa degradation with 0.5 nM APC derivatives was carried out in the presence of protein S (220 nM). D, the same as C, except that the APC concentration dependence of fVa degradation was carried out for 1 min. The plots are representative experiments out of 2–3 independent and reproducible measurements.

Figure 6.

Time course of wild-type and Leiden factor Va degradation by the APC derivatives. A, the time course of wild-type fVa (5 nM) degradation by wild-type (○) and mutant activated GDPC (●) (2.5 nM each) was carried out at room temperature in TBS containing 0.5 mg/mL BSA, 0.1% PEG 8000 and 5 mM Ca^{2+} . At each time point, small aliquots of the inactivation reaction was transferred to a 96-well assay plate and the remaining cofactor activity of fVa was determined by a prothrombinase assay using prethrombin-1 as the substrate as described under “Materials and methods”. B, the time course of fVa Leiden (5 nM) degradation by wild-type (○) and mutant APC (●) (1 nM each) was carried out on PC/PS vesicles (25 μM) at room temperature and the remaining cofactor activity of fVa was determined by a prothrombinase assay as described above. The plots are representative experiments out of 2 independent and reproducible measurements.

Figure 7. Plasma clotting activity of wild-type and mutant APC. The clotting activities of wild-type (○) and mutant APC (●) were determined as a function of different concentration of APC at 37 °C as described under “Materials and methods”. The plot is a representative experiment out of 2 independent and reproducible measurements.