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Role of the Hexapeptide Disulfide Loop Present in the γ-Carboxyglutamic Acid Domain of Human Protein C in Its Activation Properties and in the in Vitro Anticoagulant Activity of Activated Protein C†

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ABSTRACT: In order to examine whether the structural integrity of the hexapeptide disulfide loop (residues 17–22), present in the γ-carboxyglutamic acid (γ) domain of human protein C (PC), and common to all vitamin K dependent coagulation proteins, is necessary for its anticoagulant properties, we employed recombinant (r) DNA technology to generate two important variants that would address this issue. One such mutein contained aspartic acid for γ-residue substitutions at sequence positions 19 and 20 ([γ^{19}D, γ^{20}D]r-PC) in the light chain of the mature protein, and the other possessed a serine for cysteine substitution at position 22 ([C^{22}S]r-PC of the same light chain. A subpopulation of molecules of these mutant proteins, containing the maximum levels of γ-residues in each, has been purified by fast-protein anion-exchange liquid chromatography and affinity chromatography on an anti-human PC column. A study of the kinetic characteristics of the inhibition by Ca^{2+} of the thrombin-catalyzed activation rates of these variants, and the corresponding stimulation by Ca^{2+} of the thrombin/thrombomodulin-catalyzed activation rates of the same recombinant PC molecules, demonstrated that higher concentrations of Ca^{2+} were required to display these effects, when compared to wild-type (wt) r-PC and human plasma PC. This suggested that the kinetically relevant Ca^{2+} site responsible for these effects on activation of PC, and known to be present in another domain of PC, was affected by both mutations in the γ-domain. The recombinant PC variants were converted to their activated forms ([γ^{19}D, γ^{20}D]r-APC and [C^{22}S]r-APC) and assayed for their Ca^{2+}-dependent anticoagulant activities. Each of these molecules displayed less than 1% of the activity of the wtr-APC in the activated partial thromboplastin time of PC-deficient plasma and similar low activity, relative to their wt counterpart, toward inactivation of purified human coagulation factor VIII. These results suggest that γ-residues at positions 19 and 20 of PC and APC, as well as the intact disulfide loop structure encompassing residues 17–22 of these proteins, are of some importance to the Ca^{2+}-dependent kinetics properties of PC activation and of great importance to the anticoagulant properties of APC. Coupled with a previous study demonstrating that the pair of γ-residues at positions 6 and 7 of the γ-domain of APC is similarly important to its anticoagulant properties [Zhang, L., & Castellino, F. J. (1990) *Biochemistry 29*, 10828–10834], this work suggests that Ca^{2+} interaction(s) essential for the anticoagulant functions of APC is influenced by different regions of the γ-domain.

Protein C (PC)[1] is a member of the class of vitamin K dependent plasma proteins, sharing amino acid sequence homology with other zymogens of this type. The biological role of the activated form of PC (APC), resides in its ability to maintain the fluid state of blood, and this enzyme functions in this manner by at least two different mechanisms. First, its anticoagulant properties are a consequence of its ability to proteolytically inactivate, by specific limited cleavage reactions, coagulation cofactors, factor V (f-V) and factor Va (f-Va) (Kisiel et al., 1977) as well as factor VIII (f-VIII) and factor VIIIa (f-VIIIa) (Vehar & Davie, 1980), in reactions that are stimulated by Ca^{2+}, phospholipid (Kisiel et al., 1977), and protein S (Walker, 1980). Second, APC functions as a fibrinolytic agent, likely due to its ability to inactivate inhibitors of plasminogen activators (Sakata et al., 1985; Taylor & Lockhart, 1985; van Hinsberg et al., 1985). Maximal activation of PC occurs at the endothelial cell surface and results from limited proteolysis, catalyzed by thrombin, along with

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[1] Abbreviations: PC, human plasma protein C; APC, activated human protein C; [γ^{19}D, γ^{20}D]r-PC(APC); a recombinant protein C (or activated protein C) containing aspartic acid residues substituted for γ-carboxyglutamic acid residues at positions 19 and 20 of the protein C amino acid sequence; [C^{22}S]r-PC(APC), a recombinant protein C (or activated protein C) containing a serine substituted for cysteine at position 22 of the protein C amino acid sequence; TM, thrombomodulin; ATIII, antithrombin III; γ, γ-carboxyglutamic acid; βOH-D, β-hydroxyaspartic acid; r, recombinant; wt, wild-type; DodSO$_4$/PAGE, sodium dodecyl sulfate/polyacrylamide gel electrophoresis; FPLC, fast-protein liquid chromatography; HPLC, high-performance liquid chromatography; MAb, monoclonal antibody; Fmoc, [(9-fluorenylmethyl)-oxy]carbonyl; S2366, L-pyroglutamyl-L-prolyl-L-arginine-*p*-nitroanilide.

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Ca^{2+} and the membrane protein cofactor, thrombomodulin (Esmon et al., 1982).

The nucleotide sequence of the PC gene contains eight exons (Foster et al., 1985). These DNA elements code for a 42 amino acid leader sequence, followed consecutively by a 155 amino acid light chain and a heavy chain of 262 amino acids, the latter of which contains the catalytic triad of H, D, and S, common to serine proteases (Banyai et al., 1983). A dipeptide, $K^{156}R^{157}$, is present in the noncompletely processed protein that connects the two chains. Several processing events occur prior to formation of the mature protein. In the noncatalytic chain, such processing includes cleavage of the leader polypeptide (Foster et al., 1985), γ-carboxylation of nine E-residues (DiScipio & Davie, 1979), and β-hydroxylation of one D-residue (Drakenberg et al., 1983). Additionally, in both chains, glycosylation of four N-residues occurs (Kisiel, 1979) as well as cleavage of the dipeptide linker between the latent heavy and light chains (Foster & Davie, 1984; Beckmann et al., 1985). From consideration of the positions of the introns in the gene, and the amino acid sequences of the mature protein, it is clear that PC is organized into several domains. These include the γ-rich amino-terminal polypeptide, two consecutive regions homologous to epidermal growth factor, an activation peptide, and the catalytic region. This latter domain is homologous in amino acid sequence to other serine proteases (Banyai et al., 1983).

Correctly processed human r-PC (Grinnell et al., 1987; Madden et al., 1990; Zhang & Castellino, 1990) and r-APC (Erlich et al., 1989) have been expressed in mammalian cells. This allowed the possibility that the functional properties of specific γ-residues of these proteins could be determined by expression of variant r-PC molecules that contain strategic mutations at these locations. We have recently examined the roles of the highly conserved pair of γ-residues at amino acid sequence positions 6 and 7 of APC and found that they were of extreme importance to the ability of APC to display plasma anticoagulant activity and ability to inactivate coagulation factor VIII (Zhang & Castellino, 1990). We wished to extend this investigation to the hexapeptide disulfide-linked loop in the γ-region of human PC, composed of amino acid residues 17–22, which contains γ-residues at amino acid sequence positions 19 and 20. This peptide is conserved in all vitamin K dependent blood coagulation proteins and is likely important to its function. The present paper presents a summary of the pertinent results of this investigation.

Materials and Methods

Proteins and Peptides. Human 293 kidney cell expressed wtr-PC and [γ^6D, γ^7D]r-PC were generated and purified as described previously (Zhang & Castellino, 1990). Purified r-human factor VIII (f-VIII) was provided by Genentech, Inc. (South San Francisco, CA), and purified human plasma f-VIII was donated by Armour Pharmaceuticals (Kankakee, IL). Bovine factor X (f-X) (Bajaj & Mann, 1973), bovine factor IX (f-IX) (Amphlett et al., 1979), and bovine factor IXaβ (f-IXaβ) (Amphlett et al., 1979) were obtained as described in the above publications. Human plasma PC, human thrombin, and human ATIII were donated by Enzyme Research Laboratories, Inc. (South Bend, IN). Rabbit TM was provided by Dr. Charles Esmon (Oklahoma City, OK).

The dodecapeptide containing the NH_2-terminal sequence of the light chain of human PC was synthesized in our laboratory with use of standard Fmoc chemistry. The α-(*N*-Fmoc)-γ,γ′-di-t-Bu-L-Gla-OH used to incorporate γ-residues in the peptide was chemically synthesized by Dr. Sushil Sharma in this laboratory.

All r-PC and variant r-PC activations were carried out as described earlier (Zhang & Castellino, 1990), with the *Agkistrodon contortix* venom activator of PC, Protac C, purchased from American Diagnostica (New York, NY).

Murine monoclonal anti-PC, C3 (Heeb et al., 1988), was provided by Dr. John Griffin (La Jolla, CA). The Ca^{2+}-dependent murine MAb, 7D7B10, the properties of which have been described (Orthner et al., 1989), was obtained from Dr. Dudley K. Strickland (Washington, DC).

For production of sufficient amounts of the MAb 7D7B10 for use in immunoaffinity chromatography columns, hybridoma cells were propagated in 1-L-scale cell cultures. The cells were grown in HY medium, as described (Ploplis et al., 1982), except that the medium contained 20% (v/v) CPSR-3 (Sigma Chemical Co., St. Louis, MO) as a supplement instead of 10% (v/v) fetal bovine serum + 10% (v/v) fetal horse serum. Purification of 7D7B10 was accomplished on a column of protein G–Sepharose (Zymed). After application of the conditioned medium, the column was washed with a buffer containing 0.01 M sodium phosphate/0.15 M NaCl, pH 7.4 (PBS) until the absorbance at 280 nm remained constant at <0.05. Antibody was eluted from the column with a solution of 0.1 M glycine, pH 2.7. Yields of approximately 20 mg of antibody/L of conditioned media were obtained.

Restriction endonucleases were purchased from Fisher Scientific (Springfield, NJ) and BRL (Gaithersberg, MD). These enzymes were employed according to the manufacturers' recommendations.

Genes for Wild-Type and Mutant PC Molecules. The cDNA coding for wtr-PC was provided by Dr. Earl Davie (Seattle, WA) in pUC119. The changes that we incorporated into the cDNA for use with our 293 kidney cell expression system, and the plasmid expression vector employed (pCIS2M), have been described in detail (Zhang & Castellino, 1990).

The cDNA coding for the variant, [γ^{19}D, γ^{20}D]r-PC, was constructed from the cDNA coding for wtr-PC by using the mutagenic synthetic oligonucleotide primer (the lower case letters indicate the mutagenic bases)

5′-GGAGTGCATAGAcGAcATCTGTGACTTCG

Screening of mutated colonies was accomplished by observing the loss of the *Bgl*II restriction endonuclease site at the second base of the codon for E^{20}.

The cDNA coding for the variant, [C^{22}S]r-PC, was constructed from the wt-cDNA, as above, except that the mutagenic primer

5′-AGGAGATCaGTGACTTC

was employed. This primer also resulted in the loss of the *Bgl*II site at the second base of the codon for E^{20}. Therefore, screening mutant colonies was accomplished as above.

The presence of the proper nucleotide insertions in the relevant cDNAs was examined by DNA sequence analysis (vide infra) prior to their placement into the expression vectors. Insertion of the variant cDNAs into pCIS2M was accomplished as for the wt-PC gene.

Transfection in Human Kidney 293 Cells. The transfection and clonal selection, as well as cell growth procedures employed, were as described earlier (Zhang & Castellino, 1990).

Purification of the Recombinant Proteins. The chromatographic method described for purification of wtr-PC (Yan et al., 1990) was employed, with minor operational modifications (Zhang & Castellino, 1990).

The purification of [γ^{19}D, γ^{20}D]r-PC, with selection for a subpopulation of variant PC molecules that contained the

maximal level of γ-residues, was as described for a similar purification of [γ^6D, γ^7D]r-PC (Zhang & Castellino, 1990). In addition, final purification was accomplished by using affinity chromatography with a column (0.6 cm × 5 cm) of Sepharose–MAb 7D7B10. The [γ^{19}D, γ^{20}D]r-PC pool from the Fast Flow Q Sepharose (FFQ) column was adsorbed to the column in a buffer of 20 mM Tris-HCl/150 mM NaCl, pH 7.4, and was eluted from the column with a solution of 0.1 M glycine, pH 2.7. While it would have been desirable to elute the protein with $CaCl_2$ (Orthner et al., 1989), this variant r-PC (as well as the one below) did not respond to $CaCl_2$ elution in the same manner as wtr-PC.

Purification of [C^{22}S]r-PC, with selection of a subpopulation of molecules containing the maximal level of γ-residues, was accomplished as above, except that the material that eluted from the Sepharose–MAb 7D7B10 column was reapplied to the FFQ anion-exchange column, equilibrated in 20 mM Tris-HCl, pH 7.4, then washed with a solution of 20 mM Tris-HCl/200 mM NaCl, pH 7.4, and eluted with a buffer of 20 mM Tris-HCl/500 mM NaCl, pH 7.4.

γ-Carboxyglutamic Acid Determination. These procedures, as well as the manner of treatment of the chromatographic data, have been published (Zhang & Castellino, 1990).

APTT Assays and f-VIII Inactivation Assays. Our methods for performance of these assays have been described in detail (Zhang & Castellino, 1990).

Amidolytic Assays for the Effect of Ca^{2+} on the Thrombin- and Thrombin/TM-Catalyzed Activations of PC and r-PC Variants. Aliquots from stock solutions of human plasma PC and Ca^{2+} were mixed subsequent to addition of thrombin (or thrombin/TM) such that final concentrations of 1 μM (or 2 μM) HPC, 0–600 mM Ca^{2+}, and 20 nM thrombin (or 0.4 nM thrombin/8.0 nM TM, preincubated for 5 min) were present in a final volume of 100 μL, 37 °C. The buffer was 20 mM Tris-HCl/100 mM NaCl/0.1% (w/v) gelatin, pH 7.4. All solutions used were passed over a Chelex-100 column, prior to addition of Ca^{2+}. Aliquots (30 μL) were removed at times of 2, 4, and 8 min, and the thrombin (or thrombin/TM) was rapidly neutralized by addition of 10 μL of a solution of human antithrombin III (1 mg/mL)/heparin (50 μg/mL)/5 mM EDTA in a buffer of 20 mM Tris-HCl/100 mM NaCl, pH 7.4. The amount of APC present in each sample was determined by amidolytic assay with S2366, at 37 °C, employing a Cary 15 Spectrophotometer. The K_i (in the case of thrombin-catalyzed activation) or K_a (in the case of thrombin/TM-catalyzed activation) for Ca^{2+} was evaluated from usual plots of the dependence of the initial rate of activation of PC on the concentration of Ca^{2+}.

Similar experiments were conducted for wtr-PC, [γ^{19}D, γ^{20}D]r-PC, [C^{22}S]r-PC, and [γ^6D, γ^7D]r-PC (Zhang & Castellino, 1990). In cases where proteins were in particularly scarce supply, such as [γ^{19}D, γ^{20}D]r-PC and [C^{22}S]r-PC, APC assays were performed in wells of microtiter plates with a Molecular Devices (Menlo Park, CA) Thermomax plate reader and Softmax software for data reduction. This procedure allowed the volumes to be reduced to 50% of those employed in the above spectrophotometric assay. In these same cases, thrombin or thrombin/TM activations were conducted for time periods of 2 and 8 min. Additionally, no differences in the K_i or K_a values for Ca^{2+} were found for thrombin and thrombin/TM activations, respectively, of human plasma PC, wtr-PC, or [γ^6D, γ^7D]r-PC, when the spectrophotometric and microtiter plate assays were compared.

DNA Analytical Methods. Oligonucleotides were synthesized by using phosphoramidite chemistry on a Biosearch (San Rafael, CA) Cyclone two-column DNA synthesizer. All reagents were purchased from this same source. The resulting oligonucleotides were purified by using oligonucleotide purification cartridges (Applied Biosystems, Foster City, CA). cDNAs were sequenced by the dideoxy technique (Sanger et al., 1977) with use of the Sequenase reagent kit (U.S. Biochemicals, Cleveland, OH). Cell transfections were performed by the calcium phosphate method (Kingston, 1987). Plasmid DNAs were purified by CsCl/ethidium bromide (EtBr) gradient centrifugation (Moore, 1987) with a Beckman (Palo Alto, CA) L5 65 preparative ultracentrifuge and a VTi.65.1 vertical rotor. Centrifugation was allowed to proceed for 7 h at 55 000 rpm, 15 °C. EtBr was removed from the plasmid DNA by extraction into a solution of 2-propanol saturated with CsCl. The DNA was then dialyzed against a buffer of 1 mM Tris-HCl/0.1 mM EDTA, pH 7.1.

The cDNAs and cDNA fragments were purified by excising the appropriate bands after their electrophoretic separation on 1% agarose. Recombinant molecules were created by the method of Struhl (1985). Single-strand plasmid DNAs were generated as described (Vieira & Messing, 1987), and site-specific mutagenesis was conducted according to Kunkel et al. (1987).

Western Analysis. In general, protein samples were separated by $DodSO_4$/PAGE (Laemmli, 1970) on 10% (w/v) gels, and the protein bands were transferred to Immobilon-P (Millipore, Bedford, MA). PC-containing bands were labeled by the MAb, C3, the latter of which was detected enzymatically with a rabbit anti-mouse IgG/alkaline phosphatase complex, after addition of a chromogenic substrate for alkaline phosphatase. Our exact procedures have been described earlier (Zhang & Castellino, 1990).

Results

Variant forms of the cDNA for wtr-PC, one of which, when translated, would contain substitutions of D for both E-residues at positions 19 and 20 of the protein, and another that would contain a S for C substitution at position 22 of the mature protein, have been constructed and expressed in human kidney 293 fibroblast cells. In the first case, both of the normal E-residues are γ-carboxylated in the mature protein. Thus, these particular substitutions result in a recombinant mutant protein with two fewer γ-residues than wtr-PC. In the second case, the hexapeptide disulfide loop encompassing sequence positions 17–22 in the mature protein, and containing [$\gamma^{19}\gamma^{20}$], would be disrupted. In order to investigate the anticoagulant properties of these recombinant variant proteins, containing only the desired alterations, we required a subpopulation of molecules that was maximally γ-carboxylated. It had been concluded previously that the region of the protein including and surrounding amino acid residues 19 and 20 formed a recognition site for the vitamin K dependent γ-carboxylase (Price et al., 1987), and thus, it was possible that not all molecules of the variants in the total mutant r-PC pool would be completely processed with regard to γ-carboxylation.

As estimated from Western analysis of the conditioned cell medium, the expression level of wtr-PC was approximately 2–3 μg/mL. Virtually all of this adsorbed to the FFQ column and was eluted with Ca^{2+}. This chromatographic behavior suggests that a high level of γ-carboxylation has occurred in the majority of the molecules and provides a method to obtain r-PC subpopulations with at least 7 mol of γ/mol of protein (Yan et al., 1990). It has been shown previously that the wtr-PC that eluted from this column under these conditions possesses the full complement of approximately 9 mol of γ/mol of protein (Yan et al., 1990; Zhang & Castellino, 1990). Sim-

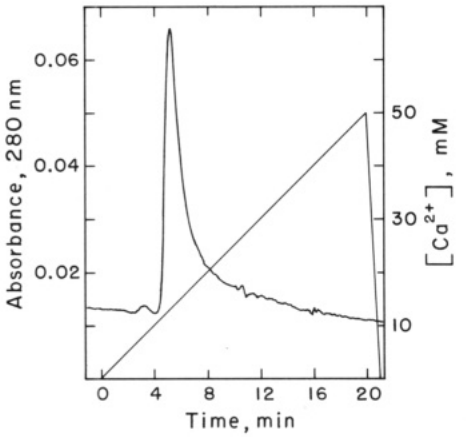

FIGURE 1: Elution from a MonoQ column of purified wtr-PC with the indicated Ca^{2+} gradient. FPLC methodology was employed.

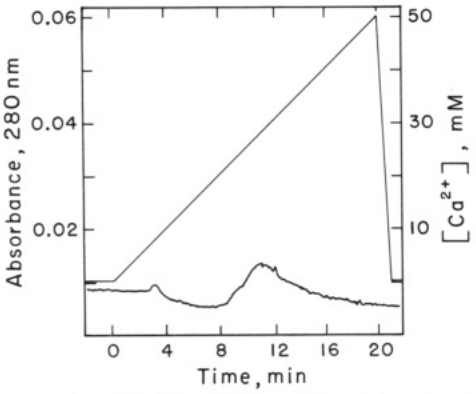

FIGURE 2: Elution of [γ^{19}D, γ^{20}D]r-PC from MonoQ as in Figure 1. The elution profile for [C^{22}S]r-PC by this method was very similar to that shown here.

ilarly, expression of another γ-variant, [γ^{6}D, γ^{7}D]r-PC, occurred at a level of approximately 1–2 μg/mL, at least 90% of which was adsorbed to FFQ and eluted with Ca^{2+}, again suggesting that the majority of material was maximally γ-carboxylated (Zhang & Castellino, 1990). Analysis of this material revealed the presence of the maximum of 7 mol of γ/mol of protein (Zhang & Castellino, 1990). However, expression levels of [γ^{19}D, γ^{20}D]r-PC were approximately 0.1–0.2 μg/mL under the same conditions, only approximately 20% of which adsorbed to the FFQ column and eluted with Ca^{2+}. This suggests that a large subpopulation of this variant possessed less than the maximal number of 7 γ-residues/mol. Even lower expression levels of [C^{22}S]r-PC were found (ca., 0.1 μg/mL), of which only approximately 15% was bound to FFQ and eluted with Ca^{2+}. Particularly in the case of this latter mutant, only very small amounts of material that contained a high level of γ-carboxylation were attainable, and final yields of this latter subpopulation of approximately 100 μg/6 L of culture fluid were obtained.

After this FFQ step, [γ^{19}D, γ^{20}D]r-PC was approximately 60% pure, as revealed by $DodSO_4$/PAGE. Final purification of [γ^{19}D, γ^{20}D]r-PC was achieved by immunoaffinity chromatography with monoclonal antibody 7D7B10. Purification of the subpopulation of [C^{22}S]r-PC that contained the highest possible number of γ-residues was accomplished in the same manner except that the product obtained after the immunoaffinity chromatography column required repassage over the FFQ column. The elution behavior of [C^{22}S]r-PC from the FFQ column occurred at a concentration of Ca^{2+} nearly identical with that of [γ^{19}D, γ^{20}D]r-PC, despite the fact (vide infra) that it contained two additional γ-residues as compared

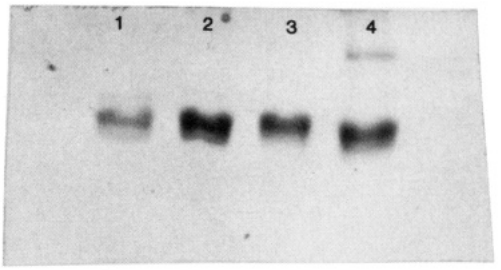

FIGURE 3: Nonreduced electrophoretic analysis of human plasma PC and recombinant PCs. The proteins (1 μg) in lanes 1–4 were separated by $DodSO_4$/PAGE and stained for protein with Coomassie Blue. Lane 1, human plasma PC; lane 2, wild-type r-PC; lane 3, [γ^{19}D, γ^{20}D]r-PC; lane 4, [C^{22}S]r-PC.

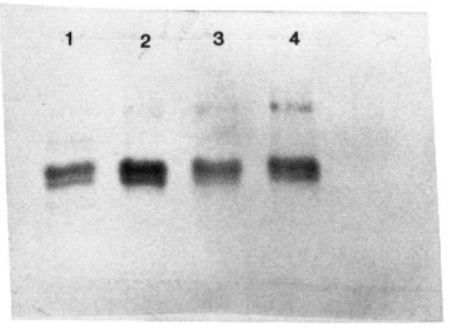

FIGURE 4: Western analysis of human plasma PC and recombinant PCs. The proteins (20 ng) in lanes 1–4 of Figure 3 were blotted from a $DodSO_4$/PAGE electrophoretic separation onto Immobilon-P, and the bands were visualized by immunoassay with the monoclonal antibody C3. Lane 1, human plasma PC; lane 2, wild-type r-PC; lane 3, [γ^{19}D, γ^{20}D]r-PC; lane 4, [C^{22}S]r-PC.

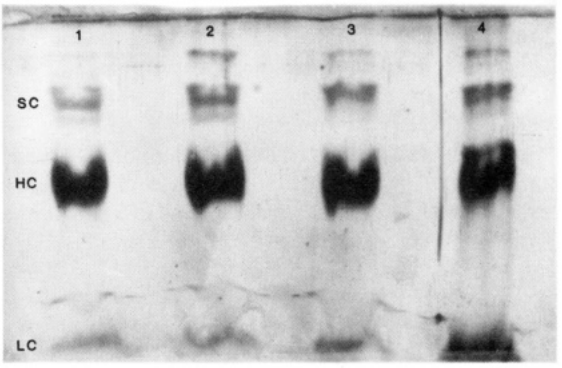

FIGURE 5: Reduced electrophoretic analysis of human plasma PC and recombinant PCs. The proteins (1 μg) in lanes 1–4 were separated by $DodSO_4$/PAGE and silver-stained for protein. Lane 1, human plasma PC; lane 2, wild-type r-PC; lane 3, [γ^{19}D, γ^{20}D]r-PC; lane 4, [C^{22}S]r-PC. SC refers to single-chain PC, and HC and LC refer to the heavy and light chains of two-chain PC, respectively.

to [γ^{19}D, γ^{20}D]r-PC. Thus, the Ca^{2+}-binding properties of [C^{22}S]r-PC appear to have been affected by this latter mutation.

Figures 1 and 2 show the FPLC elution behavior of purified wtr-PC and [γ^{19}D, γ^{20}D]r-PC, respectively, from a column of MonoQ (equivalent to FFQ) by the Ca^{2+} gradient indicated. Clearly, loss of the two γ-residues from this variant r-PC has affected its retention time on the column, suggesting that elution with Ca^{2+} does distinguish molecules with differing binding affinities and/or differing numbers of sites for this cation.

Purified [γ^{19}D, γ^{20}D]r-PC and [C^{22}S]r-PC has been subjected to protein characterization studies. $DodSO_4$/PAGE and concomitant Western analyses of both variants, as compared to wtr-PC, are shown in Figures 3 and 4, respectively. All

Table I: γ-Carboxyglutamic Acid and Acid Contents of Various Proteins

protein	Gla[a] (mol/mol) expected	obtained
PC peptide[b]	2.0	2.0
bovine f-IX[c]	12.0	12.0 ± 0.2
human PC[d]	9.0	8.8 ± 0.3
wtr-PC[e]	9.0	8.9 ± 0.3
[γ^{19}D, γ^{20}D]r-PC[f]	7.0	7.0 ± 0.2
[C^{22}S]r-PC[g]	9.0	8.5 ± 0.3

[a] γ-Carboxyglutamic acid. [b] Synthetic peptide consisting of the amino-terminal 12 residues of human plasma protein C. [c] Bovine plasma factor IX. [d] Human plasma protein C. [e] Wild-type recombinant protein C. [f] Recombinant human protein C containing D for E substitutions at positions 19 and 20. [g] Recombinant human protein C containing a S for C substitution at position 22.

proteins appear highly homogeneous. Futher, reduced Dod-SO_4/PAGE (Figure 5) demonstrates that only a very small amount of each PC exists as the single chain (SC) form and that the majority has been converted to the active site containing heavy chain (HC) and the γ-containing light chain (LC). It has been shown previously that approximately 10% of PC, purified from plasma or produced in culture from a cDNA, is not fully processed with regard to cleavage of the dipeptide linking the two chains of the protein (Yan et al., 1990), and the gels of Figure 5 are consistent with this view. In addition, the gels in Figures 3 and 4 reveal the presence of a small high molecular weight contaminant (<5%, estimated), that reacts with MAb-C3, in the [C^{22}S]r-PC sample. This may represent a disulfide-linked dimer of the protein, formed due to the presence of the free SH at amino acid sequence position 17. However, because of the very small amount of maximally γ-carboxylated [C^{22}S]r-PC that can be reasonably produced in normal laboratory-scale operations and the low relative level of this molecular weight band, we did not attempt its further removal, since we felt that further losses in yield of the desired component would not be acceptable.

It was of importance to determine the number of γ-residues present in the purified subpopulations of each of these variants to properly interpret the data from the anticoagulant assays. The values obtained for [γ^{19}D, γ^{20}D]r-PC and [C^{22}S]r-PC, as compared to wtr-PC, are shown in Table I. The data clearly demonstrate the presence of nearly the full complement of 7 γ-residues/mol and 9 γ-residues/mol in [γ^{19}D, γ^{20}D]r-PC and [C^{22}S]r-PC, respectively. In order to provide additional evidence for the accuracy of these determinations, data for similar analyses performed on a synthetic γ-containing peptide representing the sequence of the amino-terminal 12 residues of the light chain of PC, human plasma protein C, and bovine f-IX are also listed in Table I. The expected results were obtained in each case. Amino acid sequence analysis of [γ^{19}D, γ^{20}D]r-PC is presented in Table II and compared to the amino acid sequences obtained for human plasma PC and wtr-PC from 293 cells. The data demonstrate that the signal peptide and propeptide are effectively cleaved from the mutein, that the D-residues mutated into the cDNA have been translated at the level of protein in their predicted positions (19 and 20), and that γ-residues are present at position 6, 7, 14, and 16. Given the very small amounts of [C^{22}S]r-PC available to us, we were not able to obtain reliable sequence information beyond residue 23, but to that point the sequence was identical with that for wtr-PC, except for the presence of S that was mutated into the protein at position 22. From this, we conclude that γ-residues were present at positions 6, 7, 14, 16, 19, and 20 and that the S^{22} mutation was incorporated into the protein. There was approximately 2-fold more E present in cycles 14 and 19 than in wtr-PC at these same cycles (we normally observe 3–6% E at γ-residues due to partial decarboxylation of γ during sequencing), but with the small amount of [C^{22}S]r-PC that was employed for sequence analysis and the very low level of E seen, considerable noise was presented in this aspect of the data. In any case, there could not be more than 5–7% E in each of these cycles for the variant protein. Since the total number of γ-residues for [C^{22}S]r-PC is very close to nine (Table I), we are essentially performing our investigations with a fully γ-carboxylated protein, with a possibility of a small degree of incomplete γ-carboxylation at positions 14 and 19.

Table II: Amino Acid Sequence Analysis of Recombinant Protein C Molecules

cycle	sequence of PC L[a]	H[b]	amino acids identified in plasma PC	wtr-PC	[γ^{19}D, γ^{20}D]r-PC
1	A	D	A, D	A, D	A, D
2	N	T	N, T	N, T	N, T
3	S	E	S, E	S, E	S, E
4	F	D	F, D	F, D	F, D
5	L	Q	L, Q	L, Q	L, Q
6	γ[c]	E	E	E	E
7	γ[c]	D	D	D	D, E[d]
8	L	Q	L, Q	L, Q	L, Q
9	R	V	R, V	R, V	R, V
10	H	D	H, D	H, D	H, D
11	S	P	S, P	S, P	S, P
12	S	R	S, R	S, R	S, R
13	L	L	L	L	L
14	γ[c]	I	E,[d] I	E,[d] I	E,[d] I
15	R	D	R, D	R, D	R, D
16	γ[c]	G	E,[d] G'	E,[d] G	E,[d] G
17	C	K	–, K	–, K	–, K
18	I	M	I, M	I, M	I, M
19	γ[c]	T	E,[d] T	E,[d] T	D, T
20	γ[c]	R	E,[d] R	E,[d] R	D, R
21	I	R	I, R	I, R	I, R

[a] Amino acid sequence of the light chain of human plasma PC. [b] Amino acid sequence of the heavy chain of human plasma PC. [c] γ-Carboxyglutamic acid. A small amount of E is normally seen when γ-residues are present at the relevant sequence positions. [d] Trace levels.

Since potential kinetically relevant Ca^{2+}-binding regions of PC may have been affected by the nature of the alterations made in the molecule, we first examined the inhibitory effects of Ca^{2+} on the activation of PC by thrombin (Amphlett et al., 1981) and the stimulatory effects of Ca^{2+} on this same activation by the thrombin/TM complex (Esmon et al., 1982). From steady-state kinetic analysis of the effect of varying concentrations of Ca^{2+} on the initial activation rates of PC, wtr-PC, [γ^{19}D, γ^{20}D]r-PC, and [C^{22}S]r-PC, as well as of [γ^{6}D, γ^{7}D]r-PC (Zhang & Castellino, 1990), typical Lineweaver–Burk kinetic plots were obtained, an example of which is illustrated in Figure 6A, and K_i (dissociation) values for Ca^{2+} for each of these proteins are listed in Table III. This K_i value represents the relevant parameter of the Ca^{2+} site(s) responsible for the kinetic inhibitory effects and may, or may not, be one or more of the sites measured macroscopically. In any case, both PC and wtr-PC possessed similar K_i values for Ca^{2+}, and all variants possessed K_i values that demonstrated their diminished ability to be influenced by Ca^{2+} in this regard. The [C^{22}S]r-PC variant displayed behavior closest to the native proteins, and this particular property of Ca^{2+} was most affected in the [γ^{19}D, γ^{20}D]r-PC variant. A similar situation was found when the effect of Ca^{2+} on the stimulation of activation by thrombin/TM of each of the PC preparations was evaluated. Again, in this case, typical Lineweaver–Burk plots were ob-

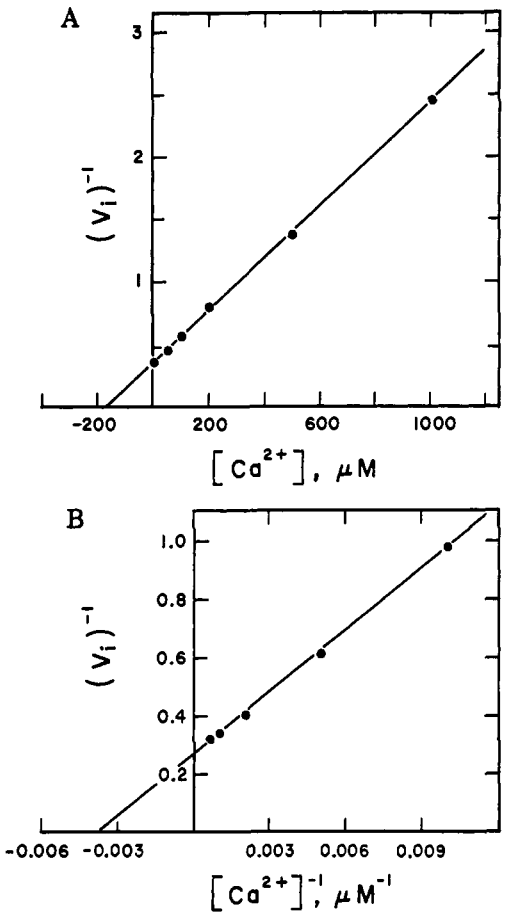

FIGURE 6: The effect of Ca^{2+} on the activation of PC. (A) Steady-state kinetic plot illustrating the concentration dependence of Ca^{2+} on the inhibition of the initial rates of the thrombin-catalyzed activation of [$C^{22}S$]r-PC. The K_i for the reaction was determined as the negative intercept of the abscissa. (B) Steady-state kinetic plot illustrating the concentration dependence of Ca^{2+} on the stimulation of the initial rates of the thrombin/TM-catalyzed activation of [$C^{22}S$]r-PC. The K_a for the reaction was determined as the negative intercept of the reciprocal of the abscissa. In both cases, initial velocities are in arbitrary units.

Table III: Effects of Ca^{2+} on the Activation of PC by Thrombin and the Thrombin/Thrombomodulin Complex

protein	Kinetic constants	
	K_i[a] (μM)	K_a[b] (μM)
human plasma PC[c]	125 ± 30	167 ± 50
wtr-PC[d]	143 ± 10	196 ± 40
[γ^6D, γ^7D]r-PC[e]	252 ± 40	299 ± 45
[γ^{19}D, γ^{20}D]r-PC[f]	330 ± 20	388 ± 80
[$C^{22}S$]r-PC[g]	174 ± 10	267 ± 30

[a] Kinetic inhibition (dissociation) constant for Ca^{2+} on the activation of the relevant PC by thrombin. [b] Kinetic activation (dissociation) constant for Ca^{2+} on the activation of the relevant PC by the thrombin/thrombomodulin complex. [c] Human plasma protein C. [d] Wild-type recombinant protein C. [e] Recombinant human protein C containing D for E substitutions at positions 6 and 7 (Zhang & Castellino, 1990). [f] Recombinant human protein C containing D for E substitutions at positions 19 and 20. [g] Recombinant human protein C containing a S for C substitution at position 22.

tained, one example of which is presented in Figure 6B. Here, the kinetic activation constants (K_a) for Ca^{2+}, provided in Table III, for the thrombin/TM-catalyzed activation of the various PC preparations, responded in parallel to the K_i values for Ca^{2+} measured for the thrombin-catalyzed activations of the same protein. In this case also, of the three variant r-PC molecules examined, [$C^{22}S$]r-PC possessed properties most closely associated with those of the native protein.

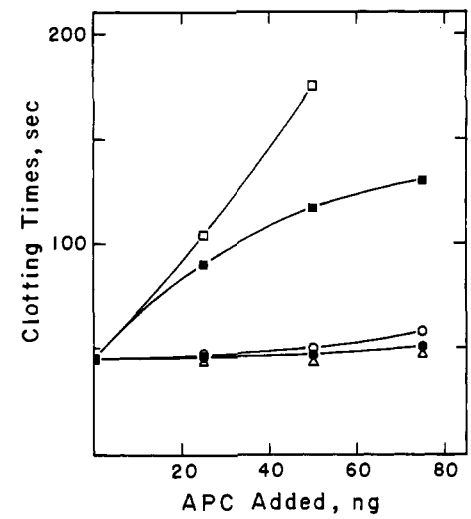

FIGURE 7: The effect of various APCs on the activated partial thromboplastin times (APTT) of PC-deficient human plasma. The APTT of PC-deficient plasma in the presence of (□) wtr-APC, (■) human plasma APC, and (○) [γ^6D, γ^7D]r-APC (Zhang & Castellino, 1990) are shown for comparison purposes; (△) clotting times in the presence of [γ^{19}D, γ^{20}D]r-APC and [$C^{22}S$]r-APC were virtually identical, and data for both enzymes are grouped under one symbol; (●) represents any of the parent PC molecules used. The clotting times of controls containing buffer alone or Protac alone were approximately 45 s (ordinate intercept). All PC molecules possessed approximately the same clotting times at each concentration, and the average values for the group were plotted as (●). For ease of comparison, the data for [γ^{19}D, γ^{20}D]r-APC and [$C^{22}S$]r-APC were placed on graphs previously published for a similar analysis of [γ^6D, γ^7D]r-APC (Zhang & Castellino, 1990).

The muteins, [γ^{19}D, γ^{20}D]r-PC and [$C^{22}S$]r-PC, have been activated to [γ^{19}D, γ^{20}D]r-APC and [$C^{22}S$]r-APC by the venom protease, Protac C, an activation reaction that does not require Ca^{2+} (in fact, Ca^{2+} is an inhibitor of the activation; Orthner et al., 1988). The Ca^{2+}-dependent anticoagulant activity of each variant APC, as measured by the APTT activity of PC-deficient plasma, is illustrated in Figure 7. As compared to wtr-APC, expressed in this same cell line, and human plasma APC, it is clear that [γ^{19}D, γ^{20}D]r-APC and [$C^{22}S$]r-APC are essentially inactive in this assay. For reference, data is illustrated from the same assay for the variant, [γ^6D, γ^7D]r-APC, which has been shown previously (Zhang & Castellino, 1990) to display a very small level (<5%) of activity in the APTT assay, compared to wtr-APC. The two disulfide loop variant APCs described in this study are even less active than [γ^6D, γ^7D]r-APC.

A direct measure of the ability of the APC variants to inactivate necessary coagulation cofactors has been assessed by employing the complete f-X activation system with purified components. In this case, the comparative activities of wtr-APC, human plasma APC, [γ^{19}D, γ^{20}D]r-APC, and [$C^{22}S$]r-APC toward the essential cofactor for this reaction, human f-VIII, are illustrated in Figure 8. We employed f-VIII, rather than f-VIIIa in this assay, fully realizing that the activity of APC toward f-VIIIa is greater than that toward f-VIII. However, the much greater instability of f-VIIIa as compared to f-VIII in the control assay precluded its effective employment for the purpose desired.

The data of Figure 8 demonstrate that both variants possess no observable activity toward f-VIII in this assay. Shown for comparison are the same results from a previous investigation with [γ^6D, γ^7D]r-APC in this same assay (Zhang & Castellino, 1990). This latter variant possessed approximately 5% of the activity of wtr-APC. Clearly, disruption of the structural integrity of the hexapeptide disulfide loop affects the activity

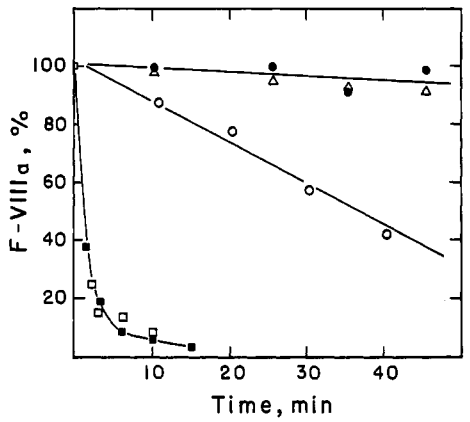

FIGURE 8: The effect of various APC on the inactivation of human r-f-VIII. The desired APC was preincubated with f-VIII, Ca^{2+}, and PC/PS for the times indicated on the abscissa. After these times, thrombin was added to convert the remaining f-VIII to f-VIIIa. Factor IXaβ was then added to stabilize f-VIIIa, and the solution was used to stimulate the activation of f-X, as described under Materials and Methods. The percentage of the original concentration of f-VIIIa present (derived from activation of remaining f-VIII), as measured by its stimulation of f-X activation, is plotted on the ordinate. (●) The control is the amount of f-VIIIa present in the absence of added APC or in the presence of any of the parent PC molecules. (■) Human plasma APC was preincubated with f-VIII. (□) wtr-APC was preincubated with f-VIII. (○) [γ^6D, γ^7D]r-APC was preincubated with f-VIII (Zhang & Castellino, 1990) and is shown for comparison purposes. (△) [γ^{19}D, γ^{20}D]r-APC and [C^{22}S]r-APC were preincubated with f-VIII. Both enzymes showed virtually identical behavior in this assay, and data for both are grouped under one symbol. For ease of comparison, the data for [γ^{19}D, γ^{20}D]r-APC and [C^{22}S]r-APC were placed on graphs previously published for a similar analysis of [γ^6D, γ^7D]r-APC (Zhang & Castellino, 1990).

even more dramatically and points to the essential nature of this structural element of APC for effective Ca^{2+}-dependent anticoagulation.

In both assays (above), all normal and variant APC concentrations were estimated from protein concentration. However, small final adjustments of the stock solutions to provide the same concentrations of each enzyme were made on the basis of equalizing their amidolytic activities, since this latter reaction has been amply demonstrated by several laboratories, including our own, to be independent of the level of γ-carboxylation. Stock solution corrections of no more than 10% were required in any case.

Discussion

The hexapeptide disulfide loop present at amino acid sequence positions 17–22 in the γ-rich domain of human PC (Foster & Davie, 1984) and containing paired γ-residues at sequence positions 19 and 20 is highly conserved in a similar sequence location in other vitamin K dependent coagulation proteins, such as prothrombin (Degen et al., 1983), f-VII (Hagen et al., 1986), f-IX (Kurachi & Davie, 1982; Yoshitake et al., 1985), f-X (Leytus et al., 1984; Fung et al., 1985), and protein S (Lundwall et al., 1986). Because of this strong sequence homology, we believed that this region of these proteins possessed an important functional property, presumably involving one interaction common to this group of proteins, i.e., functional consequences associated with Ca^{2+} binding. Thus, we decided to evaluate the role of this peptide structural element in Ca^{2+}-dependent functions of PC and APC, and did so by employing recombinant DNA technology to construct and express variants of PC containing important alterations that affected the structural integrity of the disulfide loop region of this enzyme. The functional properties that we examined involved the well-known inhibition by Ca^{2+} of the thrombin-catalyzed activation of PC (Amphlett et al., 1981) and the concomitant stimulation by Ca^{2+} of this same activation when catalyzed by the thrombin/TM complex. In addition, the Ca^{2+} dependence of the anticoagulant properties of APC in both a plasma clot system and in an assay with purified components of an essential step in clot formation that is inhibited by APC was also evaluated. Two recombinant variants PCs were generated, both of which disrupted to some degree key features of the hexapeptide disulfide loop; one in which both γ-residues in the loop were conservatively altered to D-residues, and another in which one of the paired C-residues in this region, viz., C^{22}, was altered to a S-residue. Each cDNA was successfully expressed in human kidney 293 cells.

It is not known whether alterations of critical E-residues in the γ-rich domain, or alteration of other important elements of this region, would affect γ-carboxylation of remaining E-residues. One group of investigators has published evidence that only the structure of the propeptide influences vitamin K dependent γ-carboxylation (Jorgensen et al., 1987; Ulrich et al., 1988; Hubbard et al., 1989; Huber et al., 1990), while another group believes that residues of the immature protein, primarily involving the sequence $E^{16}XXXE^{20}XC^{22}$, which encompasses the hexapeptide disulfide loop, acts as a recognition site for the vitamin K dependent carboxylase (Price et al., 1987). Other evidence in agreement with this latter view is that peptides containing the hexapeptide disulfide loop are much better substrates for the vitamin K dependent carboxylase (DeBoer-van den Berg et al., 1985; Ulrich et al., 1985) than peptides not containing this structural element (Rich et al., 1981), although this view has also been challenged (Huber et al., 1990). Given these uncertainties, we decided that effective execution of our study required examination of the level of γ-carboxylation of each of these variants and, if necessary, purification of the subpopulation of molecules with the maximal level of γ-carboxylation.

Employing an ion-exchange purification step with an FFQ column, which involved elution with Ca^{2+}, the success of which has been previously shown to depend upon the presence of at least 7 residues of γ/mol of protein (Yan et al., 1990), we were able to isolate a fraction of material for each variant, which, upon further purification by immunoaffinity chromatography, possessed nearly the maximum possible amount of γ-residues (7 mol/mol in the case of [γ^{19}D, γ^{20}D]r-PC and 9 mol/mol in the case of [C^{22}S]r-PC). Our experiences with this purification allow some qualitative comments to be forwarded on the contribution of the structural integrity of the hexapeptide disulfide loop to γ-carboxylation of pertinent PC residues. Obviously, it was possible to obtain material that was fully γ-carboxylated in the case of the two variant proteins reported here, suggesting that the vitamin K dependent carboxylase could function to some extent with mutations in E^{20} and C^{22}, residues that have been suggested to be of importance in the substrate recognition site (Price et al., 1987). However, most of the r-PC passed through the FFQ column in each case, and the relative amounts of [γ^{19}D, γ^{20}D]r-PC and [C^{22}S]r-PC that were fully γ-carboxylated (and bound to the column) represented only approximately 15%, or less, of the total r-PC antigen. In the case of wtr-PC and a previously described r-PC variant, [γ^6D, γ^7D]r-PC, greater than 90% of the total r-PC antigen was adsorbed to the FFQ column and subsequent γ-analysis demonstrated that these proteins contained the maximum levels of 9 and 7 mol of γ/mol of protein, respectively (Zhang & Castellino, 1990). Thus, we suggest that in the intact protein the hexapeptide disulfide loop may be important to substrate recognition by the vitamin K dependent

carboxylase, in addition to the well-established role of the propeptide in this process. The relative importance of these two regions toward carboxylase recognition cannot be ascertained from our data. However, E-residues at positions 6 and 7 of the protein do not appear to be as important to the γ-carboxylase recognition site as E-residues at positions 19 and 20 and as the structural integrity of the disulfide loop.

The data of Table III shows that the Ca^{2+}-related inhibition of the thrombin-catalyzed activation of PC is affected in all of the variants employed. The kinetically determined K_i for Ca^{2+} in this reaction is increased approximately 2-fold in all of the variants examined. The Ca^{2+} site responsible for this effect is believed to reside in the growth factor domain of PC (Esmon et al., 1983), which does not contain γ-residues, and we have directly observed by macroscopic binding analysis the existence of this site in the γ-independent region of PC (Hill & Castellino, 1987). If this is the same site(s) as that (those) which mediates the Ca^{2+}-dependent inhibition, then our results would suggest that the Ca^{2+} binding properties of this same site are influenced by alterations in the integrity of the γ-domain. This same phenomenon has also been observed previously, wherein it has been shown (Esmon et al., 1983) that the half-maximal rate of the thrombin-catalyzed activation of a PC derivative, in which the entire γ-domain has been proteolytically removed from the protein, is reduced to approximately 50 μM, as compared to a value of approximately 250 μM for the intact protein. While we demonstrate that mutation of paired γ-residues (viz., 6–7 and 19–20 in the γ-domain) or of a key structural element (viz., the C^{17}–C^{22} disulfide bridge, in the same γ-domain) results in a weaker ability of Ca^{2+} to inhibit the thrombin-catalyzed activation of PC, it has been shown that complete removal of the γ-domain from PC leads to an opposite effect on the ability of Ca^{2+} to inhibit this same reaction (Esmon et al., 1983). These results can be reconciled by suggesting that the γ-domain of PC does have a major influence on the functional properties of the γ-independent and kinetically relevant Ca^{2+} site, and these effects are displayed differently as a result of our more subtle modifications of the γ-domain. In any event, it should be clear that the functional Ca^{2+} binding that is present outside of the γ-region does not reside in an independent domain, and the integrity of this site is influenced by the γ-domain. Similar effects of the designed PC mutations on the functional properties of the γ-independent Ca^{2+} site(s) have been observed as a result of determination of the half-maximal concentrations (K_a) of Ca^{2+} required for stimulation of the thrombin/TM-catalyzed activation of PC. In all γ-domain variants examined here, the K_a for Ca^{2+} was increased, again showing diminished function of Ca^{2+}. It is believed that the same site on PC is involved both in the inhibition of its thrombin-catalyzed activation and in its stimulation of thrombin/TM-catalyzed activation (Esmon et al., 1983). The data of Table III tend to support this view. However, for all proteins listed in Table III, the K_a values of Ca^{2+} in the thrombin/TM-catalyzed activation are slightly and consistently higher than their counterpart K_i values in the thrombin-catalyzed activation, although the differences observed are small.

A considerably more dramatic functional role for γ-residues is observed in the Ca^{2+}-related functional properties of APC. We demonstrate herein that conservative alteration of the pair of γ-residues at sequence positions 19 and 20 of APC leads to a nearly complete inactivation of its Ca^{2+}-dependent anticoagulant activity in a clotting assay (Figure 7), while the amidolytic activity of the enzyme is not affected by these same mutations. Most likely, this is due to its inability to hydrolyze either, or both, of the cofactors for prothrombin activation, viz., f-V and f-Va, and for f-X activation, f-VIII and f-VIIIa. In addition, the interaction of APC with its cofactor, protein S, also requires Ca^{2+}, and interference with this reaction would also result in loss of activity in the APTT assay. In order to determine that such mechanisms were indeed of importance here, we investigated directly the activity of [γ^{19}D, γ^{20}D]r-APC toward f-VIII (Figure 8). As is clear from the data, this variant possessed no observable activity toward f-VIII in this assay. Thus, the two γ-residues at positions 19 and 20 are of critical importance to the ability of APC to specifically inactivate f-VIII. Although this observation could account in its entirety for the lack of activity of [γ^{19}D, γ^{20}D]r-APC in the APTT assay, it is highly likely that a parallel loss of activity toward f-V and f-Va occurs.

A similar loss of activity of APC in both functional assays is found when the disulfide bond formed between residues 17 and 22 is disrupted, as revealed by study of the variant [C^{22}S]r-APC, despite the otherwise virtual complete γ-carboxylation in the subpopulation of the molecules studied. Thus, complete γ-carboxylation is not the only criterion for the Ca^{2+}-dependent anticoagulation activity of APC. Clearly, effective Ca^{2+} binding requires integrity of certain structural elements of the γ-domain of the protein, most notably the hexapeptide disulfide loop. Examination of the X-ray crystal structure of prothrombin fragment 1 (Siriano-Garcia et al., 1989), which contains the γ-domain of this protein, reveals that the altered conformation of the peptide adopted in the presence of Ca^{2+}, mandatory to its ability to bind to phospholipid (Nelsestuen, 1976), depends upon the precise location of paired γ-residues 7–8 (6–7 in PC), 20–21 (19–20 in PC), and 26–27 (25–26 in PC). It is reasonable to expect that the disulfide bond between residues 17 and 22 in PC assists in maintenance of γ-residues 19 and 20 in the most favorable orientation for allowance of the Ca^{2+}-dependent conformational change accompanying Ca^{2+} binding. Thus, this work, as well as that published in a previous manuscript wherein we described a similar large loss in anticoagulant activity of a variant in which γ-residues at sequence positions 6 and 7 of APC were replaced with D-residues (Zhang & Castellino, 1990), provides some experimental verification for the model proposed from X-ray analysis. From our work, a strong argument can be forwarded implicating, at the least, residues 6 and 7 and 19 and 20, as well as the integrity of the disulfide bond that pairs residues 17 and 22, as structural and/or conformational determinants of the kinetically productive binding of Ca^{2+} that renders APC an effective anticoagulant.

We conclude that γ-residues 6–7 and 19–20 as well as the structural integrity of the disulfide loop present in the light chain of PC affect the functional properties of a γ-domain-independent Ca^{2+} effect required for inhibition of activation of PC by thrombin and for a parallel stimulation of this same activation by the thrombin/TM complex. This Ca^{2+} effect is not similar to the effect of Ca^{2+} associated with the γ-domain that has a profound influence on the anticoagulant properties of APC. This latter effect is more complex in nature and is influenced by different regions of the γ-domain and perhaps other parts of the molecule.

Registry No. PC, 60202-16-6; APC, 42617-41-4; Ca^{2+}, 7440-70-2; f-VIII, 9001-27-8; γ-carboxyglutamic acid, 53445-96-8.

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