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Proteolytic Formation and Properties of a Fragment of Protein C Containing the γ-Carboxyglutamic Acid Rich Domain and the EGF-like Region†

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ABSTRACT: The function of the epidermal growth factor (EGF) like domains in the vitamin K dependent plasma proteins is largely unknown. In order to elucidate the function of these domains in protein C, we have devised a method to isolate the EGF-like region from the light chain connected to the NH_2-terminal region, containing the γ-carboxyglutamic acid (Gla) residues. This was accomplished by tryptic cleavage of protein C that had been reversibly modified with citraconic anhydride to prevent cleavage at the lysine residue (in position 43) that is located between the two regions. The isolated fragment consists of residues 1-143 from the light chain of protein C connected by a disulfide bond to residues 108-131 from the heavy chain. Upon Ca^{2+} binding to the isolated Gla-EGF fragment from bovine protein C, the tryptophan fluorescence emission was quenched in a manner indicating binding to at least two classes of binding sites. These were presumably the Gla-independent Ca^{2+}-binding site located in the EGF-like region and the lower affinity sites in the Gla region. A comparison with the tryptophan fluorescence quenching that occurred upon Ca^{2+} binding to the separately isolated EGF-like and Gla regions suggested that the EGF-like region influenced the structure and Ca^{2+} binding of the Gla region. The isolated Gla-EGF fragment functioned as an inhibitor of the anticoagulant effect of activated protein C in a clotting assay, whereas no inhibition was observed with either the Gla region or the EGF-like region.

Both coagulant and anticoagulant systems are required to maintain normal hemostasis. The protein C system can be regarded as an anticoagulant counterpart to the blood clotting cascade, and it regulates the activation rates of factor X and prothrombin (Clouse & Comp, 1986; Stenflo, 1988; Esmon, 1989). The key protein of this system, protein C, is a vitamin K dependent serine protease zymogen that is activated by thrombin bound to the endothelial cell membrane protein thrombomodulin (Esmon et al., 1982). Activated protein C, in conjunction with its cofactor, protein S, rapidly inactivates factors Va and VIIIa by limited proteolysis (Walker et al., 1979; Vehar & Davie, 1980; Marlar et al., 1982; Suzuki et al., 1983). The role of protein C as an anticoagulant in vivo is shown by the fact that a deficiency of the protein leads to an increased risk of developing thrombosis (Griffin et al., 1981).

Protein C, like the other vitamin K dependent plasma proteins, is composed of domains with discrete structure and

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function (Foster et al., 1985; Plutsky et al., 1986). It consists of two polypeptide chains linked by a disulfide bond and has an NH_2-terminal γ-carboxyglutamic acid (Gla)[1] containing region in the light chain, which is followed by two domains that are homologous to domains in the epidermal growth factor (EGF) precursor. The NH_2-terminal EGF-like domain contains one *erythro*-β-hydroxyaspartic acid (Hya) residue (Drakenberg et al., 1983; McMullen et al., 1983). This modified amino acid is formed by hydroxylation of an aspartic acid residue by a 2-oxoglutarate-dependent dioxygenase (Stenflo et al., 1989; Derian et al., 1989). The heavy chain of protein C contains the serine protease part.

In order to be biologically active, protein C requires Ca^{2+} binding to the NH_2-terminal Gla-containing region (Stenflo & Suttie, 1977; Sugo et al., 1985). In addition, Ca^{2+} has to be bound to one Gla-independent site, located in the EGF-like region (Johnson et al., 1983; Öhlin & Stenflo, 1987; Öhlin et al., 1988a). This binding site must be saturated for rapid activation of protein C by the thrombin–thrombomodulin complex and for full amidolytic activity of the activated protein (Esmon et al., 1983; Hill et al., 1987). Site-directed mutagenesis of the Hya residue in the NH_2-terminal EGF-like domain suggests that the modified amino acid is involved in Gla-independent Ca^{2+} binding (Öhlin et al., 1988b). It thus appears that Ca^{2+} binding to this site induces a conformational change in the serine protease part of protein C. This notion also gains support from recent experiments in which a monoclonal antibody was found to recognize its epitope in the activation peptide region of the protein C heavy chain only in the presence of Ca^{2+} (Stearns et al., 1988).

The EGF-like domains of protein C and the related vitamin K dependent proteins presumably are involved in specific protein–protein interactions. However, the nature of these interactions is unclear (Öhlin et al., 1988b; Rees et al., 1988), although recent evidence suggests that the first EGF-like domain in factor IX binds to an endothelial cell receptor (Ryan et al., 1989). To elucidate the function of these domains, we have devised a method to isolate the two EGF-like domains with intact disulfide bonds and with the Gla domain attached, i.e., essentially the entire light chain of protein C (BpC-Gla-EGF). The method was designed to leave the Gla domain attached to the EGF-like region, which would ensure a high concentration of the fragment in the vicinity of phospholipid surfaces, where protein C exerts its function. The Ca^{2+} binding of the fragment was characterized by fluorescence emission measurements. We also report that the BpC-Gla-EGF fragment inhibits the anticoagulant effect of activated protein C in a clotting assay.

Experimental Procedures

Materials. Citraconic anhydride was obtained from Merck, and *threo*-β-hydroxyaspartic acid, diisopropyl phosphorofluoridate (DFP), and γ-carboxyglutamic acid were from Fluka. Q-Sepharose Fast Flow, cyanogen bromide activated Sepharose 4B, Sephadex G-75 SF, Sephacryl S-200, QAE-Sephadex A-50, SP-Sephadex, and molecular weight markers were from Pharmacia LKB Biotechnology Inc. and soy been trypsin inhibitor, leupeptine, α-chymotrypsin, and tosylphenylalanine chloromethyl ketone–trypsin from Sigma. The synthesis of *erythro*-β-hydroxyaspartic acid and β-carboxyglutamic acid has been described (Kornguth & Sallach, 1960; Fernlund, 1980). Chemicals used for amino acid analysis were obtained from Beckman, and those used for protein sequence determination were from Applied Biosystems.

Bovine plasma, depleted of protein S, was prepared from the same citrated plasma that was used for the APTT experiments (see below). Protein S was removed from the plasma with Sepharose 4B beads to which immunoaffinity-purified polyclonal antibodies against bovine protein S had been coupled according to the instructions of the manufacturer. Protein S could not be detected in this plasma by "rocket" immunoelectrophoresis (Laurell, 1966), indicating that it was below 5% of normal. The protein C cofactor activity of protein S in this depleted plasma, measured as prolongation of the clotting time in an APTT assay (see below), was completely restituted by the addition of purified bovine protein S.

Proteins. Bovine protein C(BpC) was purified as previously described (Stenflo, 1976; Sugo et al., 1984), with the addition of a final purification step consisting of gel chromatography on a column (1.6 × 95 cm) of Sephacryl S-200 equilibrated in 50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl. This step removes small amounts of contaminating proteins, mainly prothrombin fragment 1. Bovine protein C was activated for 4 h at 37 °C with 3% (w/w) thrombin. It was separated from thrombin by chromatography on an SP-Sephadex column (1.6 × 15 cm) equilibrated with 50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl and 2 mM EDTA. Activated protein C was collected in the break-through fraction whereas thrombin was retained on the column. Bovine thrombin was purified from prothrombin as described by Lundblad et al. (1975) following activation with Taipan snake venom (Owen & Jackson, 1973). Bovine prothrombin and protein S were purified as described previously (Stenflo & Jönsson, 1979).

The EGF homology region from bovine protein C (BpC-EGF) was isolated as previously described (Öhlin et al., 1988a). The Gla region (BpC-Gla) was obtained by α-chymotrypsin digestion of bovine protein C and subsequent chromatography on QAE-Sephadex A-50 (Esmon et al., 1983). An absorption coefficient, $A_{280}^{1\%}$, of 13.7 was used for both intact and activated protein C (Kisiel et al., 1976). For BpC-EGF the absorption coefficient was taken to be 5.8 and the molecular weight 22 000 (Öhlin et al., 1988a). The molecular weight used for protein S was 64 200, and the absorption coefficient used was 10.0 (DiScipio & Davie, 1979). The molecular weight of the isolated fragment from bovine protein C containing both the Gla and the EGF homology regions (BpC-Gla-EGF) was estimated to be 26 000 by SDS–polyacrylamide gel electrophoresis. Its absorption coefficient was determined to be 6.9 by measurements of the absorbance and subsequent amino acid analysis after acid hydrolysis of an aliquot of a protein solution that had been dialyzed against 0.1 M NH_4HCO_3.

Methods. SDS–polyacrylamide gel electrophoresis was run according to Blobel and Dobberstein (1975), but with the buffer system of Maizel (1971). Amino acid analysis was performed on acid or alkaline hydrolysates on a Beckman 6300 amino acid analyzer, as described previously (Öhlin et al., 1988a). Amino acid sequences were determined on an Applied Biosystems gas-phase sequencer using standard procedures.

The isolated BpC-EGF region was reduced and carboxymethylated as described previously (Stenflo, 1976) and then

[1] Abbreviations: Gla, γ-carboxyglutamic acid; Hya, β-hydroxyaspartic acid; EGF, epidermal growth factor; BpC, bovine protein C; BpC-Gla-EGF, isolated fragment from BpC containing residues 1–143 from the light chain and residues 108–131 from the heavy chain; BpC-Gla, isolated Gla region from BpC containing residues 1–41 from the light chain; BpC-EGF, isolated EGF-like region from BpC containing residues 42–143 from the light chain and residues 108–131 from the heavy chain; DFP, diisopropyl fluorophosphate; SDS, sodium dodecyl sulfate; HPLC, high-performance liquid chromatography; APTT, activated partial thromboplastin time.

directly subjected to HPLC on reversed-phase column (Aquapore Butyl BU-300, 2.1 × 30 mm, Brownlee Laboratories) equilibrated with 50 mM sodium phosphate, pH 6.8. An acetonitrile gradient, 0–10% in 5 min, 10–40% in 65 min, and 40–50% in 5 min in the same buffer, was used for elution at a flow rate of 0.1 mL/min. The absorbance of the column effluent was monitored at 215 nm.

Isolation of the Gla-EGF Region of Bovine Protein C. Bovine protein C (1.8–2.5 mg/mL, 12–22 mg) in 0.1 M $Na_2B_4O_7$, pH 8.2, was citraconylated by the addition of citraconic anhydride (40–75 μL, divided in at least three aliquots) at room temperature, with continuous stirring, the pH being maintained at 8.7 by the addition of 2 M NaOH (Dixon & Perham, 1968). After dialysis against 50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl and 5 mM EDTA, the citraconylated protein C was digested with trypsin (2% w/w) at 37 °C for 5 min. After the digestion was terminated by the addition of DFP to a final concentration of 10 mM, the sample was dialyzed against 50 mM Tris-HCl, pH 8.0, containing 1 mM EDTA. It was then chromatographed on a Q-Sepharose Fast Flow ion exchange column (1.6 × 16 cm) equilibrated with the same buffer. On top of this column was a layer (1.75 mL) of Sepharose 4B with immobilized soy bean trypsin inhibitor (1.5 mg/mL of gel) to remove any contaminating protease. The flow rate was 50 mL/h and 5-mL fractions were collected. The column was eluted stepwise with NaCl and $CaCl_2$ in the Tris buffer as indicated in the legend to Figure 2. The material in the peak eluted with 50 mM Tris-HCl, pH 8.0, containing 0.2 M NaCl and 20 mM $CaCl_2$ was pooled, and the lysine blocking groups were removed by lowering the pH to 3.0 by the addition of 10% HCOOH and incubating overnight at room temperature. The protein precipitated under these conditions but was readily dissolved after adjustment of the pH to 7.5 with 2 M NaOH. The sample was concentrated and subjected to chromatography on an Sephadex G-75 column (1.6 × 90 cm), equilibrated in 0.1 M NH_4HCO_3, to remove trace impurities (not shown). The flow rate was 8 mL/h, and 2-mL fractions were collected.

Fluorescence Measurements. Fluorescence was measured at 25 ± 0.2 °C in an SLM 4800 S spectrofluorometer (SLM-Aminco Instruments, Urbana, IL). All measurements were made in quartz cuvettes (1 × 1 cm) with sample volumes of 2.0 mL. Excitation was at 280 nm with a bandwidth of 2 nm. Photobleaching of the samples was minimized by shielding the protein solutions from light between measurements. Emission spectra were obtained with an emission bandwidth of 8 nm at protein concentrations of 1–10 μM in 50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl and either EDTA or Ca^{2+} as indicated. Titrations of the changes in fluorescence intensity induced by Ca^{2+} binding to BpC-Gla-EGF were monitored at an emission wavelength of 345 nm with a bandwidth of 16 nm. The protein was diluted to a concentration of 1 μM with 50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl, that had been made Ca^{2+} free by passage through a Chelex 100 column. The residual Ca^{2+} concentration in the protein solution was estimated to be below 1 μM, i.e., negligible in comparison with the observed dissociation constant of the Gla-independent Ca^{2+} binding site in protein C, for which values from 44 (Johnson et al., 1983) to 190 (Sugo et al., 1984) μM have been reported. The titrant (0.1 M $CaCl_2$) was added in portions ranging from 0.5 to 15 μL. The emission intensity was measured 2 min after each addition of titrant by averaging 30 signal readings of 0.25 s each. The data were corrected for dilution and were plotted as F/F_0, where F is the fluorescence intensity of the sample and F_0 the intensity of a control solution containing BpC-Gla-EGF at the same concentration but no Ca^{2+}.

Measurements of Anticoagulant Activity. Activated partial thromboplastin time (APTT, General Diagnostics) was used to measure the prolongation of the clotting time induced by activated protein C. Bovine citrated plasma was used as a test base. The test plasma (75 μL) was incubated at 37 °C with 75 μL of reconstituted APTT reagent for 4 min and 20 s. Then, 75 μL of 50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl and 1 mg/mL bovine serum albumin, or 75 μL of bovine activated protein C in the same buffer (final concentration 20–25 nM) was added, and the mixture was incubated for another 40 s. $CaCl_2$ (75 μL of 0.025 M solution) was finally added, and the time required for clot formation was recorded. Duplicate measurements were made. In competition experiments activated bovine protein C was preincubated at ambient temperature for 5 min with either BpC-Gla-EGF (0.02–1.7 μM final concentration), BpC-EGF (0.2–1.5 μM final concentration), or BpC-Gla (0.06–2.3 μM final concentration) before measurements of its anticoagulant activity.

Results

Isolation and Characterization of the Gla-EGF Region from Bovine Protein C. The two domains in protein C that are similar to domains in the EGF precursor contain a Gla-independent Ca^{2+} binding site (Öhlin & Stenflo, 1987; Öhlin et al., 1988a). The function of these domains in protein C was studied by isolating a fragment of the molecule that contains the EGF-like domains linked to the Gla region, thus ensuring phospholipid affinity. Tryptic cleavage of protein C was used to this end, but cleavage at the lysine residue in position 43 had to be precluded. Protein C was therefore modified with citraconic anhydride before digestion with trypsin. Tryptic cleavage of the modified protein resulted in the formation of a fragment, with an apparent M_r of approximately 28 000 on unreduced SDS–polyacrylamide gel electrophoresis (Figure 1), that was only slowly degraded further (time course of the digestion not shown). After inactivation of the trypsin with DFP, the solution was incubated at pH 3 overnight. This treatment quantitatively removed the lysine blocking groups as judged by the mobility of the fragment on SDS–polyacrylamide gel electrophoresis, in which the fragment appeared as a discrete narrow band both before and after incubation at the low pH (Figure 1). The fragment was isolated by chromatography on a Q-Sepharose column (Figure 2).

Sequence analysis of the isolated fragment gave two sequences in apparently equimolar amounts; one corresponded to residues 1–8 of the light chain of protein C, Ala-Asn-Ser-Phe-Leu-Xxx-Xxx-Leu, whereas the other one corresponded to residues 108–115 of the heavy chain, Leu-Ala-Lys-Pro-Ala-Thr-Leu-Ser (Fernlund & Stenflo, 1982; Stenflo & Fernlund, 1982). The Gla residues in positions 6 and 7 in the light chain were not seen due to poor extraction from the sequencer (Fernlund & Stenflo, 1979). The sequence analysis indicated that the isolated material was at least 95% homogeneous. There were no internal cleavages in the fragment. A heavy-chain fragment remained linked to the light chain through the cysteine residue in position 141 (Öhlin et al., 1988a). The amino acid composition of unreduced BpC-Gla-EGF was in good agreement with the sequence for residues 1–143 from the light chain together with residues 108–131 from the heavy chain (Table I). The isolated BpC-Gla-EGF was reduced and carboxymethylated, and the two chains were separated by HPLC (not shown). The material in the major peak had an amino acid composition that agreed with residues 1–143 of the bovine protein C light chain, whereas the amino

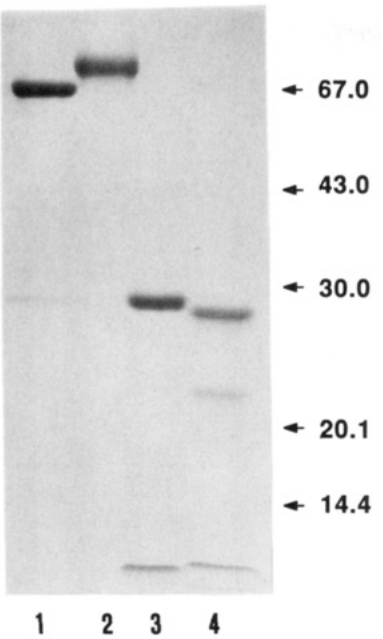

FIGURE 1: SDS–polyacrylamide gel electrophoresis of citraconylated bovine protein C before and after tryptic digestion. The lysine residues in bovine protein C were derivatized with citraconic anhydride. After dialysis against 50 mM Tris-HCl, pH 7.5, containing 0.1 M NaCl the protein was subjected to tryptic digestion (2% w/w) for 5 min at 37 °C. The reaction was terminated by the addition of DFP, and the lysine blocking groups were removed at low pH. (Lanes 1 and 2) Bovine protein C before and after citraconylation. (Lane 3) Tryptic digest before removal of the lysine blocking groups. (Lane 4) Same digest after removal of the lysine blocking groups. The migration of the molecular weight standards is indicated on the vertical axis. The samples were not reduced.

Table I: Amino Acid Composition of the BpC-Gla-EGF Region Isolated from a Tryptic Digest of Citraconylated Bovine Protein C

	intact fragment[a]		light-chain fragment, residues 1–143[b]		heavy-chain fragment, residues 108–131[b]	
residue	calcd[c]	found	calcd[c]	found	calcd[c]	found
CM-Cys	18	ND[d]	17	13.2	1	0.6
Asp	14	13.2	13	12.3	1	1.7
Thr	5	4.8	3	3.1	2	1.8
Ser	12	10.8	9	8.4	3	2.9
Glu	25	27.4	23	21.6	2	3.0
Pro	9	9.2	6	5.8	3	2.9
Gly	16	16.8	15	14.2	1	2.2
Ala	9	9.2	7	6.3	2	2.0
Val	6	6.3	5	5.0	1	1.7
Met	2	1.9	2	2.3	0	0
Ile	4	3.8	2	2.3	2	1.6
Leu	11	11.6	7	7.8	4	3.8
Tyr	3	3.5	3	2.6	0	0
Phe	8	8.4	8	7.6	0	0
His	4	4.2	4	3.5	0	0.1
Lys	4	4.4	3	2.9	1	1.0
Arg	13	14.0	12	12.0	1	2.0
Trp	2	ND[d]	2	ND[d]	0	ND[d]
Hya	1	1.1	1	1.0	0	0
Gla	11	12.7	11	6.5	0	0.3

[a] 24-h hydrolysis. Recalculated to 166 residues. [b] After reduction and carboxymethylation. [c] From Fernlund and Stenflo (1982) and Stenflo and Fernlund (1982). [d] Not determined.

acid composition of the peptide in the small peak was in reasonable agreement with residues 108–131 of the heavy chain (Table I, Figure 3). The amino acid composition indicated an overall recovery of the fragment between 15 and 25%.

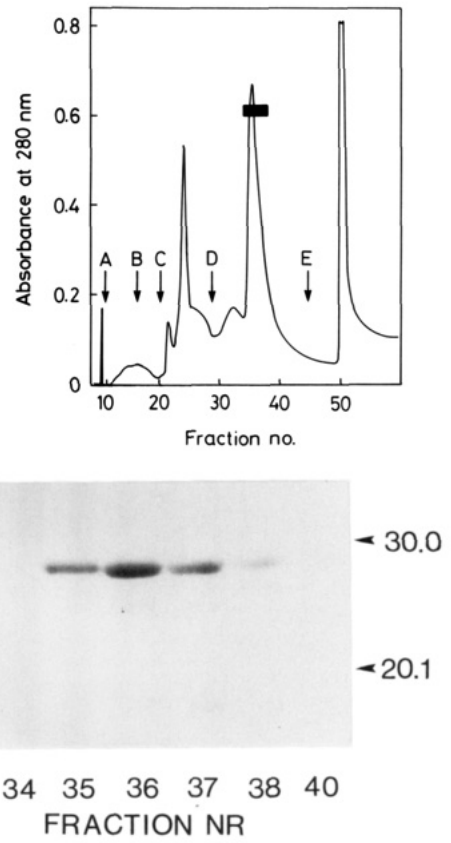

FIGURE 2: Isolation of the BpC-Gla-EGF fragment from a tryptic digest of citraconylated bovine protein C. (Top) After removal of the lysine blocking groups, the tryptic digest of protein C was chromatographed on a Q-Sepharose Fast Flow column equilibrated in 50 mM Tris-HCl, pH 8.0, containing 1 mM EDTA. Soy bean trypsin inhibitor (1.4 mg/mL of gel) coupled to cyanogen bromide activated Sepharose (1.75 mL) was layered on top of the column. Elution was accomplished with 50 mM Tris-HCl/1 mM EDTA, pH 8.0 (A) and the same Tris buffer containing 0.1 mM NaCl (B), 0.2 M NaCl (C), 20 mM $CaCl_2$ and 0.2 M NaCl (D), and 0.8 M NaCl (E). Fractions were pooled as indicated by the horizontal bar. (Bottom) SDS–polyacrylamide gel electrophoresis (10–15% polyacrylamide) of the effluent from the Q-Sepharose Fast Flow column. The migration of the molecular weight standards is indicated on the vertical axis. The samples were not reduced.

Ca^{2+}-Dependent Changes in Protein Fluorescence. It has been shown that Ca^{2+} binding to intact protein C results in a quenching of the intrinsic protein fluorescence (Johnson et al., 1983; Sugo et al., 1984). At least two classes of binding sites could be identified by fluorescence titrations, one that is half-saturated at approximately 40 μM Ca^{2+} and one that is half-saturated at >200 μM Ca^{2+}. Only the stronger of these classes of site(s) was observed in identical experiments with protein C from which the Gla region had been removed by limited chymotryptic digestion (Johnson et al., 1983; Sugo et al., 1984). We now examined the influence of Ca^{2+} binding on the intrinsic protein fluorescence of three fragments from the light chain of protein C, BpC-Gla-EGF, BpC-EGF, and BpC-Gla. In the absence of Ca^{2+}, the emission maxima were approximately 360, 345, and 340 nm for the BpC-Gla-EGF, BpC-EGF, and BpC-Gla fragments, respectively (Figure 4). At 1 μM protein concentration and 1.1–1.5 mM Ca^{2+} concentration, a Ca^{2+}-dependent decrease of the intrinsic protein fluorescence and about a 5-nm blue shift of the emission maximum were observed for BpC-Gla-EGF (Figure 4A). In contrast, Ca^{2+} did not affect the fluorescence of BpC-EGF (Figure 4B) or of BpC-Gla (not shown) at protein and Ca^{2+} concentrations comparable to those used for BpC-Gla-EGF,

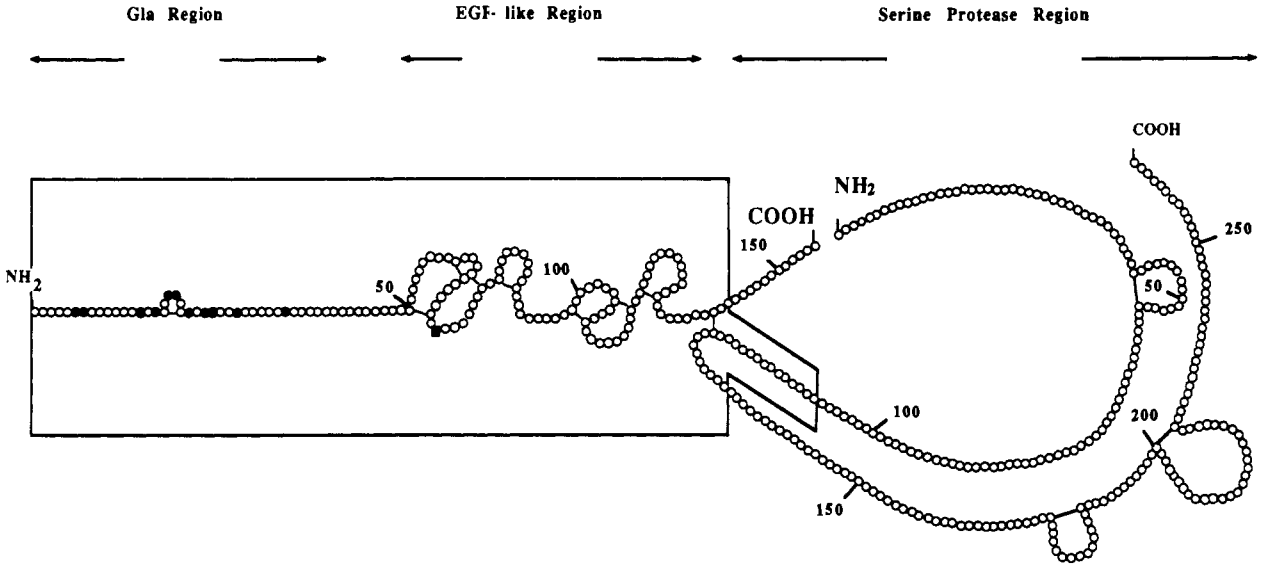

FIGURE 3: Schematic model of the bovine protein C molecule. The large *boxed* area denotes the BpC-Gla-EGF fragment of bovine protein C, isolated from a tryptic digest of the citraconylated intact protein. The filled circles denote γ-carboxyglutamic acid (Gla) residues, and the filled square denotes the β-hydroxyaspartic acid residue (Hya), position 71. The COOH termini of the light- and heavy-chain fragments are suggested from acid hydrolysis to be the first arginine residues after the interchain disulfide bridge that connects Cys-141 in the light chain with Cys-122 of the heavy chain.

despite each fragment containing one tryptophan residue, corresponding to residues 41 and 84, respectively, of intact protein C. Increasing the protein and Ca^{2+} concentrations to 5 μM and 5 mM, respectively, did not change the fluorescence intensity of BpC-EGF (not shown). However, a small Ca^{2+}-dependent fluorescence decrease and a blue shift of the emission maximum were observed for BpC-Gla (Figure 4C) at relatively high protein (10 μM) and Ca^{2+} (15 mM) concentrations. Titration of BpC-Gla-EGF with Ca^{2+} showed an initial small, but reproducible, increase in fluorescence intensity, with a maximum at approximately 0.1 mM Ca^{2+}, followed by a larger decrease in intensity at higher Ca^{2+} concentrations (Figure 5). Half-maximum change of the decrease in fluorescence intensity was observed at approximately 0.6 mM Ca^{2+}.

Inhibition of the Anticoagulant Activity of Activated Protein C. We have proposed that the calcium-stabilized conformation of the EGF-like region in the light chain of protein C is involved in the interaction between protein C and its cofactor, protein S (Öhlin et al., 1988b). The isolation of the BpC-Gla-EGF fragment enabled us to investigate whether the EGF-like region inhibits the anticoagulant effect of activated protein C. To this end, the effect of activated bovine protein C was measured in an APTT system with normal citrated bovine plasma as a test base. Activated protein C, at a final concentration of 20 nM, prolonged the clotting time from 40 to approximately 180 s (Figure 6). Addition of the BpC-Gla-EGF fragment reduced the prolongation of the clotting time. Half-maximal inhibition of the anticoagulant effect of activated protein C was reached approximately 0.4 μM BpC-Gla-EGF at 20 nM activated protein C, i.e., at a 20-fold molar ratio of the fragment to activated protein C. In contrast, addition of the BpC-EGF region, without the Gla domain, together with the activated protein C caused no inhibition in the prolongation of the clotting time even at a 100-fold molar excess of BpC-EGF. Similarly, the isolated Gla domain did not inhibit the anticoagulant activity of activated protein C.

We also made attempts to repeat the clotting experiments in bovine plasma that had been depleted of protein S to investigate whether the inhibitory effect of BpC-Gla-EGF was dependent on the presence of this protein. If so, this would argue in favor of an interaction between BpC-Gla-EGF and protein S. However, even more than a 20-fold increase in the concentration of activated protein C had no anticoagulant effect in the APTT system used when protein S depleted plasma was used as a the test base. In a control experiment, the protein C cofactor activity of protein S measured as prolongation of the clotting time in an APTT assay was completely restituted by the addition of purified bovine protein S. Accordingly, more refined experimental systems will have to be developed to elucidate these protein–protein interactions.

Discussion

Several proteins that contain EGF-like domains have recently been identified. In the low-density lipoprotein receptor, one of these domains has been shown to be required for normal recycling of the receptor (Davis et al., 1987). Moreover, in urokinase the EGF-like domain appears to be involved in binding to an endothelial cell receptor (Appella et al., 1987), and recent preliminary evidence suggests that also the first EGF-like domain in factor IX is involved in binding to such a receptor (Ryan et al., 1989). In the other proteins that contain EGF-like domains the function of these domains is largely unknown. To elucidate structure–function relationships in the EGF-like region of protein C, particularly with respect to calcium binding, we have previously devised methods to isolate these domains from tryptic digests of human and bovine protein C (Öhlin & Stenflo, 1987; Öhlin et al., 1988a). The two adjacent EGF-like domains from the light chain of bovine protein C, which are connected to a small peptide from the heavy chain by a disulfide bond, bind a single Ca^{2+} with a K_d of approximately 100 μM (Öhlin et al., 1988a). A monoclonal antibody against human protein C has been found to bind to an epitope in the EGF-like region in the presence of Ca^{2+}, but very poorly in the absence of the metal ion (Laurell et al., 1985; Öhlin & Stenflo, 1987). A titration of this interaction with Ca^{2+} indicated a K_d of 100–200 μM. Furthermore, we have expressed, in eukaryotic cells, wild-type recombinant human protein C and a protein C mutant in which the aspartic acid residue in position 71 in the NH_2-terminal EGF-like domain (which is hydroxylated in plasma protein C and in the recombinant wild-type protein C) had been changed to glutamic

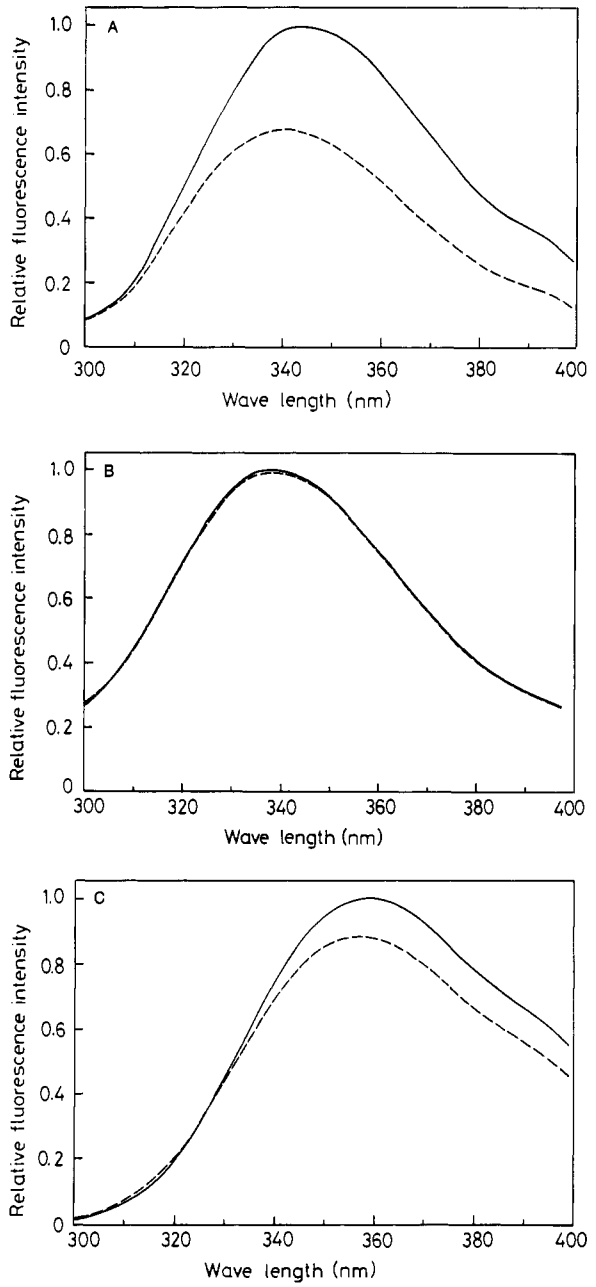

FIGURE 4: Ca^{2+}-induced changes of intrinsic protein fluorescence. Emission spectra of BpC-Gla-EGF (A), BpC-EGF (B), and BpC-Gla (C) were recorded at 25 °C in 50 mM Tris-HCl/0.1 M NaCl, pH 7.5, containing either EDTA (—) or the same EDTA concentration and Ca^{2+} (--). The excitation wavelength was 280 nm with a bandwidth of 2 nm. Protein concentrations were 1 (A and B) or 10 μM (C). The EDTA concentration was 0.1 (A and C) or 0.5 mM (B), and the total Ca^{2+} concentration was 1.1 (A), 1.5 (B), or 15 mM (C).

acid. The recombinant protein C had full biological activity, it contained Hya, and it had a Ca^{2+}-dependent epitope that was recognized by the monoclonal antibody. The mutant protein C had no Hya, it lacked the Ca^{2+}-dependent epitope, and its biological activity was reduced to 5–10% of normal. On the basis of these results, we have proposed that the Hya residue is directly involved in Gla-independent Ca^{2+} binding to protein C (Öhlin et al., 1988b).

Johnson et al. (1983) have found that the fluorescence change that results from the binding of a single Ca^{2+} ion to Gla-domain-less protein C is larger than the change associated with the binding of Ca^{2+} to the sites in the Gla domain. In intact and Gla-domain-less factor IX, the structural transition associated with Ca^{2+} binding to a Gla-independent high-affinity site causes a decrease in the intrinsic protein fluorescence (Morita et al., 1984). Finally, a Ca^{2+}-dependent quenching of the intrinsic protein fluorescence has been demonstrated for intact factor X, but was not observed for Gla-domain-less factor X (Skogen et al., 1983; Sugo et al., 1984). In this context it is interesting to note that Gla-domain-less factor X, in contrast to the Gla-domain-less forms of factor IX and protein C, does not have a Trp residue in the EGF-like region.

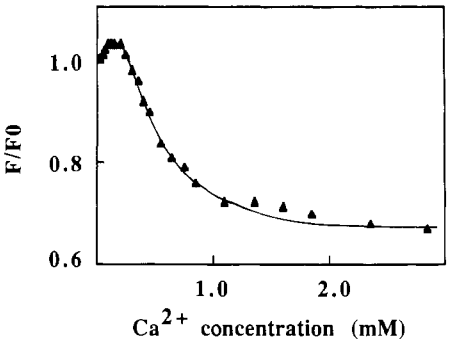

FIGURE 5: Ca^{2+} dependence of the intrinsic protein fluorescence of the isolated BpC-Gla-EGF fragment. BpC-Gla-EGF (1 μM) in 50 mM Tris-HCl/0.1 M NaCl, pH 7.5, that was made Ca^{2+} free by filtration through a Chelex 100 column, was titrated with Ca^{2+}, and the emission was monitored at 345 nm with a bandwidth of 16 nm. The excitation wavelength was 280 nm and the excitation bandwidth 2 nm. The data were corrected for dilution. They were plotted as F/F_0, where F is the fluorescence intensity of the sample and F_0 the intensity of a control solution containing BpC-Gla-EGF at the same concentration but no Ca^{2+}.

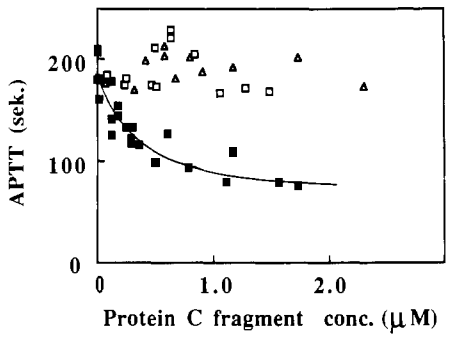

FIGURE 6: Inhibition of the anticoagulant effect of activated bovine protein C by isolated BpC-Gla-EGF. The activated partial thromboplastin time method (APTT) was used with citrated normal bovine plasma as the test base (clotting time 35–45 s). Activated bovine protein C (20–25 nM final concentration) was added to give a prolongation of the clotting time to approximately 200 s. The effect of the addition of increasing amounts of BpC-Gla-EGF (■), BpC-Gla (△), and BpC-EGF (□) on the prolongation of the clotting time caused by activated protein C was measured.

To elucidate whether the EGF-like region in protein C interacts with protein S and to characterize the Ca^{2+} binding to this region when it is linked to the Gla domain, we have devised a method to isolate the EGF-like region from bovine protein C connected to the Gla domain to ensure phospholipid affinity of the fragment. The isolated BpC-Gla-EGF had intact disulfide bonds, cysteine 141 in the light chain being connected by a disulfide bond to a small peptide from the heavy chain (Figure 3).

The BpC-Gla-EGF fragment has two Trp residues, in positions 41 and 84, whereas BpC-EGF only has the Trp residue in position 84 and BpC-Gla only the Trp residue in position 41. Titrations of BpC-Gla-EGF with Ca^{2+} showed a small increase in tryptophan fluorescence with a maximum at approximately 100 μM Ca^{2+}, while a larger decrease in fluorescence was observed at higher Ca^{2+} concentrations. The K_d for Ca^{2+} binding to the Gla-independent site in protein C

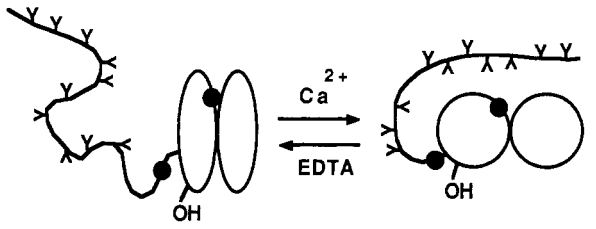

FIGURE 7: Schematic model of the putative conformational transition induced by Ca^{2+} binding to BpC-Gla-EGF. The model is discussed in the text. The filled circles denotes the two tryptophan residues present (Trp-41 and Trp-84), the Y denotes Gla residues, and –OH denotes the Hya residue.

has been reported to be between 44 and 190 μM (Johnson et al., 1983; Sugo et al., 1984). The relatively low Ca^{2+} concentration sufficient to give the initial, small increase in fluorescence emission of BpC-Gla-EGF suggests that this spectral perturbation reflects Ca^{2+} binding to the EGF-like region, resulting in a change of the environment of either Trp-41 or Trp-84. The larger decrease in fluorescence emission with a half-maximum at approximately 0.6 mM Ca^{2+} presumably arises from the weaker Ca^{2+} binding to the low-affinity sites in the Gla region (Amphlett et al., 1981), again affecting either of the two Trp residues.

Attempts to uniquely assign the fluorescence changes of the two titration steps to either Trp-41 or Trp-84 were fraught with difficulties. No Ca^{2+}-dependent change in fluorescence emission of BpC-EGF, containing only Trp-84, could be detected. Since high-affinity Ca^{2+} binding to BpC-EGF has been demonstrated with an ion-selective electrode (Öhlin et al., 1988a), the environment of Trp-84 in BpC-EGF thus appears to be unaffected by Ca^{2+} binding. This finding suggests that the small fluorescence change associated with the high-affinity Ca^{2+} binding to BpC-Gla-EGF reflects a change of the environment of Trp-41. However, it cannot be excluded that Trp-84 is responsible for the fluorescence change, if its environment in BpC-EGF has been altered by the removal of the Gla domain. No fluorescence quenching was observed on binding of Ca^{2+} to BpC-Gla, unless a high Ca^{2+} and protein concentration was used. This finding may be due to Trp-41 either not being involved in the quenching process at low Ca^{2+} and protein concentrations or having a different environment in BpC-Gla than in BpC-Gla-EGF (and in intact protein C). The latter alternative is supported by the observations made by Pollock et al. (1988) on the isolated Gla domain from prothrombin. This domain was found to bind fewer Ca^{2+} ions when free than when part of a larger protein fragment and was also shown to precipitate at high Ca^{2+} concentrations. Analogously, the structure and Ca^{2+}-binding properties of the isolated Gla domain from protein C may be entirely different from those of the Gla domain that is part of a larger molecule. This alternative also gains support from the finding that the fluorescence changes resulting from Ca^{2+} binding to the Gla domain in BpC-Gla-EGF occur at the same Ca^{2+} concentration in the fragment as in intact protein C. Our results are compatible with the schematic model shown in Figure 7, which emphasizes that the EGF-like region influences the Ca^{2+} binding and conformation of the Gla region.

The isolated BpC-Gla-EGF fragment was found to inhibit the anticoagulant effect of activated protein C in a clotting assay, although a fairly large molar ratio (approximately 15-fold) of BpC-Gla-EGF to activated protein C was needed to obtain half-maximal inhibition. In contrast, the BpC-Gla fragment and the BpC-EGF fragment did not inhibit the anticoagulant effect of activated protein C at all. The protein S concentration in the test plasma can be lowered to about 25% of normal before it becomes limiting (not shown). The amount of BpC-Gla-EGF required to inhibit the anticoagulant effect of activated protein C and the excess of protein S in the test plasma is compatible with the assumption that the two proteins interact directly, perhaps through the EGF-like domains. However, such direct interaction between protein S and activated protein C has not yet been demonstrated, possibly due to a low binding constant. Due to the numerous protein–protein interactions involved and the complexity of the system, other explanations thus are possible.

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Registry No. Gla, 53861-57-7; EGF, 62229-50-9; BPC, 60202-16-6; Ca, 7440-70-2.

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Systematics in the Interaction of Metal Ions with the Main-Chain Carbonyl Group in Protein Structures†

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ABSTRACT: An analysis of the geometry of metal binding by peptide carbonyl groups in proteins is presented. Such metal ions are predominantly calcium in known protein structures. Cations tend to be located in the peptide plane, near the C=O bond direction. This distribution differs from that observed for water molecules bound to carbonyl oxygens. Most metal ions are bound to carbonyl oxygens of peptides in turns or in regions with no regular secondary structure. More infrequent binding interactions occur at the C-terminal end of α-helices or at the edges and sides of β-sheets, where the geometrical preferences of the metal–carbonyl interaction may be satisfied. In many proteins carbonyl groups that are one, two, or three residues apart along the polypeptide chain bind to the same cation; these structures show a limited number of main-chain conformations around the metal center.

Metal ions perform a wide variety of physiological functions, such as structural stabilization, electron transfer, catalysis, transport, and storage (Williams, 1983). The main-chain carbonyl group is an important protein ligand, and the interaction of many model compounds with metal ions has been studied spectroscopically and crystallographically (Chakrabarti et al., 1981; Einspahr & Bugg, 1984). In this paper we extend the work of Einspahr and Bugg (1984) to study the geometry of such interactions in protein structures. The orientation of metal ions with respect to the peptide group, the secondary structure where such a group is located, and the folding of the peptide chain containing more than one carbonyl ligand group are also examined. Such an analysis will help to understand why a metal ion binds to a given site in a protein structure and how it functions.

Materials and Methods

The analysis is based on atomic coordinates from the Brookhaven Protein Data Bank (PDB) (Bernstein et al., 1977). Only the refined structures were included; as a result, taka-

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