Identification and Characterization of the Sodium-binding Site of Activated Protein C*

(Received for publication, September 2, 1998, and in revised form, October 29, 1998)

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Activated protein C (APC) requires both Ca$^{2+}$ and Na$^+$ for its optimal catalytic function. In contrast to the Ca$^{2+}$-binding sites, the Na$^+$-binding site(s) of APC has not been identified. Based on a recent study with thrombin, the 221–225 loop is predicted to be a potential Na$^+$-binding site in APC. The sequence of this loop is not conserved in trypsin. We engineered a Gla domainless form of protein C (GDPC) in which the 221–225 loop was replaced with the corresponding loop of trypsin. We found that activated GDPC (aGDPC) required Na$^+$ (or other alkali cations) for its amidolytic activity with dis-
the Ca\(^{2+}\) conformer possibly by Lys\(^{80}\) forming a salt bridge with Glu\(^{70}\) (6). In the current study, the catalytic activities of the activated wild type, tryp/loop, and E80K GDPC (aGDPC) derivatives were monitored by their ability to hydrolyze several chromogenic substrates in both the absence and presence of Na\(^{+}\) and Ca\(^{2+}\). We found that unlike the wild type protease, the aGDPC tryp/loop mutant lost its ability to bind Na\(^{+}\) and no longer discriminated between various monovalent cations. The affinity of the mutant protease for Ca\(^{2+}\) was also impaired ~16-fold. Interestingly, further study suggested that the affinity of the wild type aGDPC for Na\(^{+}\) in the presence Ca\(^{2+}\) was improved ~20-fold, and the binding of aGDPC E80K to Na\(^{+}\) was of the high affinity type independent of Ca\(^{2+}\). These results suggest that the 221–225 loop is a Na\(^{+}\)-binding site in APC, which is allosterically linked to the divergent catalytic-binding loop of the protease. The allosteric coupling of the two metal ion-binding loops in APC has likely played a role in the divergent evolution of this highly specific anticoagulant enzyme.

**EXPERIMENTAL PROCEDURES**

**Mutagenesis, Expression, and Purification.—**The expression of Gla domainless protein C (GDPC) and its derivatives by the RSV-FL4 expression/purification vector system in human 293 cells has been previously described (6, 20). The 221–225 loop (Gly\(^{221}\)-Tyr\(^{225}\)) of GDPC was replaced with the corresponding sequence of trypsin (GDPC tryp/loop) by the polymerase chain reaction mutagenesis approach, and the mutant molecule was expressed in the same vector system as described previously (20, 21). Accuracy of the mutations was confirmed by sequencing prior to expression. The wild type and mutantzymogens were purified from the cell culture supernatants as described previously (20).

Human plasma protein C (22), human plasma thrombin (23), human factor Va (24), bovine antithrombin (25), recombinant human prethrombin-1 (26), and recombinant human thrombomodulin fragment 4–6 (TM4–6, the minimal fragment of TM required as a cofactor for thrombin activation of protein C) (20) were prepared by the cited methods. The chromogenic substrate Spectrozyme PCAs (SpPCa) was purchased from American Diagnostics (Greenwich, CT), and S2266 and S2238 were purchased from Kabi Pharmacia/Chromogenix (Franklin, OH).

**Activation of GDPC and GDPC Trp/Loop by the Thrombin/TM4–6 Complex.—**The initial rate of protein C activation by the thrombin/TM4–6 complex (1 nM thrombin in complex with 100 nM TM) was measured as a function of different concentrations of GDPC or GDPC tryp/loop in 0.1 M NaCl, 0.02 M Tris-HCl, pH 7.5 (TBS), containing 0.1% polyethylene glycol 8000 (PEG 8000), and 2.5 mM Ca\(^{2+}\) at room temperature. After inhibition of thrombin activity by antithrombin, the initial rates of activation were measured from the rate of activated protein C generation in an amidolytic activity assay using 1 mM SpPCa in TBS buffer containing 0.1% PEG 8000 and 2.5 mM Ca\(^{2+}\). The rate of hydrolysis was measured at 405 nm at room temperature in a V\(_{\max}\) kinetic plate reader as described above. The apparent K\(_{\text{app}}\) and k\(_{\text{cat}}\) values for substrate hydrolysis were calculated from the Michaelis-Menten equation. The specificity constant for each chloride salt was expressed as the ratio of k\(_{\text{cat}}\)/K\(_{\text{app}}\). The specificity constant for the bulky monovalent cation choline (Ch\(^{+}\)) was used as a reference to determine the monovalent cation specificity as described by Dang and Di Cera (19). The concentration of S2266 ranged from 20 \mu M to 15 mM depending on the K\(_{\text{C}}\) values, and the concentration of enzymes ranged from 10 to 100 nM depending on the K\(_{\text{C}}\) values.

**Dissociation Constant (K\(_{\text{d(app)}}\) for Na\(^{+}\)—**The values for K\(_{\text{d(app)}}\) of Na\(^{+}\) binding to each protease was determined from the effect of varying concentrations of Na\(^{+}\) on the activity of the protease toward three synthetic substrates SpPCa, S2266, and S2238 in both the absence (Chelex-treated buffer) and presence of 2.5 mM Ca\(^{2+}\). In all experiments a constant ionic strength of 0.2 M was maintained by addition of choline chloride. This procedure has been commonly used in the past to study the effect of monovalent cations on the catalytic function of various enzymes (12, 19). The values for K\(_{\text{d(app)}}\) were calculated from the hyperbolic increase in the rate of substrate hydrolysis as a function of increasing Na\(^{+}\) concentrations.

**Factor Va Inactivation.—**The time course of human factor Va inactivation by activated GDPC was measured by a two-stage assay. In the first stage, factor Va (50 nM) was incubated with aGDPC (5 nM) at room temperature in 0.02 M Tris-HCl, pH 7.5, containing 0.15 M NaCl or 0.15 M KCl and 0.1% PEG 8000. This stage of the assay was carried out in both the absence and presence of 2.5 mM Ca\(^{2+}\). For this purpose, aGDPC and factor Va (in 5 mM Ca\(^{2+}\)) were passed through two separate PD-10 gel filtration columns equilibrated with Chelex-treated 0.02 M Tris-HCl, 0.1% PEG 8000 and used immediately in kinetic experiments. In the second stage, at different time intervals (0–12.5 min) the remaining activity of factor Va was determined by measuring its ability to accelerate factor Xa activation of recombinant human prethrombin-1 as described previously (23).

**Data Analysis—**The apparent K\(_{\text{C}}\) and K\(_{\text{D(app)}}\) values for substrate hydrolysis were calculated from the Michaelis-Menten equation, and the affinity of Na\(^{+}\) for each aGDPC derivative (K\(_{\text{D(app)}}\)) was determined by nonlinear regression fits of data to a rectangular hyperbola using EZFITTER (R. J. Leatherbarrow, Elsevier, Biosoft). All values are the average of at least 3–5 independent measurements ±S.D.

**RESULTS**

**Expression and Purification of Recombinant Proteins—**Recombinant wild type and mutant GDPC derivatives were expressed in 293 cells and isolated as described under “Experimental Procedures”. SDS-PAGE analysis (Fig. 1) indicated that both derivatives expressed as two subforms with identical apparent molecular weights that correspond to α and β protein C that are glycosylation variants observed previously with this protein (20). Under reducing conditions, a light chain was also observed with both derivatives. With both proteins a fraction of the protein samples remained non-reducible suggesting that
the GDPC derivatives were expressed as a mixture of single and two-chain proteins. This property has been also observed for both recombinant and plasma-derived human protein C in the past (29, 30). We have previously demonstrated that both single and double-chain APC derivatives have identical catalytic activities (31). These results suggest that the mutation does not alter the post-translational modifications or the processing of the protein. With the mutant GDPC, however, a minor band migrating at \(~35\) kDa was also observed (Fig. 1). The nature of this band was not characterized.

Comparison of the initial rate of protein C activation by the thrombin-TM4–6 complex as a function of different zymogen concentrations suggested that both GDPC derivatives were activated at a similar rate (Fig. 2). SDS-PAGE analysis of the activated products indicated that bothzymogens were completely converted to activated forms (data not shown). This was consistent with the observation that the concentrations of enzymes as determined by the active site titration were similar (within 80%) with the values calculated based on the absorbance at 280 nm.

To determine whether the mutant aGDPC can bind Na\(^+\), the effect of increasing concentration of Na\(^+\) on the activity of aGDPC derivatives toward the chromogenic substrates S2266, S2238, and SpPCa was studied. Since the catalytic domain of APC contains a Ca\(^{2+}\)-binding site (6), these studies were carried out both in the absence and presence of Ca\(^{2+}\). In the absence of Ca\(^{2+}\), the amidolytic activity of aGDPC was strongly dependent on the presence of Na\(^+\) in the reaction buffer (Fig. 3A). The amidolytic activity of aGDPC was enhanced with increasing concentrations of Na\(^+\) and reached saturation with a \(K_{d\text{app}}\) of 44.1 ± 8.6 mM. In a previous study, the interaction of Na\(^+\) with bovine aGDPC was shown to be cooperative with a Hill coefficient of 1.5 (17). A similar cooperativity for Na\(^+\) binding to human aGDPC was also observed in this study in the absence of Ca\(^{2+}\) (data not shown). However, when theionic strength of medium was adjusted to 0.2 M with choline chloride, no significant cooperativity was observed, and the nonlinear regression fits of data to a rectangular hyperbola was found to be suitable for obtaining the \(K_{d\text{app}}\) values (Fig. 3A). In the presence of Ca\(^{2+}\), no cooperativity for Na\(^+\) binding to aGDPC was observed irrespective of whether the ionic strength of medium was adjusted to 0.2 M with choline chloride. In this case an ~5–6-fold stimulation of the amidolytic activity of aGDPC was observed at saturating Na\(^+\) concentrations (Fig. 3A). Interestingly, the affinity of Na\(^+\) for the protease was also improved ~20-fold in the presence of Ca\(^{2+}\) \((K_{d\text{app}} = 2.3 ± 0.3\) mM\)), and at higher concentrations of Na\(^+\) (>60 mM) the activity of aGDPC was slightly diminished. A similar improvement in the affinity of Na\(^+\) for the protease in the presence of Ca\(^{2+}\) was observed at physiological temperature, although the \(K_{d\text{app}}\) values were slightly elevated \((14.0 ± 20.9\) and 5.3 ± 0.3 mM in the absence and presence of 2.5 mM Ca\(^{2+}\), respectively. To ensure that this effect of Ca\(^{2+}\) on the Na\(^+\) binding properties of APC was not a phenomenon related to the Gla domainless form of APC or an effect related to a particular substrate, the amidolytic activity of plasma-derived APC was monitored as a function of Na\(^+\) in both the absence and presence of Ca\(^{2+}\) with three different chromogenic substrates (S2266, S2238, and SpPCa).

In all cases, similar results were obtained (data not shown). Only the results with SpPCa hydrolysis for aGDPC at room temperature are presented in these figures. It is worth noting that in previous studies, the amidolytic
activity of bovine APC displayed a strict requirement for Na\(^+\) (16). In the current study, we noticed some base-line amidolytic activity for human aGDPC in the absence of Na\(^+\) and Ca\(^{2+}\) (Fig. 3A). However, we believe that our results are consistent with the literature since a similar base-line activity was also observed in previous studies (15, 16). It was previously suggested that the base-line amidolytic activity of APC in the absence of Na\(^+\) and Ca\(^{2+}\) may be due to the presence of other monovalent cations such as Tris\(^+\) and/or choline (Ch\(^+\)) in the reaction buffer (15, 16).

The Na\(^+\) concentration dependence of the amidolytic activity of aGDPC tryp/loop mutant was studied in a similar fashion. In the absence of Ca\(^{2+}\), no \(K_{d(app)}\) could be estimated for the Na\(^+\) interaction with the mutant since no saturation of Na\(^+\) binding to the mutant was observed up to 400 mM (data are presented for up to 200 mM NaCl in Fig. 3B). No attempt was made to increase the concentration of Na\(^+\) above 400 mM since the effect of high ionic strength on the structure of the enzyme is not known. However, in the presence of Ca\(^{2+}\), the amidolytic activity of the mutant was insensitive to the absence or presence of Na\(^+\) (Fig. 3B). These results clearly suggest that the mutant has lost its ability to bind Na\(^+\) and further suggest that Ca\(^{2+}\) binding to aGDPC has a profound effect on the ability of the protease to interact with Na\(^+\).

There are two known Ca\(^{2+}\)-binding sites on aGDPC that can influence the Na\(^+\) binding properties of the protease. The first Ca\(^{2+}\)-binding site resides in the epidermal growth factor like domain-1 of the light chain and the other was localized to the C-terminal catalytic domain of APC (6, 32). The Ca\(^{2+}\)-binding site in the catalytic domain is located on a loop between residues Glu\(^70\) and Glu\(^80\), analogous to the Ca\(^{2+}\) binding loop in trypsin (33). Previously, we prepared and characterized a GDPC derivative in which Glu\(^80\) was replaced with Lys (E80K) (6). In that study, we demonstrated that the catalytic domain of the mutant was stabilized in the Ca\(^{2+}\) conformer possibly as a result of Lys\(^80\) in the mutant forming a salt bridge with Glu\(^70\) (6). To characterize further the Na\(^+\) binding site of APC and determine the Ca\(^{2+}\)-binding site responsible for altering the Na\(^+\) binding properties of the protease, the amidolytic activity of aGDPC E80K toward SpPCa was studied as a function of different concentrations of Na\(^+\). Interestingly, the \(K_{d(app)}\) values for Na\(^+\) binding to this mutant was high affinity and insensitive to the absence or presence of Ca\(^{2+}\) (5.1 ± 0.7 mM in the absence of Ca\(^{2+}\), and 5.0 ± 0.6 mM in the presence of Ca\(^{2+}\)) (Fig. 4). The \(K_{d(app)}\) of Na\(^+\) for the mutant did not change even if the amidolytic activity of the mutant was monitored in the presence of 0.1 mM EDTA or EGTA to chelate divalent metal ions.

It is known that there is an ~20% Ca\(^{2+}\) stimulation of the amidolytic activity of aGDPC toward chromogenic substrates with a \(K_{d(app)}\) of ~50 \(\mu\)M (6). To determine whether mutagenesis of the 221–225 loop influences the affinity of the 70–80 loop for binding to Ca\(^{2+}\), the amidolytic activity of aGDPC tryp/loop was monitored as a function of increasing Ca\(^{2+}\) concentrations. Two interesting observations emerged. First, unlike the small effect of Ca\(^{2+}\) on the amidolytic activity of aGDPC, Ca\(^{2+}\) stimulated the amidolytic activity of the mutant toward the chromogenic substrates 8–10-fold (data not shown). Second, the \(K_{d(app)}\) for Ca\(^{2+}\) binding to the aGDPC tryp/loop mutant increased to ~800 \(\mu\)M, representing about 16-fold weaker interaction than that observed for Ca\(^{2+}\) binding to aGDPC. Taken together, these results suggest that occupancy of one metal-binding site of aGDPC with its specific metal cation influences the affinity of the other site for its specific metal ligand. We conclude that the 70–80 and 221–225 loops of aGDPC are allosterically linked.

It is known that other monovalent alkali cations can substitute for Na\(^+\) in stimulation of the amidolytic activity of bovine APC and that the activity increases in parallel with increasing cation radius (16, 17). To determine whether the mutant can discriminate among various alkali cations, the kinetic constants were determined for hydrolysis of SpPCa (200 \(\mu\)M) by aGDPC E80K (5 mM) in the absence or presence of 2.5 mM Ca\(^{2+}\) as measured under “Experimental Procedures.” Solid lines are nonlinear regression fits to a rectangular hyperbola. In all measurements a constant ionic strength of 0.2 \(\mu\) was maintained by addition of choline chloride.

**Fig. 4. Na\(^+\) dependence of the amidolytic activity of activated GDPC E80K in the absence or presence of Ca\(^{2+}\).** The initial rate of hydrolysis of SpPCa (200 \(\mu\)M) by aGDPC E80K (5 mM) in the absence or presence of 2.5 mM Ca\(^{2+}\) was measured as described under “Experimental Procedures.” Solid lines are nonlinear regression fits to a rectangular hyperbola.

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Na\(^{+}\) -binding Site of APC

**Fig. 5.** Comparison of the relative specificity of activated wild type and GDPC trypt/loop toward hydrolysis of the chromogenic substrate S2266. The kinetic constants, \(k_{\text{cat}}\) and \(K_{\text{m}}\), for the hydrolysis of S2266 in the presence of various monovalent cations (0.2 mM) in 5 mM Tris-HCl, pH 8.0, 2.5 mM Ca\(^{2+}\), and 0.1% PEG 8000 were calculated as described under “Experimental Procedures.” The concentration of enzymes ranged from 10 to 100 nM, and the concentration of substrate ranged from 20 nM to 150 nM. The specificity constant \(k_{\text{cat}}/K_{\text{m}}\) for each cation was calculated and expressed as relative specificity using the specificity constant for choline chloride as a reference as described by Dang and Di Cera (19).

**Fig. 6.** The time course of factor Va inactivation by activated GDPC in the presence of Na\(^{+}\) or K\(^{+}\). Human factor Va (50 nM) was incubated with activated GDPC (5 nM) in 20 mM Tris-HCl, pH 7.5, 0.1% PEG 8000 and either 150 mM Na\(^{+}\) (○), or 150 mM K\(^{+}\) (●) at room temperature. At the indicated time intervals the remaining factor Va activity was determined from the rate of thrombin generation from recombinant prethrombin-I as described under “Experimental Procedures.” The open symbols represent factor Va degradation in the absence of Ca\(^{2+}\), and the closed symbols represent factor Va degradation in the presence of 2.5 mM Ca\(^{2+}\). Factor Va degradation by aGDPC trypt/loop (▲) was carried out in the presence of Ca\(^{2+}\) and either Na\(^{+}\) or Ch\(^{+}\) under the same conditions mentioned above except that the concentration of the mutant enzyme was increased to 75 nM. Identical results were obtained with both monovalent cations.

**DISCUSSION**

The effect of Na\(^{+}\) and other monovalent cations on the catalytic activity of bovine APC has been studied in the past. It has been demonstrated that the binding of Na\(^{+}\) or another alkali metal to bovine APC is required for expression of the amidolytic activity of the protease toward tri-peptide chromogenic substrates (15, 17). In the current study, we have identified the 221–225 loop as the monovalent cation-binding site in human APC. Our strategy for the mutagenesis was based on 1) the observation that trypsin does not require Na\(^{+}\) for its catalytic activity, and 2) the report that the 221–225 loop is a Na\(^{+}\) -binding site in APC. We should also add that our qualitative results suggest that the Try\(^{225}\) → Pro mutant also lost its ability to bind Na\(^{+}\), in agreement with the proposal that Pro\(^{225}\) does not support Na\(^{+}\) binding to the 221–225 loop of serine proteases.

In the previous Na\(^{+}\) binding studies with APC, a \(K_{\text{app}}\) of 87–129 mM for Na\(^{+}\) binding to bovine APC or aGDPC was reported (15–17). In these studies, positive cooperativity (Hill coefficient = 1.5) for Na\(^{+}\) binding to bovine aGDPC was observed (17). In the current study, a similar result for the recombinant human aGDPC was observed only if choline chloride was not included in the reaction buffer to compensate for the ionic strength during Na\(^{+}\) titrations. However, minimal cooperativity for Na\(^{+}\) binding to aGDPC was observed when the total ionic strength in all measurements was adjusted to 0.2 M with choline chloride. Under these conditions, the data fit well to a hyperbolic binding equation, and a \(K_{\text{app}}\) of ~44 mM for Na\(^{+}\) binding to aGDPC was obtained. In addition to monovalent cations, previous studies by Hill and Castellino (11, 39–41) has established that divalent cations also stimulate the amidolytic activity of APC and aGDPC and that separate binding sites for these cations exist on each enzyme. Interestingly, when we included Ca\(^{2+}\) in the reaction buffer, the \(K_{\text{app}}\) for Na\(^{+}\) binding to aGDPC was improved ~20-fold suggesting that the Ca\(^{2+}\) -binding site of APC allosterically modulates the Na\(^{+}\) binding loop of the molecule. Further support for an allosteric link between the two metal-binding sites was provided by the observation that the affinity of the aGDPC trypt/loop mutant for Ca\(^{2+}\) increased ~16-fold. These results indicate that the conformation of the Na\(^{+}\) - and Ca\(^{2+}\) -binding sites are interdependent and that the two metal ions allosterically regulate the structure and function of this anticoagulant enzyme.

Previously, Hill and Castellino (11, 39, 40) reported that APC contains a single divalent cation-binding site outside of the Gla domain that is critical for the optimal expression of its amidolytic activity.
lytic activity. Recently, we demonstrated that this divalent cation-binding site is located in the catalytic domain of APC in a loop between the residues Glu70-Glu80, which is also known as the Ca$^{2+}$-binding loop in trypsin (6, 33). The binding of Ca$^{2+}$ to this site is essential for rapid activation of protein C by the thrombin-TM complex (6). In thrombin, which does not bind Ca$^{2+}$, Glu70 is not conserved, but it is replaced with Lys. In the crystal structure of thrombin, an internal salt bridge between Lys70 and Glu80 stabilizes this loop (42). Previously, we prepared and characterized a GDPC mutant in which Glu80 was replaced with Lys (E80K) so that the loop was stabilized in the Ca$^{2+}$ conformer, possibly by Lys in the mutant forming a salt bridge with Glu70 (6). Interestingly, the affinity of this mutant for Na$^{+}$ was of high affinity (5 mM) and was not sensitive to the absence or presence of Ca$^{2+}$ in the reaction buffer. Results obtained with this mutant support the hypothesis that the conformation of the monovalent and divalent cation-binding sites are allosterically linked. Furthermore, no cooperativity for Na$^{+}$ binding to this mutant was observed in either absence (10 μM EDTA) or presence of 2.5 mM Ca$^{2+}$. It is possible that the cooperativity observed for the Na$^{+}$ binding to aGDPC in the absence of Ca$^{2+}$ is due to binding of Na$^{+}$ to the 70–80 loop and partially substituting for Ca$^{2+}$. In support of this hypothesis, cooperativity is observed only for Na$^{+}$ (ionic radius 1.16 Å) which has similar ionic radius as Ca$^{2+}$ (1.14 Å), but not with any other alkali cation. Several previous studies by Di Cera and co-workers (12, 18) suggest that Na$^{+}$ binding to the 221–225 loop of thrombin modulates the activity and specificity of this enzyme in an allosteric fashion. These authors believe that the Na$^{+}$-bound form (fast form) has improved catalytic activity toward chromogenic substrates and specifically cleaves fibrinogen, whereas the Na$^{−}$-free form (slow form) specifically activates protein C (12). It is interesting to note that the catalytic activity of the loop mutant of aGDPC was impaired but that the activation of the zymogen form of the mutant by the thrombin-TM complex was not impaired and rather improved slightly. These results suggest that Na$^{+}$ binding to either thrombin or protein C is not required for recognition and rapid activation by the thrombin-TM complex. It is not known if APC, similar to thrombin, can exist in two distinct slow and fast forms in plasma. The concentration of Na$^{+}$ in blood is very close to the K$_{d_{app}}$ of this cation for thrombin, so that the two forms of thrombin may exist in equilibrium under physiological conditions (12, 14). In the case of APC, however, the K$_{d_{app}}$ for Na$^{+}$ is −25–30-fold lower than the concentration of this cation in blood, which suggests that APC may predominantly exist in the fast form under physiological conditions.

The other interesting observation of this study is that the catalytic activity of aGDPC in the presence of K$^{+}$ was 2–3-fold more efficient than Na$^{+}$. This was true whether the activity was monitored by hydrolysis of chromogenic substrates or by the inactivation of the natural substrate factor Va. Both thrombin and factor Xa, unlike APC, function more efficiently in the presence of Na$^{+}$. The molecular basis for the improved catalytic property of APC in the presence of K$^{+}$ was not understood. Since K$^{+}$ is primarily an intracellular metal ion, it is not known if it can play a role in up-regulation of the catalytic activity of APC in plasma. However, it is worth noting that similar to Na$^{+}$, the affinity of K$^{+}$ for binding to aGDPC was improved dramatically in the presence of Ca$^{2+}$. The local concentration of K$^{+}$ at the site of clot formation and/or inflammation due to K$^{+}$ release from aggregated platelets and/or lysed cells may increase to a level that could specifically activate the APC anticoagulant pathway. Further study will be required to determine whether there is validity for this hypothesis.

Finally, the Ca$^{2+}$ binding loop on the N-terminal β-barrel and the Na$^{+}$ binding loop on the C-terminal β-barrel of APC are located 15–20 Å away from the catalytic triad and −30 Å apart from each other (Fig. 7). The observation that stabilization of these loops by various metal ions allosterically modulates the catalytic function of APC provides further insight into how coagulation proteases and other more specialized serine proteases may have evolved from a common ancestral fold into highly specific enzymes with very diverse functions. Whereas the key catalytic residues are conserved in all serine proteases, other residues at the vicinity of the primary specificity pocket (S1) or remote from this pocket have diversified. There is overwhelming evidence in the literature that variant residues restrict the specificity of coagulation proteases by enabling them to specifically interact with additional residues surrounding the P1 site of various peptide substrates. In recent years, homology modeling based on the known crystal structures and subsequent mutagenesis studies have identified a number of variant residues critical for determination of the P3-P3′ specificity of coagulation proteases (27, 43–47). It is also becoming clear that the binding of specific cofactors or substrates to variant residues and loops remote from the catalytic pocket allosterically regulates the specificity and function of coagulation serine proteases (48–51). However, except for a few studies with small synthetic substrates and inhibitors, the molecular basis of the coagulation protease specificity with the natural macromolecular substrates has remained largely elusive.

The results presented in this study suggest that this may partly be due to evolution of complex allosteric links among different surface loops in coagulation serine proteases that
are not easily identified by homology modeling based on the crystal structures.

Acknowledgment—We thank Drs. Charles Esmon for the plasma proteins; Omid Safa for human factor Va; Deborah Stearns-Kurosawa for critical reading of the manuscript; and Xiao Qiang Wang from the crystallography department of Oklahoma Medical Research Foundation for preparation of Fig. 7. The excellent technical assistance of Mei Cheng is also appreciated.

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