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Enhancing the Activity of Protein C by Mutagenesis To Improve the Membrane-Binding Site: Studies Related to Proline-10[†]

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ABSTRACT: Bovine and human protein C show high homology in the amino acids of their GLA domains (amino-terminal 44 residues), despite the about 10-fold higher membrane affinity of the human protein. A proposed membrane contact site and mechanism suggested that this difference was largely due to the presence of proline at position 10 of bovine protein C versus histidine at position 10 of human protein C [McDonald, J. F., Shah, A. M., Schwalbe, R. A., Kisiel, W., Dahlback, B., and Nelsestuen, G. L. (1997) *Biochemistry 36*, 5120−5127]. This study examined the impact of replacing proline-10 in bovine protein C with histidine, and the reverse change in human protein C. In both cases, the protein containing proline-10 showed lower membrane affinity, about 10-fold lower for bovine protein C and 5-fold lower for human protein C. As expected, activated human protein C (hAPC) containing proline at position 10 showed 2.4−3.5-fold lower activity than wild type hAPC, depending on the assay used. Most interesting was that bovine APC containing histidine-10 displayed up to 15-fold higher activity than wild type bAPC. This demonstrated the ability to improve both membrane contact and activity by mutation. This general strategy should be applicable to other vitamin K-dependent proteins, providing opportunities to study function as well as to produce proteins that may find use as promoters and inhibitors of blood coagulation in pathological states.

Vitamin K-dependent proteins contain 9−13 γ-carboxyglutamic acids within the amino-terminal 45 residues (GLA domain[1]) that are needed for calcium and membrane binding. Despite great homology among amino acid sequences (*1*, *2*) and structures of calcium-bound proteins (*3*−*6*), members of this protein family display a 1000-fold range in membrane affinity (*7*, *8*). Since no vitamin K-dependent protein displays the maximum potential membrane-binding affinity of a GLA domain, all must contain amino acids whose purpose is to reduce binding affinity.

Lowered membrane affinity could serve several purposes. High affinity is accompanied by slow exchange, which may limit reaction rates. For example, the prothrombinase enzyme, when assembled on membranes with high affinity for substrate, was limited by protein exchange from the

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[1] Abbreviations: GLA domain, residues 1−44 of protein C; Gla, γ-carboxyglutamic acid; APC, activated protein C.

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membrane rather than by enzyme catalysis (*9*). Alternatively, adjustment of membrane affinity by nonoptimum amino acids may balance the competing processes of procoagulation [factor X, IX, and VII and prothrombin (*10*)] and anticoagulation [protein C and S (*11*)]. If membrane sites on one arm of these competing processes were improved, the result may be too much or too little blood coagulation. Adjustment of the membrane affinity by mutation may have provided a simple method of balancing these forces through natural selection.

Although membrane affinities of native proteins may be optimal for normal states, improvements in the membrane contact site may produce proteins that are useful for *in vitro* study and which may offer improved mechanisms for regulating blood clotting in pathological conditions. To date, most alterations in the GLA domain have replaced amino acids that are thought to be important to membrane contact (*12*−*25*]. The modifications have resulted in either neutral or negative impacts on membrane affinity, with analogous but not identical impacts on enzyme activity. While the GLA domain of factor IX was substituted into human protein C without an apparent effect on protein C activity (*13*), these proteins are essentially isomorphous with respect to membrane affinity (*8*) so that no influence on this basis would be expected.

Proteolysis of intact proteins has shown that the membrane contact site is expressed within residues 1−37 of factor X (*26*). The 3 amino-terminal residues of prothrombin can be removed with only a small impact on membrane affinity (*27*). Both of these properties applied at saturating calcium levels, where actual membrane contact may be studied in a manner that is independent of events such as the calcium-induced protein conformational change.

A recently proposed membrane contact site, involving an isolated protein−phospholipid ion pair, is localized within residues 4−37 (*8*). This site provided specific suggestions of amino acids that may be responsible for the diverse membrane affinities of members of this protein family. An example was proline-10. This residue is located on the edge of the suggested binding site. It is found in bovine protein C, human factor VII, and bovine factor VII, the proteins with the lowest affinity for membranes. Histidine is found at position 10 of human protein C.

This study reports the outcome of replacing proline-10 of bovine protein C with histidine, and the introduction of proline-10 into human protein C. As predicted by the binding mechanism (*8*), bovine protein C-H10 had enhanced membrane affinity relative to that of the wild type protein, while human protein C-P10 had reduced affinity. In both cases, activated protein C containing His-10 had higher enzyme activity both in a purified factor Va inactivation assay and in a clotting assay. The extent of impact varied from about 2.4- to 15-fold, depending on the protein and assay conditions used. These results provided support for the membrane-binding mechanism which predicted an impact of proline-10 (*8*). They also illustrated the success of a strategy for improving membrane contact by site-directed mutation. The approach appeared to offer many opportunities for exploitation in research, and possibly for *in vivo* regulation of both arms of the coagulation cascade.

MATERIALS AND METHODS

cDNA Clones. A full-length human protein C cDNA clone was the generous gift of J. Stenflo (Department of Clinical Chemistry, University Hospital, Malmö, Sweden). The bovine protein C cDNA clone was kindly provided by D. Foster (ZymoGenetics, Inc., Seattle). Both human and bovine protein C cNDAs were cleaved by restriction enzymes, and then cloned to the expression vector pRc/CMV after digesting with *Hin*dIII and *Xba*I.

Primers. Site-directed mutagenesis was performed by the PCR method. For human protein C mutagenesis at position 10, the following oligonucleotides were synthesized: A, 5′-AAA TTA ATA CGA CTC ACT ATA GGG AGA CCC AAG CTT-3′ (corresponding to nucleotides 860−895 in the vector pRc/CMV) to create a *Hin*dIII site between pRc/CMV and protein C; and B, 5′-GCA CTC CCG CTC CAG GCT GCT GGG ACG GAG CTC CTC CAG GAA-3′ (corresponding to amino acid residues 4−17 in human protein C; the eighth residue in this sequence was mutated from that for human protein C to that of bovine protein C, as indicated by the underline). These oligonucleotides were used to replace histidine at position 10 of human protein C with proline.

For bovine protein C mutagenesis, the following oligonucleotides were synthesized: A, as above, corresponding to nucleotides 860−895 in the vector pRc/CMV, to create a *Hin*dIII site between pRc/CMV and protein C; C, 5′-ACG CTC CAC GTT GCC GTG CCG CAG CTC CTC TAG GAA-3′ (corresponding to amino acid residues 4−15 in bovine protein C; the sixth amino acid was mutated from that for bovine protein C to that of human protein C, as indicated by the underline); D, 5′-TTC CTA GAG GAG CTG CGG CAC GGC AAC GTG GAG CGT-3′ (corresponding to amino acid residues 4−15 in bovine protein C; the seventh amino acid was mutated from that for bovine protein C to that of human protein C, as indicated by the underline); and E, 5′-GCA TTT AGG TGA CAC TAT AGA ATA GGG CCC TCT AGA-3′ (corresponding to nucleotides 984−1019 in the vector pRc/CMV), creating a *Xba*I site between pRc/CMV and protein C. The above oligonucleotides were used to replace proline at position 10 of bovine protein C with histidine.

In Vitro Mutagenesis and Construction of Expression Plasmids. Both human and bovine protein C cDNA (full-length) were cloned in the vector pRc/CMV cleaved by two restriction enzymes *Hin*dIII and *Xba*I. PCR amplification of the target DNA was performed as follows. Human protein C cDNA containing 5′ terminal to the amino acid at position 17 was amplified with intact human protein C cDNA and primers A and B. The mixture for PCR was 100 μL containing 0.25 μg of template DNA, each of the deoxyribonucleoside triphosphates at 200 μM (dATP, dCTP, dGTP, and dTTP), each primer at 0.5 μM, and 2.5 units of Pwo-DNA polymerase (Boehringer Mannheim) in Tris-HCl buffer (10 mM Tris, 25 mM KCl, 5 mM $(NH_4)_2SO_4$, and 2 mM $MgSO_4$ at pH 8.85). The sample was subjected to 30 cycles of PCR consisting of a 2 min denaturation period at 94 °C, a 2 min annealing period at 55 °C, and a 2 min elongation period at 72 °C. After amplification, the DNA was electrophoresed on an 0.8% agarose gel in 40 mM Tris-acetate buffer containing 1 mM EDTA. The PCR products were purified with the Geneclean III Kit (BIO 101, Inc., Vista,

CA); the human protein C cDNA containing respective mutations was cleaved by *Hin*dIII and *Bsr*BI, and then this fragment was cloned to the pRc/CMV vector which was cleaved by *Hin*dIII and *Xba*I and the human protein C fragment (*Bsr*BI, 3′ terminal) to produce human protein C full-length cDNA with the respective mutation.

Bovine protein C full-length cDNA was cloned in the vector pRc/CMV cleaved by two restriction enzymes *Hin*dIII and *Xba*I. PCR amplification of the target DNA was performed with the following steps. Bovine protein C cDNA containing the amino acid at position 10 was amplified with intact bovine protein C cDNA and primers A and C. Bovine protein C cDNA from amino acid 7 to the 3′ terminal was amplified with intact bovine protein C cDNA and primers D and E. These two cDNA fragments were used as templates to amplify full-length bovine protein C cDNA containing mutated amino acids with primers A and E. The PCR mixture and program were described above. The PCR product of the bovine protein C cDNA fragment containing the respective mutations was cleaved by *Hin*dIII and *Bsu*36I, and then the fragment of *Hin*dIII and *Bsu*36I was cloned to the pRc/CMV vector which contains intact bovine protein C fragments (*Bsu*36I, 3′ terminal) to produce bovine protein C full length cDNA with the respective mutation. All mutations were confirmed by DNA sequencing prior to transfection.

Cell Culture and Expression. The adenovirus-transfected human kidney cell line 293 was grown in DMEM supplemented with 10% fetal calf serum, 2 mM L-glutamine, 100 units/mL of penicillin, 100 units/mL streptamycin, and 10 μg/mL vitamin K_1. Transfection was performed using the lipofectin method (*28*). Two micrograms of DNA was diluted to 0.1 mL with DMEM containing 2 mM L-glutamine medium. Ten microliters of Lipofectin (1 mg/mL) was added to 100 μL of DMEM containing 2 mM L-glutamine medium. DNA and lipofectin were mixed and left at room temperature for 10−15 min. Cell monolayers (25−50% confluence in a 5 cm petri dish) were washed twice in DMEM with 2 mM L-glutamine medium. The DNA/lipid mixture was diluted to 1.8 mL in DMEM containing 2 mM L-glutamine medium and added to the cells, and the cells were incubated for 16 h. The cells were fed with 2 mL of complete medium containing 10% calf serum and left to recover for another 48−72 h. They were then trypsinized and seeded into 10 cm dishes with selection medium (DMEM containing 10% serum and 400 μg/mL Geneticin) at 1/5 (*29*). Geneticin-resistant colonies were obtained after 3−5 weeks of selection. Twenty-four colonies of each DNA transfection were picked and grown to confluence, and the media were screened for protein C expression with a dot-blot assay using monoclonal antibody HPC4 (for human protein C) and monoclonal antibody BPC5 (for bovine protein C). High-producing clones were isolated and grown until confluence in the presence of 10 μg/mL vitamin K_1.

Purification of Recombinant Wild Type and Mutated Protein. The purifications of bovine recombinant protein C and its mutant were based on the method described previously (*30*) with some modifications. Conditioned serum-free medium from stable transfected cells was centrifuged at 5000 rpm at 4 °C for 10 min. The supernatant was filtered through 0.45 μm of cellulose nitrate membranes (Micro Filtration Systems, Japan). EDTA (5 mM final concentration) and PPACK (0.2 μM final concentration) were added to the conditioned medium of 293 cells, and then this solution was passed through a Pharmacia FFQ anion exchange column at room temperature using Millipore Con Sep LC100 (Millipore, Bedford, MA) and eluted with a $CaCl_2$ gradient (starting solution, 20 mM Tris-HCl/150 mM NaCl at pH 7.4; limiting solution, 20 mM Tris-HCl/150 mM NaCl/30 mM $CaCl_2$ at pH 7.4). The $CaCl_2$ was removed by dialysis and Chelex 100 treatment, and the protein was readsorbed to a second FFQ column, after which it was eluted with an NaCl gradient (starting solution, 20 mM Tris-HCl/150 mM NaCl at pH 7.4; limiting solution, 20 mM Tris-HCl/500 mM NaCl at pH 7.4). At this point in the purification, the wild type and its mutant recombinant bovine protein C were homogeneous as determined by SDS−PAGE.

The first column used for purification of wild type and mutant recombinant human protein C was the same as that described for bovine protein C. The chromatographic method described by Rezair and Esmon (*30*) was employed with some modifications described for the method of protein S purification (*31*). Fractions containing protein C from anion exchange chromatography were identified by dot-blot. Positive fractions were pooled and applied to an affinity column containing the Ca^{2+}-dependent antibody HPC-4 (*30*). The column was equilibrated with 20 mM Tris-HCl/150 mM NaCl (pH 7.4) containing 5 mM benzamidine hydrochloride and 2 mM $CaCl_2$. After application, the column was washed with the same buffer containing 1 M NaCl and Protein C was then eluted with 20 mM Tris-HCl/150 mM NaCl/5 mM EDTA (pH 7.4) containing 5 mM benzamidine hydrochloride. After purification, the purities of all human and bovine recombinant protein C preparations were determined by SDS−PAGE followed by silver staining. Proteins were concentrated using YM 10 filters (Amicon), then dialyzed against buffer (50 mM Tris-HCl and 150 mM NaCl at pH 7.4) for 12 h, and stored at −70 °C. The concentrations of proteins were measured by absorbance at 280 nm.

Preparation of APC. Activated protein C was generated by thrombin cleavage in standard buffer (0.05 M Tris and 0.1 M NaCl at pH 7.5), using identical conditions for both the wild type and mutant proteins. Approximately 150 μg of the various protein C preparations (1 mg/mL) was mixed with bovine thrombin (3 μg) and incubated at 37 °C for 5 h. The reaction product was diluted to 0.025 M Tris buffer/0.05 M NaCl and applied to a 1 mL column of SP-Sephadex C-50. The column was washed with 1 mL of the same buffer, and the flow-through was pooled as activated protein C. The yield was 65−80% of the protein applied to the column. APC activity was determined by proteolysis of S2366 (0.1 mM) at 25 °C. The preparations were compared to standard preparations obtained on a larger scale. Standard human APC was a gift from W. Kisiel. For bovine proteins, the standard was a large-scale preparation of thrombin-activated APC. The activity of bovine APC was consistent for all preparations of normal and mutant proteins (±5%). Two preparations of bovine APC were used for comparisons. Human APC generated from thrombin was 55−60% as active as the standard. The concentrations reported in this study were based on activity toward S2366, relative to that of the standard.

Coagulation Assays. The standard APTT test used bovine or human plasma and the standard APTT reagent provided by Sigma Chemical Co. according to the manufacturer's instructions. Alternatively, phospholipid was provided in the

form of vesicles formed from highly purified phospholipids. In this assay, plasma (0.1 mL) was incubated with either kaolin (0.1 mL of a 5 mg/mL solution in 0.05 M Tris buffer/0.1 M NaCl at pH 7.5) or ellagic acid (0.1 mM in buffer) for 5 min at 35 °C. Phospholipid and APC were added (0.1 mL), and coagulation was initiated with 0.1 mL of 25 mM $CaCl_2$ in buffer. The time required to form a clot was recorded by a manual technique. The amount of phospholipid was designed to be the limiting component in the assay and to give the clotting times shown. The phospholipids used were small unilamellar vesicles (45 μg/0.4 mL in the final assay, 10/90 PS/PC) or large unilamellar vesicles (120 μg/0.4 mL in the final assay, 25/75 PS/PC).

Factor Va inactivation was assayed by the method of Nicolaes et al. (*32*). Briefly, for bovine proteins, bovine plasma was diluted 1000-fold by 0.05 M Tris, 0.1 M NaCl, 1 mg/mL bovine serum albumin, and 5 mM calcium at pH 7.5. Phospholipid vesicles (5 μg/0.24 mL assay) and 5 μL of 190 nM thrombin were added to activate factor V. After a 10 min incubation at 37 °C, APC was added and the incubation was continued for 6 min. Bovine prothrombin (10 μM final concentration) and factor Xa (0.3 nM final concentration) were added, and the reaction mixture was incubated for 1 min at 37 °C. A 20 μL sample of this activation reaction mixture was added to 0.38 mL of buffer (0.05 M Tris, and 0.1 M NaCl, 5 mM EDTA at pH 7.5) containing S2288 substrate (60 μM). The amount of thrombin was determined by the change in absorbance at 405 nM ($\epsilon = 1.0 \times 10^4$ M^{-1} s^{-1}, k_{cat} for thrombin = 100 s^{-1}). For human proteins, human protein S-deficient plasma (Biopool Canada, Inc., Burlington, ON) was diluted 100-fold, factor Va was activated by human thrombin, and the factor Va produced was assayed with the reagents used for the bovine proteins.

Vesicle Preparation and Measurement of Protein−Phospholipid Interaction. Large (*33*) and small (*34*) unilamellar phospholipid vesicles were prepared by methods described previously. Highly pure phosphatidylserine (bovine brain) and phosphatidylcholine (from egg, Sigma Chemical Co.) were mixed in organic solvent. The solvent was removed by a stream of nitrogen gas. The dried phospholipids were suspended in buffer. Small unilammelar vesicles were formed by sonication and gel filtration, while large unilamellar vesicles were formed by the freeze−thaw technique and extrusion. Phospholipid concentrations were determined by organic phosphate assay assuming a phosphorus/phospholipid weight ratio of 25.

Light scattering at 90° to the incident light and the general procedure of Nelsestuen and Lim (*35*) were used to quantitate protein−membrane binding. Briefly, the light scattering intensities of phospholipid vesicles alone (I_1) and after addition of protein (I_2) were measured and corrected for background from buffer and unbound protein. The molecular weight ratio of the protein−vesicle complex (M_2) to that of the vesicles alone can be estimated from the relationship in eq 1, where $\partial n/\partial c$ is the refractive index of the respective species, estimated as described.

$$I_2/I_1 = (M_2/M_1)^2(\partial n/\partial c_2/\partial n/\partial c_1)^2 \qquad (1)$$

If phospholipid and protein concentrations are known, the concentration of bound P*PL and free protein P can be estimated. These values, together with the maximum protein binding capacity ([P^*PL_{max}]) of the vesicles (assumed to be 1.0 g/g for all proteins) can be used to obtain the equilibrium constant for protein−membrane interaction by the relationship in eq 2, where all concentrations are expressed as molar protein or protein binding sites.

$$K_D = [P][P^*PL_{max} - P^*PL]/[P^*PL] \qquad (2)$$

Protein Sequences. Sequences of the amino-terminal residues of some relevant proteins include human protein C [hC (*1*)], bovine protein C [bC (*2*)], bovine prothrombin [bPT (*36*)], bovine factor X [bX (*37*)], and human factor VII [hVII (*38*)] are given for reference, where X is Gla or Glu:

bPT, ANKGFLXXVRK$_{11}$GNLXRXCLXX$_{21}$PCSRXX-AFXA$_{31}$LXSLSATDAF$_{41}$WAKY; **bX**, ANS-FLXXVKQ$_{10}$-GNLXRXCLXX$_{20}$ACSLXXARXV$_{30}$FXDAXQTDXF$_{40}$-WSKY; **hC**, ANS-FLXXLRH$_{10}$SSLXRXCIXX$_{20}$ICDFXX-AKXI$_{30}$FQNVDDTLAF$_{40}$WSKH; **bC**, ANS-FLXXLRP$_{10}$-GNVXRXCSXX$_{20}$VCXFXXARXI$_{30}$FQNTXDTMAF$_{40}$-WSFY; and **hVII**, ANA-FL XXLRP$_{10}$GSLXRXCKXX$_{20}$-QCSFXXARXI$_{30}$FKDAXRTKLF$_{40}$WISY.

RESULTS

Association of Normal and Mutant Protein C Molecules with Membranes. Bovine protein C containing histidine at position 10 interacted with membranes with about 10-fold higher affinity (K_D = 960 nM, Figure 1A) than wild type protein (K_D = 9200 nM). The difference in affinity corresponded to about 1.4 kcal/mol at 25 °C. In fact, membrane affinity of bovine protein C-H10 was almost identical to that of native human protein C (660 nM, Figure 1B). This suggested that proline-10 formed the major basis for differences between the membrane binding site of human and bovine proteins.

The reverse substitution, replacement of His-10 of human protein C by proline, decreased membrane affinity (Figure 1B). The impact of proline introduction (K_D = 660 nM for wild type versus 3340 nM for the mutant) was only slightly less than that of proline in the bovine proteins.

Impact of Proline-10 on Activity of Activated Protein C. The anticoagulant activity of activated protein C was tested in several assays. Figure 2 shows the impact on the APTT assay, conducted with limiting phospholipid. Under the conditions of this assay, coagulation times decreased in a nearly linear, inverse relationship with phospholipid concentration. Approximately 14 times as much wild type bovine APC was needed to equal the effect of bovine APC-H10.

Parts of the study in Figure 2 were repeated for membranes of PS/PC (25/75, LUV). Again, activity was limited by phospholipid, and its concentration was adjusted to give a control clotting time of 360 s (120 μg of 25% PS in the 0.4 mL assay). Approximately 15-fold more wild type enzyme was needed to equal the impact of the H10 mutant (data not shown). Finally, standard APTT reagent (Sigma Chemical Co., standard clotting time of 50 ± 2 s) was used. Approximately 10.0 ± 0.7 nM wild type enzyme was needed to double the coagulation time to 102 ± 5 s. The same impact was produced by 2.2 ± 0.1 nM bovine APC-H10. Phospholipid is not rate-limiting in the standard assay, so a smaller impact of membrane affinity may be expected.

Results for human proteins are shown in Figure 2B. About 2.5 times as much human APC containing proline-10 was

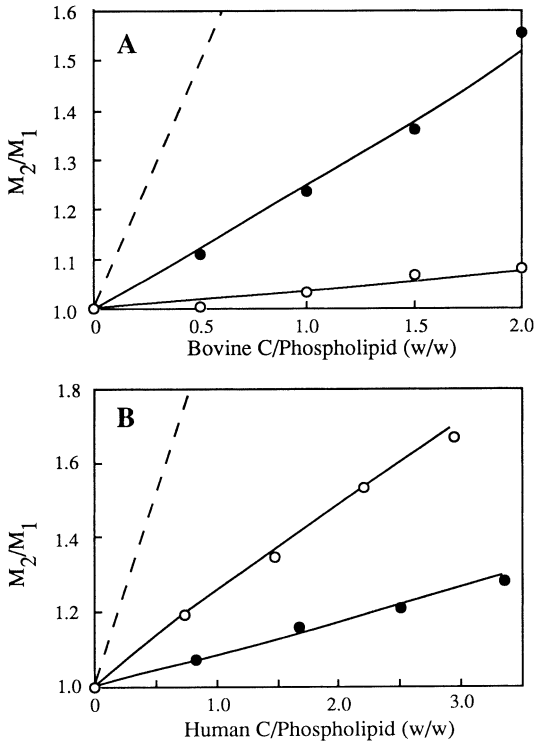

FIGURE 1: Membrane interaction by normal and modified proteins. (A) Wild type bovine protein C (○) and bovine protein C-H10 (●) interaction with vesicles (25 μg/mL PS/PC, 25/75) at 5 mM calcium (0.05 M Tris buffer/0.1 M NaCl at pH 7.5). Membrane interaction was determined by light scattering intensity, as described in Materials and Methods. M_2 is the molecular weight of the protein−membrane complex and M_1 that of the vesicles alone. When fit to eq 2, the data gave K_D values of 930 ± 80 nM for protein C-H10 and 9200 ± 950 nM for wild type protein C. (B) Wild type human protein C (○) and human protein C-P10 (●) interaction with membranes as described in panel A. When fit to eq 2, these data gave K_D values of 660 ± 90 nM for wild type human protein C and 3350 ± 110 nM for human protein C-P10. In both cases, the dashed line indicates the result if all of the added protein was bound to the membrane.

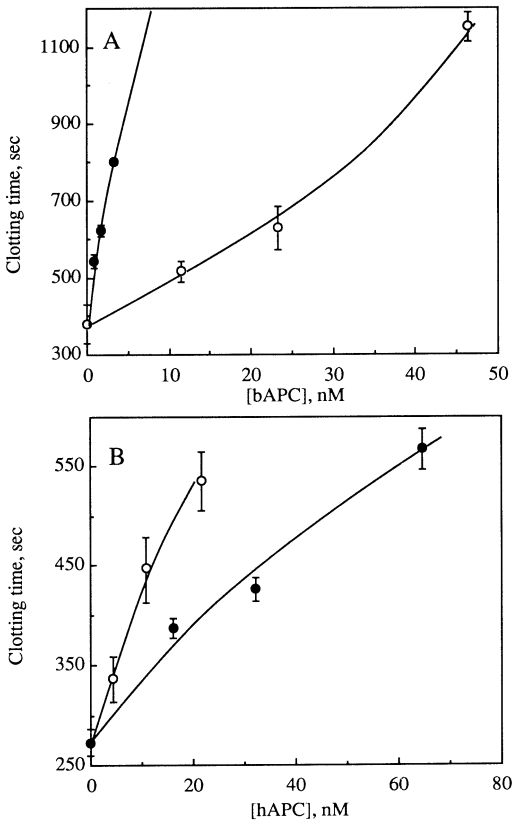

FIGURE 2: Influence of activated protein C on clotting times. (A) Bovine proteins. A modified coagulation test was used, which consisted of 0.1 mL of bovine plasma that was incubated with 0.1 mL of kaolin (5 mg/mL) or ellagic acid (0.1 mM) for 5 min at 35 °C. Coagulation was started by adding 0.1 mL of buffer containing phospholipid (45 μg of SUV, 10/90 PS/PC) and the amounts of APC shown, followed by 0.1 mL of 25 mM calcium chloride. All reagents were in standard buffer (0.05 M Tris buffer/0.1 M NaCl at pH 7.5). The average and standard deviation for three determinations are shown for wild type bovine APC (○) and for bAPC-H10 (●). An average of a 14-fold higher concentration of wild type bAPC was needed to duplicate the impact of the H10 mutant. Coagulation time at 10 nM bAPC-H10 was greater than 120 min. The standard clotting time for the plasma, using APTT reagent (Sigma Chemical Co.) and instructions provided by the manufacturer, was 50 s at 35 °C. (B) Human proteins were assayed with human plasma in the manner described for the bovine system in panel A. The average and standard deviation of three replicates for the wild type (○) and human APC-P10 (●) are shown. Standard APTT reagent gave a clotting time of 61 s (35 °C) with this plasma.

required to prolong coagulation to the extent of that of wild type APC. A lower impact of proline-10 introduction may reflect the smaller differences in membrane affinity of the human proteins (Figure 1B).

Inactivation of factor Va was also measured. In this case, bovine APC-H10 was 9.2-fold more active than the wild type (Figure 3A). As for membrane binding (above), the impact of proline-10 was less with the human proteins, with an average 2.4-fold difference between the curves drawn for the wild type and the P-10 mutant (Figure 3B). Similar results were obtained with normal human plasma (not shown).

DISCUSSION

A unique aspect of this study was the demonstration that amino acid substitution could improve the membrane contact site of bovine protein C. While introduction of proline into human protein C was interesting and suggested an impact of this residue on membrane contact, this modification showed loss of function by mutation, an outcome that has been observed in many other cases. Loss of function may arise in many ways, including incomplete protein processing. Proteins with improved function should not suffer from the latter problem, and the positive outcome is more easily interpreted. Improved protein function by site-directed mutation of the membrane contact site should be applicable to all of the vitamin K-dependent proteins, and this approach opens many new avenues for future study of blood clotting mechanisms and events.

Proline-10 was targeted as a residue responsible for lowered membrane affinity by a recently proposed membrane contact mechanism. The peptide backbone of residue 10 was suggested to participate in formation of a pore in the protein that may function to isolate a protein−phospholipid ion pair from the aqueous medium (*8*). Proline may reduce the efficacy of ion pair isolation. The structural change introduced by proline at position 10 appeared to be small. Molecular modeling of prothrombin, into which proline was introduced at position 11 (homologous to position 10 of protein C), showed that the α-carbon displacement of this residue was only about 0.1 Å compared to that of native

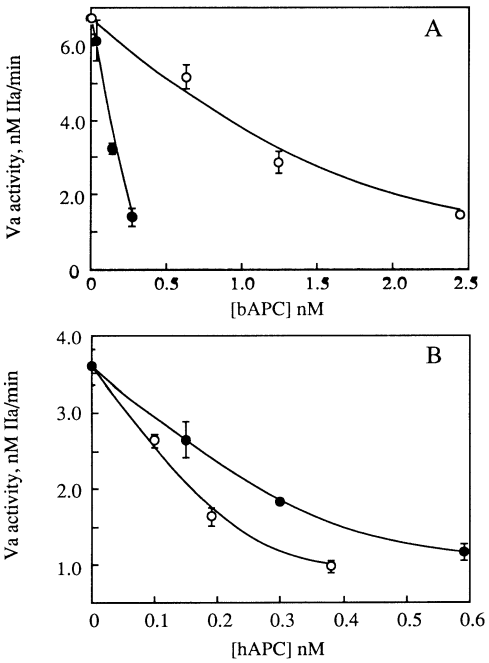

FIGURE 3: Inactivation of factor Va by bovine and human APC. (A) Studies with bovine proteins. Wild type bovine APC (○) and bovine APC-H10 (●) inactivation of factor Va. The thrombin was generated in a 1 min prothrombinase assay, after incubation of the diluted, Va-containing plasma with activated protein C, as described in Materials and Methods. The curves were offset by an average of 9.2-fold. (B) Inactivation of human factor Va by human APC. Wild type human APC (○) and human APC-H10 (●) inactivation of factor Va in protein S-deficient plasma were conducted as described for bovine proteins in panel A and Materials and Methods. The curves were offset by an average of 2.4-fold.

prothrombin.[2] In addition, the X-ray structures of GLA domains of bovine prothrombin, which contains lysine at the homologous position (*3*, *4*), and factor VII, which contains proline-10 (*6*), are very similar. This includes the placement of seven calcium ions, the nature of the hydrophobic cluster (Phe-4, Leu-5, and Leu-8 for factor VII versus Phe-5, Leu-6, and Val-9 for prothrombin), and a root mean square deviation of α-carbons 1−40 of only 0.64 Å (*6*). A 10-fold impact of proline-10 (1.4 kcal/mol free energy change) might be rationalized if the membrane contact site involved position 10 directly and involved a very sterically restricted interaction, similar to that of many substrate−enzyme binding sites. The calcium-lined pore and univalent ionic interaction appear to fit these criteria (*8*). Further studies are needed to fully describe the binding phenomenon.

Replacement of proline-10 of bovine protein C by histidine resulted in a membrane affinity nearly identical to that of human protein C (K_D = 930 versus 660 nM). This suggested that 12 additional amino acid differences in the 44 amino-terminal 44 residues (sequences given in Materials and Methods) had virtually no role in direct membrane contact. A number of the differences reside in residues 35−44. A low impact supported the suggestion that the membrane contact site is located within residues 1−37 (*26*) and that other parts of the GLA domain serve to stabilize protein structure and reduce the calcium concentration needed for proper protein folding.

As indicated above, natural selection of different membrane affinity may serve several purposes. In the case of proteins C, membrane affinity may underlie variations in cofactor involvement in bovine versus human species. The higher membrane affinity of wild type human APC allows greater activity in the absence of protein S, which appears to function primarily when factor Xa is present (*39*). Factor Xa protects factor Va from cleavage by APC. In the bovine system, the low membrane affinity of APC requires that protein S enhance APC association with the membrane under all conditions (*11*, *40*). Thus, improvements in the membrane contact site of APC by site-directed mutation may create enzymes that are independent of protein S and which may be highly potent, even in the presence of factor Xa. Such molecules should assist in studies of protein S function, and may constitute valuable proteins for regulation of coagulation, *in vivo*. Substitution of other amino acids in protein C are suggested to increase affinity by an additional 20-fold or more (*8*). It will be interesting to determine whether APC activity increases in a parallel fashion.

The general approach of improving the membrane contact site can be applied to virtually any of the vitamin K-dependent proteins, since most show less than the maximum affinity (*8*). Interesting applications may include the enzymes of procoagulation (factors VIIa, IXa, and Xa). Active enzymes with improved membrane contact should bind very tightly to their respective cofactor proteins (tissue factor, factor VIIIa, and factor Va), creating superior procoagulant proteins which may be used in treatment of coagulation deficiency states. Especially interesting may be factor VII, where administration constitutes a potential treatment for some forms of hemophilia (*41*). Improvement of the membrane contact site of factor VII may also increase activity with the soluble tissue factor (*42*), due to an improved membrane anchor.

Enhanced membrane binding could be combined with blocked active sites to produce proteins that displace the normal enzymes and create potent inhibitors of virtually any step of coagulation. Even APC, with an enhanced membrane site and modified active site, may displace factors Xa and IXa from their respective cofactors (factors VIIIa and Va). Active site-modified protein C functions as an inhibitor of the prothrombinase complex (*43*), suggesting the potential for success in this application. Thus, the new membrane binding mechanism proposed for vitamin K-dependent proteins (*8*) appears to offer many new approaches for studying coagulation reactions and may be pivotal in creating agents for treatment of coagulation pathologies.

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