Tryptophans 231 and 234 in Protein C Report the Ca^{2+}-Dependent Conformational Change Required for Activation by the Thrombin−Thrombomodulin Complex†

Alireza R. Rezaie*,‡ and Charles T. Esmon‡,§,||

Cardiovascular Biology Research Program, Oklahoma Medical Research Foundation, Departments of Pathology, Biochemistry, and Molecular Biology, University of Oklahoma Health Sciences Center, and Howard Hughes Medical Institute, Oklahoma City, Oklahoma 73104

Received March 14, 1995; Revised Manuscript Received July 18, 1995⊗

ABSTRACT: Human protein C circulates as both single- and two-chain zymogens. Activation by the physiological activation complex, thrombin−thrombomodulin, generates the anticoagulant enzyme, activated protein C. Ca^{2+} binding to the protease domain of protein C is accompanied by 5.5 ± 0.2% quenching of intrinsic fluorescence that correlates with the conformational change required for the rapid activation by the thrombin−thrombomodulin complex. To map which Trp residues report this Ca^{2+} binding, candidate Trp residues at positions 84, 115, 145, 205, 231, and 234 were changed individually to Phe within a protein C deletion mutant lacking the Gla domain (GDPC). Of these, the Trp to Phe mutation at position 231 (W231F) eliminated the Ca^{2+}-induced fluorescence quenching, and the Trp 234 to Phe mutation (W234F) increased the maximum quenching in protein C to 9.4 ± 0.4%. Upon Ca^{2+} binding, the fluorescence emission intensity of the W231F mutant was increased 3.4% ± 0.6%. The K_d for this site (84 ± 20 μM) was similar to that of GDPC (K_d = 39 ± 4 μM). To compare the properties of single- and two-chain protein C, we replaced the Lys156-Arg157 dipeptide cleavage site in protein C with Thr and Gln to form GDPCKR/TQ. GDPCKR/TQ and the two-chain form of protein C were activated at the same rate with the thrombin−thrombomodulin complex, they exhibited similar Ca^{2+} dependence for both activation and fluorescence quenching, and these enzymes had the same chromogenic activity. In contrast to the zymogen form, activated human Gla-domainless protein C did not undergo a Ca^{2+}-induced fluorescence change. These results indicate that the environment of Trp 231 and 234 within the Ca^{2+} binding loop of the protein C zymogen are perturbed by Ca^{2+} binding to the zymogen.

Protein C is a plasma glycoprotein zymogen of the anticoagulant serine protease, activated protein C. The zymogen circulates in plasma as single- and two-chain forms. Protein C contains several discrete domains: the vitamin K-dependent Gla domain, a helical stack of hydrophobic residues, two epidermal growth factor (EGF)-like domains, and a protease domain (Beckmann et al., 1985). Ca^{2+} is critical for protein C to function, and Ca^{2+} binding sites have been detected in the Gla, EGF, and protease domains. Ca^{2+} binds to multiple sites within the Gla domain that elicit conformational changes, which are required for membrane interaction (Zhang & Castellino, 1993). The helical domain appears to facilitate the formation of the appropriate Ca^{2+}-dependent conformation of the Gla domain (Colpitts & Castellino, 1994). The first EGF domain also binds Ca^{2+} (Öhlin et al., 1988). This interaction also seems to favor appropriate folding of the Ca^{2+} stabilized conformation of protein C (Öhlin et al., 1990). A Ca^{2+} binding site involving Glu 70 and 80 (chymotrypsin numbering system) is located in the protease domain (Rezaie et al. 1994). Although there are many Ca^{2+} interactions within protein C, only Ca^{2+} binding to the protease domain is critical for the Ca^{2+}-induced conformational change required for recognition by the thrombin−thrombomodulin (TM) activation complex. To simplify analysis of the metal-dependent changes in protein C, mutants have been constructed in which the Gla domain is deleted to form Gla-domainless protein C (GDPC). This deletion eliminates the Ca^{2+}-dependent fluorescence quenching that occurs when Ca^{2+} binds to the Gla domain (Johnson et al. 1983). With the deletion mutant, Ca^{2+} occupancy of the site within the protease domain correlates with changes in Trp intrinsic fluorescence. Therefore, if one were able to map critical Trp residue(s) within the deletion mutant, it would be possible to infer whether the Ca^{2+}-induced changes were local or global in nature.

Factor X and protein C both contain a single Ca^{2+} binding site in their protease domains (Rezaie & Esmon, 1994a; Rezaie et al. 1994). Studies with conformational-dependent antibodies have indicated that the protease domain of factor Xa also undergoes a conformational change upon binding Ca^{2+} (Persson et al., 1993). Unlike protein C, however, the

† The research discussed herein was supported by grants awarded by the National Heart, Lung, and Blood Institute of the National Institutes of Health (Grants R37 HL 30340 and R01 HL 29807 to C.T.E.). C.T.E. is an investigator of the Howard Hughes Medical Institute.

* Corresponding author telephone: (405) 271-7264; fax: (405) 271-3137.

‡ Oklahoma Medical Research Foundation.

§ University of Oklahoma Health Sciences Center.

|| Howard Hughes Medical Institute.

⊗ Abstract published in *Advance ACS Abstracts,* September 15, 1995.

[1] Abbreviations: Gla, 4-carboxyglutamic acid; GDPC, a deletion mutant of protein C in which the Gla domain corresponding to residues 1−45 has been deleted by the recombinant DNA methods; GDPCW321F and GDPCW234F, GDPC derivatives in which the tryptophan residue at the indicated position is replaced with phenylalanine; GDPCKR/TQ, a GDPC derivative in which the dibasic Lys-Arg at position 156−157 is replaced with Thr-Gln; TM, thrombomodulin; TM4−6, a truncated form of soluble thrombomodulin containing EGF domains 4−6; PCR, polymerase chain reaction; TBS, Tris-buffered saline.

0006-2960/95/0434-12221$09.00/0

intrinsic fluorescence of factor Xa is not altered by Ca^{2+} occupancy of the high-affinity site in the protease domain.

Early studies demonstrated that the first EGF domain of protein C and factor X could bind Ca^{2+} (Stenflo, 1991). A single Trp residue is located at position 84 within the first EGF domain of protein C. Factor X lacks any Trp residues in this EGF domain, and Phe is the corresponding residue at position 84 in factor X. This difference makes Trp84 a candidate for reporting the Ca^{2+}-dependent conformational changes in protein C. This possibility was made unlikely by the observation that protein C deletion mutant lacking the Gla and first EGF domains exhibited a Ca^{2+}-dependent fluorescence quenching comparable to forms containing both of the EGF domains (Rezaie et al. 1992). Another candidate Trp residue in protein C that is not conserved in factor X is Trp 205. This residue is conserved in factor IX and protein C but replaced with Tyr in factor X. Based on this and the observation that the intrinsic protein fluorescence of Gla-domainless bovine factor IX (Morita et al. 1984) and Gla-domainless bovine and human protein C (Stearns et al. 1988; Johnson et al. 1983; Sugo et al. 1984) but not Gla-domainless bovine factor X (Sugo et al. 1984) is quenched upon Ca^{2+} binding, Stenflo's group has proposed Trp 205 in protein C as a candidate for the reporting Trp (Persson et al. 1993). Other studies, based on the molecular modeling of the protease domain of protein C (Fisher et al. 1994) suggested Trp 231 and 234 as candidates for reporting Ca^{2+} binding to protein C. In this model, Trp 231 and 234 are located within the Ca^{2+} binding loop and are solvent exposed. These residues are not conserved in factor X.

Human protein C circulates in plasma as a mixture of ≈80% two-chain and 20% single-chain forms (Foster et al. 1990; Grinnell et al. 1991). The two-chain form arises from an endoproteolytic cleavage at the dibasic Lys-Arg residues that join the light and heavy chains in the precursor (Foster et al. 1990). Expression of recombinant human protein C in 293 cells results in ≈10−20% single-chain form (Foster et al. 1990). The ratio of single-chain protein C may increase depending on cell line, culture conditions, and mutagenesis (Foster et al. 1990; Rezaie & Esmon, 1994b). The properties of single-chain protein C with respect to activation and Ca^{2+} binding have not been carefully explored.

In this study, we have changed candidate Trp residues to Phe within human GDPC and examined the properties of the resultant mutants with respect to activation by thrombin, the thrombin−TM complex, and Ca^{2+}-dependent changes in intrinsic fluorescence intensity. To evaluate the properties of a single-chain protein C and assure that the varying ratios of single- to two-chain molecules in the expressed GDPC derivatives do not influence the functional assays, we replaced the KR dipeptide (Lys-Arg residues 156−157) with Thr-Gln in GDPC to prevent proteolytic processing to two-chain protein C. The results indicate that the activation, fluorescence properties, and amidolytic activity of activated single-chain GDPC are identical to those of wild-type GDPC.

MATERIALS AND METHODS

Mutagenesis, Expression, and Purification of Recombinant Proteins. Construction and expression of GDPC in the RSV-PL4 vector was described previously (Rezaie & Esmon, 1992). Trp to Phe or Lys-Arg to Thr-Gln substitutions in GDPC were performed by the polymerase chain reaction (PCR) using standard protocols (Higuchi et al. 1988). The sequence of all PCR primers used for mutagenesis in this study are shown in Table 1. Primers 1 and 2 are the outside sense and antisense primers used for the amplification of all DNA fragments. The inside mutagenic sense and antisense primers are as follows: 3 and 4 for W84F, 5 and 6 for W115F, 7 and 8 for W145F, 9 and 10 for W205F, 11 and 12 for W231F, 13 and 14 for W234F, and 15 and 16 for the KR→TQ mutation. *Bst*EII and *Apa*I are two unique restriction enzyme sites in human protein C cDNA that encompass all of these mutations. After PCR amplification with the appropriate mutant primers, the *Bst*EII and *Apa*I restriction fragment of the wild-type GDPC in the RSV-PL4 vector (Rezaie & Esmon, 1992) was replaced with the identical fragments obtained by the PCR amplification of protein C cDNA with appropriate mutagenic primers (Table 1) and their subsequent digestion with the *Bst*EII and *Apa*I restriction enzymes. After confirmation of the mutations by DNA sequencing (Sanger et al. 1977), all the expression vectors were transfected into human embryonic kidney 293 cells, and the mutant proteins were isolated from the cell culture supernatants by immunoaffinity chromatography as described previously (Rezaie & Esmon, 1992).

Table 1: Sequence of Oligonucleotide Primers Used for Mutagenesis[a]

no.	primer sequence
1	5′−ATGTGGCAGCTCACAAGCCT−3′
2	5′−CTAAGGTGCCCAGCTCTTCT−3′
3	5′−TGCCGCAGCGGCTTTGAGGGCCGCTTC−3′
4	5′−TAGAGGAGGTGGGCTTTCGGCGCTGTAGCTGT−3′
5	5′−ACAGCTACAGCGCCGAAAGCCCACCTCCTCTA−3′
6	5′−GAAGCGGCCCTCAAAGCCGCTGCGGCA−3′
7	5′−ATCCACCCCTCCTTTGTGCTGACAGCG−3′
8	5′−CGCTGTCAGCACAAAGGAGGGGTGGAT−3′
9	5′−CGGGGAGACAGCCCCTTTCAGGTGGTCCTGCTG−3′
10	5′−CAGCAGGACCACCTGAAAGGGGCTGTCTCCCCG−3′
11	5′−ATGACCTGCGGCGCTTTGAGAAGTGGGAGCTG−3′
12	5′−CAGCTCCCACTTCTCAAAGCGCCGCAGGTCAT−3′
13	5′−CGCTGGGAGAAGTTTGAGCTGGACCTGGACA−3′
14	5′−TGTCCAGGTCCAGCTCAAACTTCTCCCAGCG−3′
15	5′−CGCAGTCACCTGACACAAGACACAGAAGAC−3′
16	5′−GTCTTCTGTGTCTTGTGTCAGGTGACTGCG−3′

[a] The oligonucleotides 1 and 2 are outside sense and antisense primers used together with each pair of the inside mutagenic sense (odd numbers) and antisense (even numbers) to prepare protein C mutants used in this study (see text for more details).

Activation by Thrombin and the Thrombin−Thrombomodulin 4−6 Fragment (TM4−6) Complex. Previous studies have shown that the Ca^{2+} dependence of protein C activation by thrombin or the thrombin−TM complex correlates with occupancy of a Ca^{2+} binding site in protein C (Johnson et al. 1983) and that mutation of this site eliminates the Ca^{2+} requirement for protein C activation (Rezaie et al. 1994). The affinity of Ca^{2+} for GDPC and the GDPC mutants was, therefore, inferred from plots of the initial rates of protein C activation by thrombin or thrombin−TM4−6 complex as a function of Ca^{2+} concentration. With thrombin alone, GDPC and its derivatives (1 μM) were incubated at 37 °C for 8 min with thrombin (19 nM) in 0.1 M NaCl and 20 mM Tris-HCl, pH 7.5 (TBS), containing 0.1% gelatin and different concentrations of Ca^{2+}. In the presence of TM4−6 (200 nM), GDPC and all of the derivatives (1 μM) were incubated at 37 °C for 6 min with thrombin (1 nM) in the same buffer. All zymogens were dialyzed extensively in

TBS-containing Chelex beads (Bio-Rad) before activation. Thrombin present in the reaction mixtures was inhibited with antithrombin (final concentration of 100 μg/mL) before the amidolytic activity of the activated GDPC derivatives was determined. Amidolytic activity was measured with 200 μM Spectrozyme PCa (SPCa) (American Diagnostica, Greenwich, CT) as described (Rezaie & Esmon, 1992). The rates were monitored in TBS at 405 nm at room temperature in a V_{max} kinetic plate reader (Molecular Devices, Melano Park, CA). The concentration of activated GDPC and the mutant derivatives in the reaction mixtures were determined from standard curves generated at the time of the experiments that were prepared by total activation of each derivative with 5 nM thrombin complexed to 200 nM TM4−6 in TBS containing 0.1% gelatin and 5 mM Ca^{2+} for 90 min at 37 °C. The relative amidolytic activity of totally activated zymogens agreed within 5% for all derivatives. Steady-state SPCa hydrolysis by each activated GDPC derivative was monitored by incubating 2 nM each enzyme with different concentrations of chromogenic substrate (ranging from 20 to 2500 μM) at room temperatures in TBS buffer. The K_m and k_{cat} values were calculated by nonlinear regression analysis of data using the Michaelis−Menton equation.

Fluorescence Measurements. An SLM-8000 (SLM-Aminco Instrument, Inc., Urbana, IL) was used for protein fluorescence measurements at 22 °C. The excitation and emission wavelengths were 285 and 340 nm, respectively. The bandwidths were set at 4 nm for excitation and 8 nm for emission. Ca^{2+} titration was performed by adding 5 μL of Ca^{2+} solution into protein C samples. The protein samples were allowed to incubate for 5 min with Ca^{2+} ion. This time was sufficient to reach equilibrium. The concentration of each protein was 1 μM, and all samples were dialyzed extensively in TBS-containing Chelex beads (Bio-Rad). In all experiments, the background signal due to solvent was subtracted, and all data were corrected for dilution due to addition of titrant. F/F_o was calculated as the fluorescence intensity of the sample with ligand (F) divided by the intensity of control protein solution containing no Ca^{2+} (F_o). To prevent photodegradation, the slits were kept closed, and the samples were exposed to the exciting light only during measurements.

Electrophoresis. Sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) was performed on a 10% polyacrylamide gel as described by Laemmli (Laemmli, 1970) and stained with Coomassie Blue R-250.

Data Analysis. The affinity of Ca^{2+} for each derivative of protein C was analyzed by nonlinear regression analysis using the one-site ligand binding equation in Enzfitter program (Elsevier-Biosoft, London) with simple weighting. Data from three different experiments were combined, and the K_d values as well as the standard errors were calculated by the Enzfitter computer program.

RESULTS

Preparation and Characterization of Recombinant Proteins. Recombinant GDPC, all the Trp to Phe (W→F) and Lys156-Arg157 to Thr-Gln (KR→TQ) substitution mutants were expressed and isolated as described under Materials and Methods. SDS−PAGE analysis of GDPC and the W→F mutants (Figure 1) and GDPC and the KR→TQ mutant (Figure 2) indicate that all the purified recombinant proteins

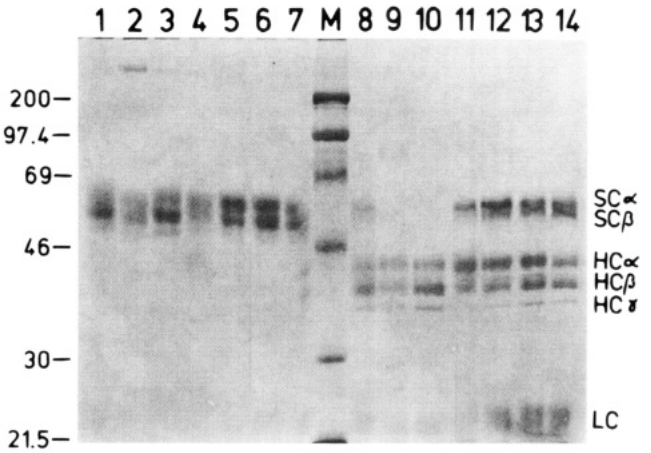

FIGURE 1: SDS−PAGE analysis of recombinant protein C mutants expressed in 293 cells. Under nonreducing conditions: lane 1, GDPC; lane 2, GDPCW84F; lane 3, GDPCW115F; lane 4, GDPCW143F; lane 5, GDPCW205F; lane 6, GDPCW231F; lane 7, GDPCW234F. Under reducing conditions: lane M, protein standards; lanes 8−14 GDPC and the WF mutant derivatives in the same order as in nonreducing conditions. SCα and SCβ are α and β single-chain protein C; HCα, HCβ and HCγ are heavy chain of two chain α, β, and γ protein C; LC, light chain protein C. The numbers at the left show the molecular mass in kDa of the protein standards.

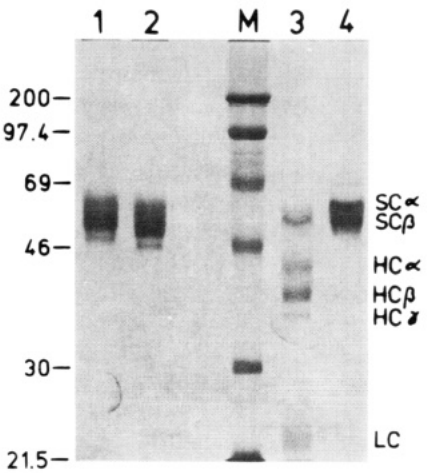

FIGURE 2: SDS−PAGE analysis of recombinant GDPC and the single-chain mutant expressed in 293 cells. Under nonreducing conditions: lane 1, GDPC; lane 2, GDPCKR/TQ. Under reducing conditions: lane M, protein standards; lane 3, GDPC; lane 4, GDPCKR/TQ. The symbols are the same as those in Figure 1.

are essentially pure. Under nonreducing conditions, all protein C derivatives have identical apparent molecular masses and exhibit three subforms, which correspond to α (≈55 kDa), β (≈52 kDa), and γ (≈50 kDa) protein C that are glycosylation variants observed previously with this protein (Miletich & Broze, 1990). As shown in Figure 1, under reducing conditions, in addition to the α, β, and γ subforms a light chain is also observed in all lanes, indicating that 293 cells process recombinant GDPC derivatives in a manner similar to wild-type protein C. However, under reducing conditions (Figure 1, lanes 8−14), variable amounts of GDPC derivatives remained as the single-chain form, indicating that the KR dipeptide is not fully processed in these proteins. These results are consistent with the previous reports that 293 cells secrete 10−20% of recombinant protein C as a single-chain molecule into the cell culture supernatants (Foster et al. 1990) and the ratio of single- to two-chain protein C may increase in some mutants of protein C (Rezaie

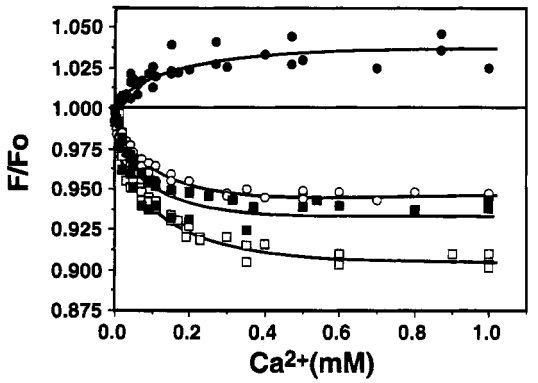

FIGURE 3: Influence of Ca^{2+} on the intrinsic fluorescence of recombinant GDPC, GDPCW231F, GDPCW234F, and GDPCKR/TQ mutant. GDPC (○), W231F (●), W234F (□), and KR/TQ (■) (1 μM each) were titrated with Ca^{2+}, and the intrinsic protein fluorescence was determined as described in Materials and Methods. Each curve is the best fit to the data from three independent measurements. F and F_o correspond to the emission intensity with and without Ca^{2+} present, respectively.

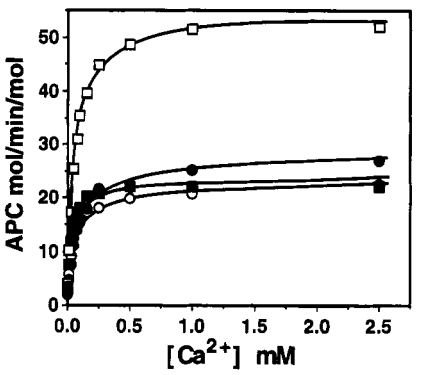

FIGURE 4: Influence of Ca^{2+} on the activation of recombinant GDPC, GDPCW231F, GDPCW234F, and GDPCKR/TQ mutant by the thrombin-TM4−6 complex. GDPC (○), W231F (●), W234F (□), and KR/TQ (■) (1 μM each) were incubated at 37 °C with 1 nM thrombin in complex with 200 nM TM4−6 in TBS buffer containing 0.1% gelatin at the Ca^{2+} concentrations indicated. The reaction was stopped by the addition of 100 μg/mL antithrombin. The reaction rates were linear for all protein C derivatives for the duration of experiments. Activation rates for GDPC, W231F, W234F, and KR/TQ were 22.48, 26.92, 51.93, and 22.02 mol of activated protein C (APC) min^{-1} (mol of activation complex)$^{-1}$ at 2.5 mM Ca^{2+}, respectively. Each curve is the representative of one of three independent measurements for each protein C derivative.

& Esmon, 1994b). When the KR dibasic peptide cleavage site is replaced by TQ, the processing enzyme(s) no longer cleave protein C at this site and a single-chain molecule is produced as demonstrated by lack of formation of heavy and light chains under reducing conditions (Figure 2). The removal of the KR dipeptide, however, does not influence the glycosylation of the single-chain protein C, since all of the subforms are observed (Figure 2, lanes 2 and 4).

Fluorescence Properties. We previously demonstrated that titration of native or recombinant GDPC with increasing Ca^{2+} concentrations results in a saturable 5 ± 1% decrease in the intrinsic protein fluorescence emission intensity with a K_d of ≈30 μM (Rezaie & Esmon, 1992). Similar to GDPC, the GDPC mutants of W84F, W115F, W145F, and W205F all underwent a saturable Ca^{2+}-dependent fluorescence quenching of ≈5% with a similar K_d (data not shown). These GDPC mutants were not further characterized. However, as shown in Figure 3, in contrast to wild-type GDPC, which underwent a 5.5 ± 0.2% (mean ± SD, n = 3) decrease in fluorescence emission upon Ca^{2+} titration, the emission intensity of the W231F mutant was increased 3.4 ± 0.6% (mean ± SD, n = 3) with a K_d similar to wild-type GDPC [39 ± 4 μM for GDPC as measured by a decrease and 84 ± 20 μM (mean ± SE) for W231F as measured by an increase in fluorescence intensity]. The W234F mutant exhibited a maximum Ca^{2+}-dependent decrease in intrinsic fluorescence of 9.4 ± 0.4% (mean ± SD, n = 3) with K_d = 70 ± 5 μM (mean ± SE) (Figure 3). The Ca^{2+}-dependent fluorescence quenching of the single-chain KR→TQ mutant was 6.7 ± 0.5% (mean ± SD, n = 3) with K_d = 34 ± 5 μM (mean ± SE), which is similar to that of wild-type GDPC (Figure 3). In all experiments, the fluorescence changes were completely reversed by the addition of 2 mM EDTA to samples containing 1 mM Ca^{2+}.

Since activated GDPC also binds Ca^{2+}, we next examined the changes in the fluorescence properties of activated GDPC and the activated KR→TQ mutant. Unlike bovine protein C, which undergoes Ca^{2+}-dependent fluorescence quenching in both zymogen and enzyme forms (Johnson et al. 1983), neither activated human GDPC nor the activated single-chain mutant exhibited any fluorescence change upon Ca^{2+} binding (data not shown).

Activation by the Thrombin−TM4−6 Complex. Ca^{2+} plays a critical role in protein C activation by the thrombin−TM complex. In the presence of TM or TM4−6 fragment, Ca^{2+} accelerates protein C activation by thrombin (Rezaie & Esmon, 1992). We previously demonstrated that the half-maximal rate of protein C activation by the thrombin−TM complex occurs at a Ca^{2+} concentration of approximately 50 μM (Johnson et al. 1983; Rezaie & Esmon, 1992). To assure that mutations did not adversely affect the conformation of the recombinant protein C molecules, we examined the initial rate of activation of the GDPC derivatives by thrombin and thrombin−TM complex as a function of Ca^{2+} concentration. The representative data for the initial rates of GDPC, GDPCW231F, GDPCW234F, and the single-chain KR→TQ mutant activation by thrombin−TM4−6 are shown in Figure 4. The W234F mutant was activated ≈2-fold faster than GDPC. GDPC, W231F, and the single-chain KR→TQ mutant were activated at similar rates at all Ca^{2+} concentrations. Kinetic analysis suggested that the 2-fold enhanced rate of activation of the W234F mutant is due to an increase in the V_{max} value (data not shown). The half-maximal rate of activation by the thrombin−TM4−6 complex occurred at a Ca^{2+} concentration of 38 ± 4 μM for GDPC, 82 ± 18 μM for GDPCW231F, 58 ± 6 μM for GDPCW234F and 22 ± 5 μM for the KR→TQ mutant (mean ± SE, n = 3). In the absence of TM, Ca^{2+} inhibits protein C activation by thrombin (Rezaie & Esmon, 1992). The half-maximal Ca^{2+} dependence of inhibition during activation by thrombin alone occurred at a Ca^{2+} concentration of 49 ± 3 μM for GDPC, 145 ± 11 μM for GDPCW231F, 141 ± 23 μM for GDPCW234F, and 37 ± 3 μM for the single-chain KR→TQ mutant (mean ± SE, n = 3). The representative Ca^{2+}-dependent inhibition data during activation by thrombin alone for one of the experiments is shown in Figure 5. Activated GDPC, GDPCW231F, GDPCW234F, and GDPCKR/TQ hydrolyzed the synthetic substrate SPCa at similar rates (Table 2).

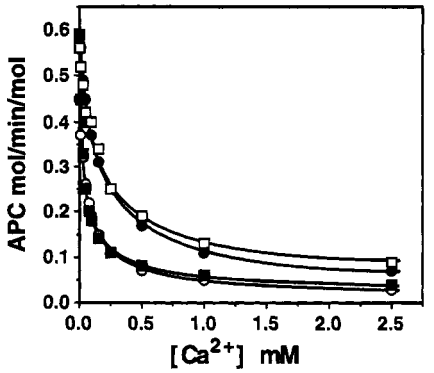

FIGURE 5: Inhibitory influence of Ca^{2+} on the activation of recombinant GDPC, GDPCW231F, GDPCW234F, and GDPCKR/TQ mutant by thrombin alone. GDPC (○), W231F (●), W234F (□), and KR/TQ (■) (1 μM each) were incubated at 37 °C with 18 nM thrombin in TBS buffer containing 0.1% gelatin at the Ca^{2+} concentrations indicated. The reactions were performed as described in the legend to Figure 4. Activation rates for GDPC, W231F, W234F, and KR/TQ were 0.03, 0.07, 0.09, and 0.04 mol of APC min^{-1} (mol of thrombin)$^{-1}$ and 0.47, 0.58, 0.59, and 0.60 mol of APC min^{-1} (mol of thrombin)$^{-1}$ at 2.5 mM and no Ca^{2+}, respectively. Each curve is the representative of one of three independent measurements for each protein C derivative.

Table 2: Steady-State Kinetics of Spectrozyme PCa Hydrolysis by Activated Recombinant Protein C Derivatives

activated protein C derivative	K_m (μM)	k_{cat} (s^{-1})
GDPC	162	10.4
W231F	171	11.1
W234F	160	10.3
KR/TQ	150	10.8

DISCUSSION

The present studies were all performed with Gla-domainless protein C. While this compromises to some extent extrapolation to structure function properties of the intact molecule, the approach facilitates interpretation of the specific domains and residues involved in Ca^{2+}-dependent interactions. Specifically, our previous results have indicated that the occupancy of a single high-affinity Ca^{2+} binding site in the protease domain is associated with the conformational change that is required for the thrombin−TM activation of protein C (Rezaie et al., 1992, 1994). Binding of Ca^{2+} to this high-affinity site in the protease domain is also accompanied by a 5 ± 1% quenching in intrinsic fluorescence of protein C (Rezaie & Esmon, 1992). With the Gla domain present, additional changes in intrinsic fluorescence result from Ca^{2+} binding to the Gla domain (Zhang & Castellino, 1993; Johnson et al., 1983). Since the Gla domain is not essential for recognition by the soluble thrombin−TM4−6 complex, using Gla-domainless derivatives facilitates analysis of the Trp residues that report the conformational changes associated with recognition by the activation complex.

The results of this study indicate that the fluorescence intensity of many of the candidate Trp residues in human protein C are not sensitive to Ca^{2+} binding. Only Trp to Phe mutations at residues 231 and 234 (76 and 79 in the chymotrypsin numbering system) influenced the Ca^{2+}-dependent fluorescence emission changes of GDPC. Both of these residues are located within the Glu 70−Glu 80 Ca^{2+} binding loop in the protease domain of protein C. The Trp 231 to Phe mutation resulted in 3.4 ± 0.6% increase in fluorescence intensity. Conversely, the Trp 234 to Phe mutation increased the maximum Ca^{2+}-dependent fluorescence quenching from 5.5 ± 0.2% to 9.4 ± 0.47%. With the W231F mutant, the Ca^{2+} concentration dependence as measured by the increase of the fluorescence emission intensity and the activation by the thrombin−TM4−6 complex correlated relatively well with those of the wild-type GDPC and W234F mutant as measured by the decrease in their fluorescence emission intensities and activation by the thrombin−TM4−6 complex. These results, together with the observation that the amidolytic activities of activated GDPC and the Trp to Phe substitution mutants toward chromogenic substrate SPCa were similar, indicate that the structure or the geometry of the Ca^{2+} binding site, the conformation of the activation peptide, and the conformation of the active site pocket in the GDPC mutants are not adversely affected by the mutations.

Our previous mutagenesis studies with protein C (GDPCE80K) indicated that the high-affinity Ca^{2+} binding site, critical for activation by the thrombin−TM complex, is located within the Glu 70 and Glu 80 loop of the protease domain (Rezaie et al. 1994). Several lines of evidence suggest that occupancy of this Ca^{2+} binding site in the protease domain induces a conformational change in the activation peptide of protein C that is required for rapid activation. One line of evidence in support of this concept is that a monoclonal antibody (HPC4), whose 12 residues epitope spans the scissile bond on protein C, recognizes only the Ca^{2+}-stabilized conformer of Gla-domainless protein C (Stearns et al. 1988). Second, Ca^{2+} binding to the high-affinity site in the protease domain inhibits protein C activation by thrombin, suggesting that Ca^{2+} changes the conformation of protein C near the scissile bond region (Esmon et al. 1983; Amphlett et al. 1981). In the presence of TM, however, thrombin undergoes a conformational change in the active-site pocket that recognizes the Ca^{2+}-induced conformer of protein C better (Esmon et al. 1983; Stearns et al. 1988).

Since the fluorescence intensity of the tryptophan residues 231 and 234 are sensitive to Ca^{2+} binding within the protease domain of the protein C zymogen but not the enzyme, it follows that the Ca^{2+}-dependent environmental or conformational changes near these residues must differ between enzyme and zymogen. Since Ca^{2+} binding to the protease domain of protein C changes the conformation of the activation peptide, it is possible that this conformational change moves the acidic residues in the activation peptide into close proximity with Trp 231, which could explain the fluorescence quenching. The concept that acidic residues in the activation peptide contribute to the fluorescence quenching is supported by the observation that mutation of Asp 167 and 172 near the scissile bond in protein C decreases the maximum Ca^{2+}-dependent fluorescence quenching 2-fold (Rezaie & Esmon, 1992). The ≈3% increase in the fluorescence intensity of the W231F mutant and ≈9% quenching of the W234F mutant suggest that the conformation and/or environment of both of these Trp residues are altered by Ca^{2+} binding to the protease domain. The net fluorescence quenching of ≈6%, which is similar to that of wild-type GDPC (≈6%), is consistent with this hypothesis. Interestingly, with the GDPCW234F mutant, the activation

by the thrombin−TM4−6 complex was 2-fold faster than that of GDPC. Kinetic analysis indicates that the increase in the rate of activation is due to an improvement in the k_{cat} of the thrombin−TM complex for the reaction, possibly suggesting that mutation of Trp 234 results in additional conformational change in the activation peptide. The altered conformation of the activation peptide in GDPCW234F probably approximates the transition state better than GDPC and improves the complimentarity to the thrombin−TM complex. All of these observations are consistent with the hypothesis that the conformation of the activation peptide and the Ca^{2+} loop within the protease domain of protein C are coupled.

In the protein C expression system, there is considerable variability in the efficiency with which cells carry out the processing of different mutants of protein C to the two-chain form. This has raised the question of whether the ratio of the single to two-chain protein C influences functional properties. The results of this study indicate that the single-chain protein C mutant has activation, Ca^{2+} binding, and amidolytic activity similar to the two-chain form, and hence, the presence of single-chain protein C is unlikely to influence functional studies.

ACKNOWLEDGMENT

We thank Gary Ferrell and Steven Carpenter for help with cell culture, Barbara Carpenter and Bronson Sievers for isolation of recombinant proteins used in this study, and Jeff Box, Karen Deatherage, and Julie Wiseman for assistance with preparation of the manuscript.

REFERENCES

Amphlett, G. W., Kisiel, W., & Castellino, F. J. (1981) *Biochemistry 20*, 2156.

Beckmann, R. J., Schmidt, R. J., Santerre, R. F., Plutzky, J., Crabtree, G. R., & Long, G. L. (1985) *Nucleic Acids Res. 13*, 5233.

Colpitts, T. L., & Castellino, F. J. (1994) *Biochemistry 33*, 3501.

Esmon, N. L., DeBault, L. E., & Esmon, C. T. (1983) *J. Biol. Chem. 258*, 5548.

Fisher, C. L., Greengard, J. S., & Griffin, J. H. (1994) *Protein Sci. 3*, 588.

Foster, D. C., Sprecher, C. A., Holly, R. D., Gambee, J. E., Walker, K. M., & Kumar, A. A. (1990) *Biochemistry 29*, 347.

Grinnell, B. W., Walls, J. D., Gerlitz, B., Berg, D. T., McClure, D. B., Ehrlich, H., Bang, N. U., & Yan, S. B. (1991) in *Protein C and related anticoagulants*, Advances in Applied Biotechnology Series, Vol. 11 (Bruley, D. F., & Drohan, W. N., Eds.) pp 29−63, Gulf Publishing Company, Houston.

Higuchi, R., Krummel, B., & Saiki, R. (1988) *Nucleic Acids Res. 16*, 7351.

Johnson, A. E., Esmon, N. L., Laue, T. M., & Esmon, C.T. (1983) *J. Biol. Chem. 258*, 5554.

Laemmli, U. K. (1970) *Nature 227*, 680.

Miletich, J. P., & Broze, J., Jr. (1990) *J. Biol. Chem. 265*, 11397.

Morita, T., Isaacs, B. S., Esmon, C. T., & Johnson, A. E. (1984) *J. Biol. Chem. 259*, 5698.

Öhlin, A-K., Linse, S., & Stenflo, J. (1988) *J. Biol. Chem. 263*, 7411.

Öhlin, A-K., Bjork, I., & Stenflo, J. (1990) *Biochemistry 29*, 644.

Persson, E., Hogg, P. J., & Stenflo, J. (1993) *J. Biol. Chem. 268*, 22531.

Rezaie, A. R., & Esmon, C. T. (1992) *J. Biol. Chem. 267*, 26104.

Rezaie, A. R., & Esmon, C. T. (1994a) *J. Biol. Chem. 269*, 21495.

Rezaie, A. R., & Esmon, C. T. (1994b) *Blood 83*, 2526.

Rezaie, A. R., Esmon, N. L., & Esmon, C. T. (1992) *J. Biol. Chem. 267*, 11701.

Rezaie, A. R., Mather, T., Sussman, F., & Esmon, C. T. (1994) *J. Biol. Chem. 269*, 3151.

Sanger, F., Micklen, S., & Coulson, A. R. (1977) *Proc. Natl. Acad. Sci. U.S.A. 74*, 5463.

Stearns, D. J., Kurosawa, S., Sims, P. J., Esmon, N. L., & Esmon, C. T. (1988) *J. Biol. Chem. 263*, 826.

Stenflo, J. (1991) *Blood 78*, 1637.

Sugo, T., Bjork, I., Holmgren, A., & Stenflo, J. (1984) *J. Biol. Chem. 259*, 5705.

Zhang, L., & Castellino, F. J. (1993) *J. Biol. Chem. 268*, 12040.

BI950576E