Mutation of Glu-80 → Lys Results in a Protein C Mutant That No Longer Requires Ca\(^{2+}\) for Rapid Activation by the Thrombin-Thrombomodulin Complex*

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Binding Ca\(^{2+}\) to a high affinity site in protein C and Gla-domainless protein C (protein C lacking residues 1–44) results in a conformational change that is required for activation by the thrombin-thrombomodulin complex, the natural activator of protein C. Protein C modeling studies suggested the single high affinity Ca\(^{2+}\) binding site might be present in a loop in the protease domain and involve Glu-70 and -80 (chymotrypsin numbering system). This loop, which is a known Ca\(^{2+}\)-binding site in trypsin, is also conserved in other coagulation proteases, including factors VII, IX, and X. In thrombin, which does not bind Ca\(^{2+}\), Glu-70 is replaced by Lys, creating an internal salt bridge with Glu-80. We constructed and expressed a Gla-domainless protein C mutant in which Glu-80 is replaced with Lys. The activation of the resultant mutant is accelerated by thrombomodulin in a Ca\(^{2+}\)-independent fashion. Unlike wild type Gla-domainless protein C, Ca\(^{2+}\) no longer inhibits activation of the mutant by free thrombin, and Ca\(^{2+}\) stimulation of chromogenic activity is also absent. The characteristic Ca\(^{2+}\)-dependent quenching of Gla-domainless protein C intrinsic fluorescence is also absent in the mutant. We conclude that the high affinity Ca\(^{2+}\)-binding site in protein C critical for zymogen activation involves Glu-80. The Glu-80 to Lys mutation probably results in a salt bridge with Glu-70 that stabilizes protein C zymogen in a conformation similar, if not identical, to the Ca\(^{2+}\)-stabilized conformation favorable for rapid activation by the thrombin-thrombomodulin complex.

Activation of the vitamin K-dependent zymogen, protein C, is catalyzed by a complex between thrombin and thrombomodu-

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∥∥∥∥∥ The abbreviations used are: GDPC, Gla-domainless protein C; protein C from which the amino-terminal 44 residues were removed by deletion mutagenesis; GPC 280K, Gla-domainless protein C in which residue 80 in the chymotrypsin numbering system (residue 235 in the mature protein C sequence inferred from the cDNA sequence) is replaced with Lys by site-specific mutagenesis; TM, thrombomodulin; EGF, epidermal growth factor.
mutant protein referred to as GDPC E80K. Unlike GDPC, the GDPC E80K activation rate was essentially independent of Ca\textsuperscript{2+} with either the thrombin-thrombomodulin complex or free thrombin.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—Mutagenesis was performed using the polymerase chain reaction as described (13). Expression and purification of GDPC E80K and recombinant wild type GDPC were carried out as described in detail previously (15). The mutation sense primer was 5'-GGGAAAGTGGAACTGGACACGTG-3' and the antisense primer was 5'-CAGGTCAGGTCTTCACCTTCC-3'. The mutation was confirmed by DNA sequencing (16). The overall recovery of protein was approximately 1 mg from 20 liters of conditioned medium.

**Protein Purification**—Rabbit lung thrombomodulin (TM) (2), human TM composed of EGF domains 4–6 (15), and human thrombin (17) were purified by published methods.

**Amidolytic Activity Assay**—Amidolytic activity of activated GDPC E80K and Glu-domainless protein C was monitored by hydrolysis of the synthetic substrate SpecCa (American Diagnostica, Greenwich, CT) in 0.1 M NaCl, 20 mM Tris-HCl, pH 7.5, containing 0.1% gelatin. The rate of hydrolysis was monitored at 405 nm at room temperature in a Vmax kinetic plate reader (Molecular Devices, Menlo Park, CA). The concentration of activated GDPC or GDPC E80K present in reaction mixtures was determined by reference to standard curves that were prepared by total activation of 1 μM GDPC E80K or 1 μM GDPC with 2 nM thrombin complexed to 20 nM TM and 5 μM Ca\textsuperscript{2+} for 90 min at 37 °C at the time of experiments. Under this experimental condition, a maximum amidolytic activity is obtained in <30 min for both zymogen forms and the amidolytic activity is stable. The concentration of active enzymes was also determined by active site titration with p-nitrophenyl-p'-guanidinobenzoate as described by Chase and Shaw (18). The activity of totally purified protein was confirmed by DNA sequencing described in detail previously with recombinant and plasma-derived protein C (21, 22).

**Activation of Glu-domainless Protein C and GDPC E80K**—The amidolytic activity of fully activated GDPC and GDPC E80K agreed within 20%. The initial rates of activation by a complex of thrombin with TM 4–6 were analyzed as a function of increasing Ca\textsuperscript{2+} concentration. GDPC activation increased as a function of Ca\textsuperscript{2+} in a simple, saturable fashion (Kd = 76 ± 16 μM) (Fig. 2A). GDPC E80K activation was as fast as the wild type in saturating Ca\textsuperscript{2+} even when the mutant was activated in Chelex-treated buffer with or without 10 μM EDTA. Ca\textsuperscript{2+} did not increase the activation rate of GDPC E80K.

Calcium has an inhibitory effect on the activation of wild type protein C or GDPC by thrombin alone (23). To test whether Ca\textsuperscript{2+} has a similar inhibitory effect on the activation of GDPC E80K, the rate of activation of GDPC E80K by thrombin was studied as a function of increasing Ca\textsuperscript{2+} concentration (Fig. 2B). Unlike GDPC, which exhibited a Ca\textsuperscript{2+} concentration-dependent decrease in activation rate (Kd = 80 ± 4 μM), GDPC E80K activation with thrombin alone was essentially insensitive to Ca\textsuperscript{2+}.

**Ca\textsuperscript{2+} Binding to GDPC E80K**—**Analysis by Intrinsic Protein Fluorescence Changes**—Increasing Ca\textsuperscript{2+} concentration resulted in a saturable, 6 ± 1% decrease in the intrinsic fluorescence emission intensity of GDPC (Kd = 18 ± 3 μM). In contrast, GDPC E80K exhibited no change in fluorescence emission in up to 0.2 mM Ca\textsuperscript{2+} and an extremely small change at higher Ca\textsuperscript{2+} that was not reversible with EDTA (Fig. 3). Thus, the ion-dependent fluorescence change that correlates with protein C activation properties is eliminated in GDPC E80K.

**Influence of Ca\textsuperscript{2+} on the Activity of Activated GDPC and GDPC E80K**—Ca\textsuperscript{2+} results in a small (~20%) enhancement of activity of activated protein C or GDPC toward peptide chromogenic substrates (Kd = 49 ± 9 μM), which correlates with the occupancy of the high affinity site in zymogen GDPC (Kd = 76 ± 16 μM). This Ca\textsuperscript{2+}-dependent change in activated GDPC activity was not observed with activated GDPC E80K mutant (Fig. 4). Kinetic analysis indicated that the 20% increase in chromogenic activity toward SpecCa of activated GDPC in response to Ca\textsuperscript{2+} is due to a comparable change in Vmax (data not shown). After correcting for the 20% lower number of active sites in the activated mutant, kinetic analysis of activated GDPC E80K revealed that the mutant and wild type had similar specific activities in the absence of Ca\textsuperscript{2+}. Thus, the mutation does not appear to replace the Ca\textsuperscript{2+} influence on chromogenic

![Fig. 1. Comparisons of the Ca\textsuperscript{2+} binding site in trypsin (---) and the protein C model (-----) with a comparison of the equivalent site in thrombin (-----) with the Ca\textsuperscript{2+} site replaced by the Lys70→Glu80 salt bridge. The sequences from residues 68–84 are given below the stereo projection. HPC, human protein C; HTHR, human thrombin; BTRY, bovine trypsin.](image)
Ca²⁺-binding site and Ca²⁺-dependent activation properties

The reaction was stopped by the addition of a binding site in protein C. Binding to this site elicits a conformational change that appears to play a critical role in the activation process. Furthermore, the amidolytic activity of the mutant factor VIIa was undetectable in the absence of tissue factor and was significantly diminished (to 30%) when bound to tissue factor. Finally, unlike the protein C mutant described above, higher Ca²⁺ concentrations were required for the factor VII mutant than for wild type factor VII for optimal interaction with tissue factor. Thus, it was more difficult to determine the role of Ca²⁺ in the factor VII activation process. Furthermore, the amidolytic activity of the mutant factor VIIa was undetectable in the absence of tissue factor and was significantly diminished (to 30%) when bound to tissue factor. Finally, unlike the protein C mutant described here, higher Ca²⁺ concentrations were required for the factor VII mutant than for wild type factor VII for optimal interaction with tissue factor. Thus, the factor VII mutant does not totally alleviate membrane-independent Ca²⁺ requirements for the interaction with tissue factor. Studies by Stafford’s group (26) have indicated that the EGF repeats of factor VIIa are successful for interaction with tissue factor. In other vitamin K-dependent proteins, the first EGF domain binds Ca²⁺. Furthermore, the binding affinity of this EGF domain is influenced by the Glu domain (27, 28). Such domain-domain interactions may be impaired with the E80K mutation. If mutagenesis strategies similar to those used with protein C and factor VII are successful with factor X, these approaches may help us to determine the exact role of Ca²⁺ in the membrane-independent molecular interactions critical to the assembly of the coagulation complexes.

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REFERENCES
