The Conformation of the Activation Peptide of Protein C Is Influenced by Ca\(^{2+}\) and Na\(^{+}\) Binding

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‡The abbreviations used are: TM, thrombomodulin; R169W, protein C mutant in which Arg\(^{169}\) at the P1 position (in the nomenclature of Ref. 32) has been replaced by Trp by the recombinant DNA methods; GD-PC, γ-carboxyglutamatic acid-domainless protein C from which residues 1–45 were removed by recombinant DNA methods; thrombin D189S, thrombin mutant in which Asp\(^{189}\), the intrinsic fluorescence of the mutant decreases ~30%, as opposed to only 5% for the wild-type, indicating that Trp\(^{169}\) is directly influenced by the divalent cation. The \(K_d\), affinity for Na\(^{+}\) binding increased ~5-fold in the presence of Ca\(^{2+}\). These findings suggest that Ca\(^{2+}\) changes the conformation of the activation peptide of protein C and that protein C is also capable of binding Na\(^{+}\), although with weaker affinity compared with the mature protease. The mutant protein C can no longer be activated by thrombin but remarkably it can be activated efficiently by chymotrypsin and by the thrombin mutant D189S. Activation of the mutant protein C by chymotrypsin proceeds at a rate comparable to the activation of wild-type protein C by the thrombin-thrombomodulin complex.

**Experimental Procedures**

Construction and Expression of Recombinant Proteins—Construction, expression, and purification of wild-type protein C in the γ-carboxyglutamatic acid-domainless form (GD-protein C) in the RSV-PL4 expression purification vector system has been described previously (18). The P1 Arg\(^{169}\) to Trp (R169W) mutant of protein C was constructed by PCR mutagenesis methods and expressed in the same vector system in HEK293 cells as described (18). Both wild-type and mutant protein C were purified by immunoaffinity chromatography using the Ca\(^{2+}\)-dependent monoclonal antibody, HPC4, linked to Affi-Gel 10 (Bio-Rad) as described (18). Following purification to homogeneity, both wild-type and mutant protein C were desalted by passing through a PD-10 gel filtration column (Amersham Biosciences) equilibrated with Chelex-treated (Bio-Rad) 5 mM Tris-HCl (pH 8.0) buffer containing 0.1% polyethylene glycol (PEG) 8000 and stored at ~80 °C. Following activation by chymotrypsin or thrombin D189S, the activated GD-PC mutant exhibited identical amidolytic activity toward Spectrozyme PCa suggesting that a Trp at the C-terminal end of the light chain does not alter the catalytic property of the mutant protein. The expression, purification...
tion, and characterization of the GD-PC mutant in which the Na+–
binding loop of the zymogen from residues Gly192 to Tyr195 was replaced with the corresponding sequence of trypsin (GD-PC trypsin loop) has been described previously (8). The expression, purification, and character-
ization of the Asp189 to Ser substitution mutant of thrombin (D189S) has been described previously (19). Recombinant human thrombin (20), thrombomodulin fragment 458 (TM456) (18), and antithrombin (21) were expressed in mammalian cells and purified to homogeneity as described in the cited methods. Bovine α-chymotrypsin and hirudin were purchased from Sigma, and Spectrozyme PCAs was purchased from American Diagnostica (Greenwich, CT).

Zymogen Activation—The initial rates of concentration dependence of wild-type and R169W GD-PC activation by thrombin or chymotrypsin were monitored in both the absence and presence of Na+ and Ca++ as described (22). The activation reactions were carried out in 20 mM Tris-HCl (pH 7.5), 0.1 mM NaCl (TBS) or 0.1 mM choline chloride containing 1 mg/ml bovine serum albumin and 0.1% PEG 8000. In both cases, the activation of wild-type GD-PC (0.3–10 μM) by thrombin (5–50 nM) and R169W (0.3–10 μM) by chymotrypsin (1 μM) was monitored in the absence or presence of 2.5 mM CaCl2 for 5–30 min at room temperature. After inhibition of thrombin activity by antithrombin, the initial rates of activations were measured from the rate of activated protein C generation in an amidolytic activity assay using 400 μM Spectrozyme PCAs in TBS as described (22). Under the experimental conditions used for the activation of GD-PC R169W, chymotrypsin did not exhibit a measurable amidolytic activity toward Spectrozyme PCAs. The rate of amidolytic activity was measured at 405 nm at room temperature in a Vmax, kinetic plate reader (Molecular Devices, Menlo Park, CA). The concentration of active pro-
tein C in each reaction was determined by reference to a standard curve, which was prepared by total activation of each protein C derivative at the time of experiments. This was accomplished by activation of 1–2.5 μM wild-type GD-PC with 10 nM wild-type thrombin in complex with 500 nM TM456 and R169W GD-PC with 100 nM thrombin D189S in complex with 500 nM TM456 in TBS containing 2.5 mM CaCl2 for 2–4 h at 37°C. Under these conditions both zymogens were completely activated to their enzymatic forms. The Km and kc,C values were calculated from the Michaelis-Menten equation. The initial rate of concentra-
tion dependence of GD-PC activation was also evaluated by the thrombin D189S mutant. In this case, GD-PC R169W (0.8–25 μM) was activated by the thrombin mutant (50 nM) in complex with TM456 (500 nM) in TBS containing 2.5 mM CaCl2. After 10–20 min of incubation at room temperature, the activity of thrombin D189S was inhibited by hirudin (200 nM) and the rate of activated protein C generation was measured by an amidolytic assay as described above. The TM456 dependence of GD-PC activation suggested that the TM456 concentration was saturating under these conditions. The initial rate of the activation of the protein C mutant by the thrombin mutant was also measured in the absence of TM in both the absence and presence of CaCl2. In this case, the activation conditions were the same as above except that the concentration dependence of the zymogen activation was monitored for 3 h at room temperature in TBS containing either 5 mM EDTA or 5 mM CaCl2.

Fluorescence Measurements—Direct binding of Na+ and Ca++ to protein C derivatives was evaluated from the changes in the intrinsic protein fluorescence associated with the binding of the cations to the zymogens. Equilibrium dissociation constants were determined by flu-
orescence titration with a Spex FluoroMax-3 spectrophotometer (Jobin-Yvon, Edison, NJ). The excitation and emission wavelengths were 295 and 345 nm, respectively. The titration experiments with monovalent cations were carried out in 50 mM Tris-HCl (pH 8.0), 1% PEG 8000 at an ionic strength of 800 mM at 25°C as described (19). Titrations were carried out by adding aliquots of wild-type or R169W GD-PC (1 μM) in solution containing [M] = 800 mM Cl−/salt to a solution containing the same concentration of each zymogen in 800 mM chloride. Monovel-
cation binding profiles were obtained in both the absence (1 mM EDTA) and presence of 2.5 mM CaCl2. In all titrations, the ionic strength of protein concentration and [Cl−] of the samples was held constant, whereas the monovalent cations [M−] were varied. The Ca++–binding titrations were carried out in 0.1 mM NaCl, 20 mM Tris-HCl, pH 7.5, at 25°C as described (15). The values of the intrinsic fluorescence (F) for the protein C derivatives as a function of each metal ion concentration [M] were fit according to the equation (19),

\[
F = \frac{F_c + F_i}{1 + \frac{[M]}{K_{c,M}}} (\text{Eq. 1})
\]

where Fc and Fi are the values of fluorescence in the absence and presence of saturating concentrations of the monovalent or divalent cations and Kc,M is the equilibrium dissociation constant for the metal ion binding.

RESULTS

Protein C Activation—GD-PC activation by thrombin in both the absence and presence of TM and Ca++ has been extensively studied in the past (13, 18). It is known that Ca++ in the presence of TM, accelerates protein C or GD-PC activation by thrombin but functions as a potent inhibitor of protein C activ-
ation in the absence of TM (1). Consistent with these findings, Ca++ inhibited GD-PC activation by thrombin in the absence of TM in the presence or absence of Na+ (Fig. 1). Individual Michaelis-Menten parameters could not be resolved in the presence of Ca++ up to 10 μM GD-PC. However, Km and kcat values of 10 ± 3 μM and 3.0 ± 0.4 min/mM for GD-PC activation by thrombin in the absence of Ca++ were obtained in NaCl- and choline chloride-containing buffers (Table I). GD-PC activation in the presence of a saturating concentration of TM456 showed similar catalytic rates in NaCl- and choline chloride-containing buffers, confirming that the activation of protein C by thrombin does not require Na+ (23, 24).

The R169W mutant of GD-PC was not activated by thrombin in either the absence or presence of Ca++ or TM but was activated by chymotrypsin with comparable rates in the absence or presence of Ca++ and/or Na+ (Fig. 2, Table I). Remarkably, the Km and kcat values for the activation of mutant GD-PC by chymotrypsin in the presence of Ca++ (5.3 μM and 13.1 min−1, Table I) were the same as those obtained for wild type GD-PC activation by the thrombin-TM complex in the presence of Ca++ (6.8 μM and 13.8 min−1 (22)). Both the Km and kcat values for the mutant GD-PC activation by chymotrypsin in the absence of Ca++ and Na+ were reduced ~3-fold (Table I). Be-
Metal Ion-induced Conformational Change in Protein C

The kinetic parameters were determined from the initial rate of concentration dependence of GD-PC (0.6–10 μM) by thrombin (10 nM) or chymotrypsin (1 nM) in Tris-HCl buffer containing either 0.2 M NaCl or choline chloride in the absence or presence of 1 mM Ca\(^{2+}\) as described under “Experimental Procedures.” All values are averages of 2–3 measurements ± S.E. Wt, wild type.

|                | Na\(^{+}\) |          |          |                  | Ch\(^{+}\) |          |          |
|----------------|-----------|----------|----------|------------------|-----------|----------|----------|
|                | \(K_m\)   | \(k_{cat}\) | \(k_{cat}/K_m\) | \(K_m\)       | \(k_{cat}\) | \(k_{cat}/K_m\) |
| Thrombin       |           |          |          |                  |           |          |          |
| Wt (−Ca\(^{2+}\)) | 10.0 ± 3.2 | 3.2 ± 0.2 | 0.32     | 9.8 ± 2.0       | 2.8 ± 0.4 | 0.29     |
| Wt (+Ca\(^{2+}\)) | ND\(^a\) | ND       | 0.010    | ND              | ND        | 0.0080   |
| Chymotrypsin   |           |          |          |                  |           |          |          |
| R169W (−Ca\(^{2+}\)) | 6.7 ± 1.0 | 9.0 ± 0.7 | 1.3      | 1.8 ± 0.1       | 3.6 ± 0.2 | 2.0      |
| R169W (+Ca\(^{2+}\)) | 5.3 ± 0.3 | 13.1 ± 0.4 | 2.5      | 3.1 ± 0.5       | 9.7 ± 0.9 | 3.1      |

\(^a\) The \(k_{cat}/K_m\) values are derived from slopes of linear kinetic data in Fig. 1, A and B.

\(^b\) ND, not determined; the activation reaction remained linear for up to 10 μM GD-PC.

Fig. 2. Concentration dependence of GD-PC R169W activation by chymotrypsin in the absence and presence of metal ions. Upper panel, the initial rate of activation of increasing concentrations of the GD-PC mutant by chymotrypsin (1 nM) was measured at room temperature in 20 mM Tris- HCl buffer containing 200 mM NaCl, 0.1% PEG 8000, and 1 mg/ml bovine serum albumin in the presence or absence of 1 mM Ca\(^{2+}\). Following 5 min activation, the rate of activated GD-PC generation was measured as described under “Experimental Procedures.” Lower panel, the same as above, except that instead of NaCl, the activation buffer contained 200 mM choline chloride. Solid lines in both panels are best fit of data to the Michaelis-Menten equation. APC, activated protein C.

cause chymotrypsin does not bind Ca\(^{2+}\) or Na\(^{+}\), the effects on the kinetic parameters must be due to cations binding to the substrate. The effect of Ca\(^{2+}\) on the mutant protein C activation by chymotrypsin was more pronounced in the absence of Na\(^{+}\), suggesting that the binding of Ca\(^{2+}\) and Na\(^{+}\) elicits distinct conformational changes around the P1 residue of the activation peptide that are detected by chymotrypsin.

The activation of the mutant GD-PC was also studied in the presence of the thrombin mutant D189S, where the residue in the primary specificity site was converted to that of chymotrypsin. Under saturating conditions of TM456, activation required Ca\(^{2+}\) with a half-maximal value of 90 ± 12 μM (Fig. 3A) as opposed to 15 μM seen for wild-type thrombin (25). This observation suggests that the R169W mutation has affected the ability of protein C to interact with Ca\(^{2+}\). The Michaelis-Menten curve for activation of the mutant GD-PC by the thrombin mutant D189S in the presence of TM456 also indicates a drastically increased \(K_m\) relative to wild-type (Fig. 3B). In the absence of TM, the thrombin mutant showed negligible catalytic activity toward the mutant GD-PC. The value of \(k_{cat}/K_m\) toward the mutant GD-PC was ~250-fold slower compared with wild type for the thrombin mutant and also ~310-fold slower compared with chymotrypsin. Hence, the thrombin mutant D189S in the presence of TM shows significant activity toward the mutant GD-PC, as opposed to the complete absence of activity of the wild-type, but it is not as active as chymotrypsin. This observation strongly suggests that thrombin, unlike chymotrypsin, has structural determinants that undermine its ability to interact efficiently with protein C and that part of this defect is the inability of protein C to penetrate the primary specificity pocket of thrombin. The defect is corrected by inter-
action with TM through a mechanism that remains to be identified.

**Fluorescence Measurements**—Binding of Ca$^{2+}$ to GD-PC caused an $-5\%$ decrease in intrinsic fluorescence, as previously reported (15), with a $K_D$ of 26.6 $\mu\text{M}$ (Fig. 4). On the other hand, binding of Ca$^{2+}$ to the mutant GD-PC decreased the intrinsic fluorescence $-30\%$, with a $K_D$ of 111 $\mu\text{M}$. The more pronounced change in intrinsic fluorescence can be assigned to the introduction of Trp$^{169}$ in the mutant protein C. An important conclusion from this observation is that Ca$^{2+}$ binding to GD-PC induces a conformational change in the activation peptide. The weaker Ca$^{2+}$ affinity of the mutant indicates that the P1 residue of the activation peptide is linked energetically to the Ca$^{2+}$ binding site in the zymogen. These results are in agreement with previous mutagenesis data where substitution of the P3, P2, or P3$'$ residues of the activation peptide also impaired the Ca$^{2+}$ affinity of the mutant proteins (18, 26). Interestingly, the conformation of the activation peptide was also influenced by Na$^+$ binding. Na$^+$ enhanced the intrinsic fluorescence of the mutant GD-PC $-9\%$ in the absence of Ca$^{2+}$, with a $K_D$ of $-285 \text{mM}$ (Fig. 5A). The Na$^+$ effect was abrogated by replacement of the 220-loop of protein C with that of trypsin, as seen for activated protein C (8). K$^+$ binding showed an even higher affinity ($K_D = 104 \text{nM}$) and a similar change in intrinsic fluorescence (Fig. 5A). On the other hand, Li$^+$ did not elicit any fluorescence change. The presence of Ca$^{2+}$ increased the affinity for Na$^+$ $-5$-fold and for K$^+$ $-2$-fold, and both monovalent cations caused a decrease in Ca$^{2+}$ fluorescence (Fig. 5B, Table II). These results suggest that the Ca$^{2+}$ and Na$^+$ binding sites are energetically linked, not only in activated protein C but also in the zymogen.

Similar to Ca$^{2+}$, the effect of the monovalent cations can be directly attributed to changes in the environment/conformation of the P1 Trp$^{169}$ in the activation peptide of the mutant protein because the metal ions exhibited opposite effects with the wild-type GD-PC, thus decreasing the emission intensity in the absence of Ca$^{2+}$ $10-35\%$ depending on the metal ion (Fig. 6A). In the presence of Ca$^{2+}$, the decrease in protein fluorescence of GD-PC varied from $-5$ to $25\%$ for the monovalent cations (Fig. 6B). The $K_D$ values for interaction with the metal ions under different conditions are presented in Table II.

**Discussion**

The results presented here shed light on how Ca$^{2+}$ enhances activation of protein C by thrombin in the presence of TM. Ca$^{2+}$ influences the conformation of the activation peptide of protein C and perhaps makes it more complementary to the active site of thrombin. There are several lines of evidence in support of this hypothesis: 1) the molecular modeling data based on the crystal structures of the protease domain of activated protein C and chymotrypsinogen have predicted electrostatic interactions between the charged residues of the activation peptide and those of the 70-loop (27, 28), 2) the mutagenesis of residues surrounding the scissile bond is associated with changes in the affinity of mutant zymogens for binding Ca$^{2+}$ (18, 26), and 3) it is known that the GD-PC zymogen but not activated GD-PC undergoes a $5 \pm 1\%$ quenching in the intrinsic protein fluorescence upon binding Ca$^{2+}$ (15). These results together with kinetic data have supported the hypothesis that the conformations of the activation peptide and the Ca$^{2+}$-binding 70-loop of protein C are allosterically linked. Nevertheless, direct evidence for this hypothesis was missing until now because the Trp residues responsible for reporting the intrinsic fluorescence of GD-PC upon binding Ca$^{2+}$ have been mapped to the two Trp residues 231 and 234 (76 and 79 in chymotrypsin numbering), which are both located in the Ca$^{2+}$-loop of the zymogen (15). Thus, the 5% fluorescence quenching of GD-PC upon interaction with Ca$^{2+}$ could not have been directly attributed to changes in the conformation of the activation peptide of the zymogen. The observation of this study that the binding of Ca$^{2+}$ is associated with a markedly higher quenching of the intrinsic fluorescence ($-30\%$) of the P1 R169W mutation directly establishes for the first time that the conformation of the activation peptide of protein C is altered upon interaction with Ca$^{2+}$. The observation that the affinity of the R169W mutant for binding Ca$^{2+}$ (111 $\mu\text{M}$) is impaired $-4$-fold shows that these two sites are energetically linked. This study also demonstrates that the Na$^+$ binding site of activated protein C is partially folded in the zymogen form and
The $K_d$ values were determined from changes in the emission intensity of intrinsic protein fluorescence using excitation and emission wavelengths of 295 and 345 nm. Titrations were carried out by adding aliquots of wild-type (WT) or R169W GD-PC (1 μM) in solution containing [M$^+$] = 800 mM Cl$^-$ salt to a solution containing the same concentration of eachzymogen in 800 mM choline chloride in the absence and presence of 2.5 mM CaCl$_2$, as described under “Experimental Procedures.” All values are derived from the fitting of data presented in Figs. 5 and 6 to Equation 1. All values are averages of 2–3 measurements ± S.E.

|       | Na$^+$  | +Ca$^{2+}$ | −Ca$^{2+}$ | +Li$^+$  | −Li$^{+}$ |
|-------|---------|------------|------------|---------|-----------|
| WT    | 660 ± 14| 111 ± 29   | 314 ± 13   | 30 ± 1  | 628 ± 27  |
| R169W | 285 ± 40| 59 ± 14    | 104 ± 6    | 60 ± 9  | ND*       |

* ND, less than 1% change in intrinsic protein fluorescence was observed in the absence of Ca$^{2+}$.

![Fig. 6. Monovalent cation binding to wild-type GD-PC in the absence and presence of Ca$^{2+}$. Monovalent cation binding curves for GD-PC were generated from changes in intrinsic fluorescence as a function of cation concentration [M$^+$]. Data are expressed relative to the value of fluorescence determined in the absence of M$^+$ for the sake of comparison. Solid lines were drawn using Equation 1 with best-fit parameter values $F_p = 0.9034 ± 0.004, F_a = 0.660 ± 0.002, K_{p} = 0.00628 ± 0.02661 × (Li^+), K_{a} = 1.002 ± 0.001, F_p = 0.6611 ± 0.0470, K_{p} = 0.660 ± 0.014 × (Na^+), F_a = 0.9983 ± 0.0024, F_a = 0.9030 ± 0.020, K_{a} = 0.314 ± 0.013 × (K^+), F_p = 0.9984 ± 0.0015, F_a = 0.993 ± 0.0037, K_{a} = 0.1108 ± 0.0293 × (Li^+), F_p = 0.9984 ± 0.0015, F_a = 0.993 ± 0.0037, K_{a} = 0.1108 ± 0.0293 × (Na^+), F_a = 0.1000 ± 0.001, F_a = 0.9109 ± 0.006, K_{a} = 0.030 ± 0.001 × (K^+), F_p = 0.9984 ± 0.0015, F_a = 0.993 ± 0.0037, K_{a} = 0.1108 ± 0.0293 × (Li^+).]

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**Table II**

$K_d$ (in mM) for interaction of wild-type and R169W GD-PC with monovalent cations

|       | −Ca$^{2+}$ | +Ca$^{2+}$ | −Ca$^{2+}$ | +Li$^+$  | −Li$^{+}$ |
|-------|------------|------------|------------|---------|-----------|
| WT    | 660 ± 14   | 111 ± 29   | 314 ± 13   | 30 ± 1  | 628 ± 27  |
| R169W | 285 ± 40   | 59 ± 14    | 104 ± 6    | 60 ± 9  | ND*       |

* ND, less than 1% change in intrinsic protein fluorescence was observed in the absence of Ca$^{2+}$.

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**Figure 6.** Monovalent cation binding to wild-type GD-PC in the absence and presence of Ca$^{2+}$. Monovalent cation binding curves for GD-PC were generated from changes in intrinsic fluorescence as a function of cation concentration [M$^+$]. Data are expressed relative to the value of fluorescence determined in the absence of M$^+$ for the sake of comparison. Solid lines were drawn using Equation 1 with best-fit parameter values $F_p = 0.9034 ± 0.004, F_a = 0.660 ± 0.002, K_{p} = 0.00628 ± 0.02661 × (Li^+), K_{a} = 1.002 ± 0.001, F_p = 0.6611 ± 0.0470, K_{p} = 0.660 ± 0.014 × (Na^+), F_a = 0.9983 ± 0.0024, F_a = 0.9030 ± 0.020, K_{a} = 0.314 ± 0.013 × (K^+), F_p = 0.9984 ± 0.0015, F_a = 0.993 ± 0.0037, K_{a} = 0.1108 ± 0.0293 × (Li^+), F_p = 0.9984 ± 0.0015, F_a = 0.993 ± 0.0037, K_{a} = 0.1108 ± 0.0293 × (Na^+), F_a = 0.1000 ± 0.001, F_a = 0.9109 ± 0.006, K_{a} = 0.030 ± 0.001 × (K^+), F_p = 0.9984 ± 0.0015, F_a = 0.993 ± 0.0037, K_{a} = 0.1108 ± 0.0293 × (Li^+).
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