Thermodynamic Linkage between the S1 Site, the Na\(^+\) Site, and the Ca\(^{2+}\) Site in the Protease Domain of Human Activated Protein C (APC)

SODIUM ION IN THE APC CRYSTAL STRUCTURE IS COORDINATED TO FOUR CARBONYL GROUPS FROM TWO SEPARATE LOOPS*

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The serine protease domain of activated protein C (APC) contains a Na\(^+\) and a Ca\(^{2+}\) site. However, the number and identity of the APC residues that coordinate to Na\(^+\) is not precisely known. Further, the functional link between the Na\(^+\) and the Ca\(^{2+}\) site is insufficiently defined, and their linkage to the substrate S1 site has not been studied. Here, we systematically investigate the functional significance of these two cation sites and their thermodynamic links to the S1 site. Kinetic data reveal that Na\(^+\) binds to the substrate-occupied APC with \(K_d\) values of \(-24\) mM in the absence and \(-6\) mM in the presence of Ca\(^{2+}\). Sodium-occupied APC has \(-100\)-fold increased catalytic efficiency (\(-4\)-fold decrease in \(K_m\) and \(-25\)-fold increase in \(k_{cat}\)) in hydrolyzing S-2288 (H-\(\beta\)-Ile-Pro-Arg-p-nitroanilide) and Ca\(^{2+}\) further increases this \(k_{cat}\) slightly (\(-1.2\)-fold). Ca\(^{2+}\) binds to the protease domain of APC with \(K_d\) values of \(-438\) \(\mu\)M in the absence and \(-105\) \(\mu\)M in the presence of Na\(^+\). Ca\(^{2+}\) binding to the protease domain of APC does not affect \(K_m\) but increases the \(k_{cat}\) \(-10\)-fold, and Na\(^+\) further increases this \(k_{cat}\) \(-3\)-fold and decreases the \(K_m\) value \(-3.7\)-fold. In agreement with the \(K_m\) data, sodium-occupied APC has \(-4\)-fold increased affinity in binding to p-aminobenzamidine (S1 probe). Crystallographically, the Ca\(^{2+}\) site in APC is similar to that in trypsin, and the Na\(^+\) site is similar to that in factor Xa but not thrombin. Collectively, the Na\(^+\) site is thermodynamically linked to the S1 site as well as to the protease domain Ca\(^{2+}\) site, whereas the Ca\(^{2+}\) site is only linked to the Na\(^+\) site. The significance of these findings is that under physiologic conditions, most of the APC will exist in Na\(^{2+}\)-APC-Ca\(^{2+}\) form, which has \(-110\)-fold increased proteolytic activity.

Activated protein C (APC) circulates in blood as an inactive zymogen, protein C (PC), which is a disulfide-linked two-chain vitamin K-dependent glycoprotein with a \(M_r\) value of \(-62,000\) (1, 2). The N-terminal light chain of PC contains a \(\gamma\)-carboxyglutamic acid (Gla) domain and two epidermal growth factor domains (EGF1 and EGF2), whereas the heavy chain contains the latent serine protease domain (3–5). Although \(\alpha\)-thrombin alone can activate PC to APC, the rate is inappropriately slow for a physiologically important reaction (1). The physiologic activator for conversion of PC to APC is the thrombin-thrombomodulin complex at the endothelial surface that may also involve the endothelial PC receptor (6–8). During conversion of PC to APC, a short peptide consisting of 12 residues in human (2) and 14 residues in bovine (9) is removed from the N terminus of the heavy chain. The resultant enzyme is a serine protease that inactivates two key cofactors of coagulation, namely, factors Va and VIIIa (8). This represents a major anticoagulant mechanism involved in the regulation of hemostasis (8).

The Gla domain of the light chain of APC contains several Gla residues, which are required for calcium binding to this domain (10). In addition, the EGF1 domain contains a high affinity Ca\(^{2+}\)-binding site in the native PC (or APC) molecule (8). Further, Gla domainless PC also contains a single high affinity Ca\(^{2+}\)-binding site (11) and by mutagenesis experiments this site is implicated to be located in the protease domain (12). Thus, it appears that removal of the Gla domain may impair binding of calcium to the EGF1 domain. The protease domain of APC also contains a Na\(^{+}\)-binding site, and residue c225 has been implicated in determining the Na\(^-\) induced allosteric regulation of catalytic activity of this as well as of other serine proteases (13).

The proteolytic activity of APC, including the hydrolysis of amide and ester substrates, is regulated by Na\(^+\) and Ca\(^{2+}\).

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1 The abbreviations used are: APC, activated protein C; PC, protein C; S-2288, H-\(\beta\)-Ile-Pro-Arg-p-nitroanilide; PEG, polyethylene glycol 8000; Gla, \(\gamma\)-carboxyglutamic acid; Ch\(_2\), choline; PFRck, D-Fhe-Pro-Arg-chloromethylketone; PABA, p-aminobenzamidine; pNA, p-nitroaniline; EGF, epidermal growth factor; APC(S), APC saturated with S-2288.

2 For comparison, the chymotrypsin amino acid numbering system is used. Thus, the numbers with the prefix “c” (e.g., c225) refer to the chymotrypsin equivalents for the protease domain of APC. Where insertions occur, the chymotrypsin numbering is followed by a capital letter, such as A.
(13–19). Initially, Castellino and co-workers (14–18) published a series of elegant kinetic and biophysical studies describing the potentiality of amidolytic and esterolytic activities of bovine APC. In these studies, they observed a sigmoidal dependence of the reaction velocity at a subsaturating concentration of substrate as a function of Na$^+$. In the absence of structural information available at that time, these authors proposed that APC contained two sites or classes of Na$^+$ sites that may be allosterically linked. In this report, we present data that support a heterotropic allosteric linkage of the single Na$^+$-binding site in the protease domain to the S1 site of the substrate. Further, in a recent study, He and Rezaie (19) attempted to establish linkage between the protease domain Ca$^{2+}$ site and the Na$^+$ site. Here, we present data which strongly indicate that occupancy of the S1 site in APC increases the affinity of Na$^+$ → 4-fold. He and Rezaie (19) did not address this point and only measured the affinity of Na$^+$ at a single subsaturating concentration of substrate. Such data led to obtaining a high $K_d$ app value for Na$^+$ binding and therefore precluded determination of an accurate linkage between the Na$^+$ site and the Ca$^{2+}$ site (19). Moreover, although it is recognized that the c221–c225 loop in APC is most likely involved in binding to Na$^+$ (13, 19), the site is not structurally defined. Here, we present crystallographic evidence that the Na$^+$ site in APC involves not only the c221–c225 loop but also the c183–c189 loop. Based upon our findings, we predict that the Na$^+$ site in factors VIIa and IXa will be similar to that in APC but not thrombin.

**EXPERIMENTAL PROCEDURES**

**Reagents—H-O-Ile-Pro-Arg-p-nitroanilide (S-2288) was obtained from Diapharma Inc. Polyethylene glycol 8000 (PEG) and p-aminobenzamidine (PABA) were purchased from Sigma. d-Ph-Pro-Arg-chloromethylketone (FPRck) and diisopropyl fluorophosphate were obtained from Calbiochem. Human PC was purified as a by-product of the purification of factor VII (20, 21). A total of 41,000 and 21,000 Da) on reduced SDS-PAGE (22). For Ca$^{2+}$ binding studies, FPRck-APC and Gla domainless FPRck-APC were prepared and purified using Mono Q fast flow anion exchange resin as outlined by Esmon et al. (23, 24) and Mather et al. (25). Decarboxylated FPRck-APC was obtained by thermal decarboxylation of Gla residues as described earlier for prothrombin (26) and factor X (27). For kinetic studies, human APC was obtained either from Enzyme Research Laboratories Inc. or from Hematologic Technologies, Inc. Both proteins gave identical results. Gla domainless APC was prepared by chymotrypsin treatment of APC as outlined by Hill and Castellino (18). It was also purified according to their method with the exception that we used a Mono Q fast protein liquid chromatography column instead of the Q Sepharose column (18). Each protein sample was freed of Na$^+$ by dialysis and/or by a desalting column exactly as described by Wells and Di Cera (28). Although this step resulted in some loss of APC activity, it was necessary for preparing APC free of Na$^+$. The final concentration of Na$^+$ after these steps was <1 mM as measured by a conductivity meter as outlined by Wells and Di Cera (28). The proteins were frozen at −80 °C in 20-μl aliquots, thawed, and used immediately. This freezing and thawing step did not result in a measurable loss of activity. All of the final kinetic data presented in this paper were obtained using APC obtained from Enzyme Research Laboratories or Hematologic Technologies, Inc.

**Amino Acid Sequencing and Gla Analysis—Automated Edman degradation of each protein component was performed using an Applied Biosystems model 477A gas phase sequencer. Approximately 0.5 nmol of protein was loaded on the filter cartridge. The proteins from SDS gels were transferred to polyvinylidene difluoride membranes as described by Rosenberg (29). The N-terminal sequence analysis of Glu domainless APC or Gla domainless FPRck-APC revealed two sequences of approximately equimolar amounts, one corresponding to the heavy chain (Leu-Lle-Asp-Gly-Lys) and the other corresponding to the modified light chain (Ser-Lys-His-Val-Asp). This indicates that Gla domainless APC does not contain the Gla domain (N-terminal 41 residues of the light chain) of APC. Decarboxylated APC contained <0.5 Gla/mol as compared with APC, which contained 7.9 Gla/mol as measured by the specific 3H incorporation (30). APC concentrations were determined from the absorbance at 280 nm using an extinction coefficient of 14.5 for 1% solution (3).

**SDS-PAGE Analysis—SDS gel electrophoresis was performed using the Laemmli buffer system (22). The acrylamide concentration was 15%, and the gels were stained with Coomassie Brilliant Blue dye. All of the proteins used in the present study were ~98% pure.

**Measurement of S-2288 Amidolytic Activity of APC Proteins**—The concentration of APC or Gla domainless APC was used between 4 and 40 nm. The S-2288 concentration ranged from 10 μM to 3 mM. The buffer used was 50 mM Tris-HCl, pH 7.4, containing 0.1% PEG and various salt combinations given in the legends to the appropriate figures. Chloride salt of choline (ChCl), a bulky monovalent cation, was used to keep the ionic strength constant to 0.2 M. p-Nitroaniline (pNA) release was measured continuously (ΔA$_{405}$ nm/min) for up to 30 min using a Beckman DU/55 spectrophotometer equipped with a Soft-Pac kinetics module. An extinction coefficient of 9.9 mM$^{-1}$ cm$^{-1}$ at 405 nm was used in fitting the data to the model described by --- (31). All of the reactions were performed in triplicate. The $K_d$ and $V_{max}$ values were obtained using the Enzyme Kinetics program from Erithacus Software. The $K_d$ app of binding of Na$^+$ or Ca$^{2+}$ to the substrate-bound APC was calculated from the kinetic data using the following equation.

$$v = b + \frac{(V-b)(x)}{K_{d,app} + x}$$

where $V$ is the apparent maximum velocity under a specified cation concentration represented by $x$, $V$ is the $V_{max}$ at saturating concentration of the cation, $b$ is the maximum velocity at 0 concentration of the cation, and $K_{d, app}$ is the $K_d$ of the specified cation for the APC(S) (substrate-bound APC). The values for the correlation coefficient ($r$), a measure of the strength of the goodness of fit, were obtained using the program SPSS, version 10.0.

**Ca$^{2+}$ Binding to Decarboxylated FPRck-APC and Gla Domainless FPRck-APC**—Calcium ion activity was determined by using a Ca$^{2+}$-specific electrode and a model 601A digital ionizer (Orion Research). Titrations of the protein at 29 μM in 4 mL of Tris/NaCl, pH 7.4 (50 mM Tris-HCl, 200 mM NaCl), were performed by adding 1–2-μl increments of 40 mM CaCl$_2$ at room temperature. In these titrations, bound Ca$^{2+}$ was taken as the difference between the measured free Ca$^{2+}$ concentration and the total added. The data were fitted to the following equation using the program GraFit from Erithacus Software.

$$C_{Ca} = \frac{C_{Ca} - C_{Ca}^0}{K_{Ca} + C_{Ca}}$$

where $K_{d, app}$ is the dissociation constant for binding of Ca$^{2+}$ to the protein, $C_{Ca}^0$ is the maximum Ca$^{2+}$ binding capacity, and $C_{Ca}$ and $C_{Ca}^0$ represent the free and bound concentrations of Ca$^{2+}$, respectively.

**Determination of $K_{f,PARA}$ of Binding of PABA to APC**—The $K_{d, app}$ of binding of PABA to APC was determined by its ability to competitively inhibit S-2288 hydrolysis in the absence and presence of Na$^+$ and/or Ca$^{2+}$. The details are provided in the legend to Fig. 6. The $C_{PABA}$ (concentration of PABA required for 50% inhibition) was determined by fitting the data to the following $IC_{50}$ four-parameter logistic equation from Halfman (32) given below.

$$y = \frac{a}{1 + (x/IC_{50})^b}$$

where $y$ is the rate of pNA release in the presence of a given concentration of PABA represented by $x$, $a$ is the maximum rate of pNA release in the absence of PABA, and $s$ is the slope factor. Each point was weighted equally, and the data were fitted to Equation 3 using the nonlinear regression analysis program GraFit from Erithacus Software. To obtain $K_{f,PARA}$ values for the interaction of PABA with APC, we used the following equation as described by Cheng and Prusoff (33) and further elaborated by Craig (34),

$$K_{f,PARA} = \frac{IC_{50}}{1 + (S/K_m)}$$

where $S$ is the S-2288 concentration. The $K_{f,PARA}$ values obtained under different conditions (listed in Table II) were used to obtain the $K_{f,PARA}$ value.

**Further Refinement of the Gla Domainless FPRck-APC Structure**—The X-ray intensity data and the atomic coordinates (Protein Data Bank code 1aut) used for refinement were provided earlier by Mather et al.
The crystallization drop contained 500 μl total calcium concentration, which could provide sufficient free Ca$^{2+}$ for it to bind to the c70–c80 loop in the protease domain. Examination of the $(2F_{o} - F_{c})$ and $(F_{o} - F_{c})$ electron density maps revealed that Ca$^{2+}$ and appropriate solvent water molecules could be added to the structure. Similarly, based upon the Na$^{+}$ site in thrombin (30) and factor Xa (36), we added Na$^{+}$ and appropriate solvent water molecules in that region of the $(2F_{o} - F_{c})$ electron density map. The structure was refined using XPLOR (37) and iterative use of computer graphics using the program O (38). In all, three rounds of refinement were performed. The resultant structure has an R factor of 17.8% compared with the starting R factor of 18.6%. The final structure has 149 water molecules, one calcium ion, and one sodium ion. All of the water molecules had significant electron density when the $(2F_{o} - F_{c})$ electron density maps were contoured at the 1σ level. The coordinates are being deposited with the Research Collaboratory for Structural Bioinformatics.

**RESULTS**

**Na$^{+}$ Potentiation of S-2288 Hydrolysis by APC in the Absence of Ca$^{2+}$**—In earlier studies, Na$^{+}$ has been shown to be the physiologically relevant and an effective monovalent ion in potentiating the amiodamic activity of APC (13–15, 18). To further investigate the mechanism of Na$^{+}$-mediated potentiation of S-2288 hydrolysis, we determined $K_{m \, app}$ and $V_{max}$ at several concentrations of NaCl. We used 200 mM NaCl as the highest salt concentration in these experiments. When the concentration of Na$^{+}$ was less than 200 mM, Cl$^{-}$ was used as a compensating ion to keep the ionic strength constant to 0.2 M. One mM EDTA was present in each buffer to eliminate the effect of divalent cations. These data are presented in Fig. 1A. The values of $K_{m \, app}$ and $V_{max}$ were calculated for each salt concentration using the Enzyme Kinetics program from Erithacus Software. The results indicate that Na$^{+}$ affects both the $K_{m \, app}$ and $V_{max}$ of this reaction (Fig. 1A). The $K_{m \, app}$ and $V_{max}$ values obtained at each Na$^{+}$ concentration are given in Table I. When $V_{max}$ are plotted as a function of Na$^{+}$ concentration (Fig. 1B), the midpoint of the curve should yield $K_{d \, app}$ of interaction of Na$^{+}$ with APC saturated with S-2288 (APC(S)) in the absence of Ca$^{2+}$; this value was calculated to be 23.9 ± 2 mM.

**Ca$^{2+}$ Potentiation of S-2288 Hydrolysis by APC in the Absence of Na$^{+}$**—These data are presented in Fig. 2. The kinetic data indicate that Ca$^{2+}$ does not change the $K_{m \, app}$ but increases the $k_{cat}$-10-fold (Table II). From the data of Fig. 2B, we calculate the $K_{d \, app}$ of Ca$^{2+}$ in its interactions with APC(S) to be 438 ± 31 μM.

**Na$^{+}$ Potentiation of S-2288 Hydrolysis by APC in the Presence of Ca$^{2+}$**—These data are presented in Fig. 3. The kinetic data indicate that Na$^{+}$ decreases the $K_{m \, app}$-3.7-fold and increases the $k_{cat}$-2.8-fold. Importantly, Ca$^{2+}$ decreases the $K_{d \, app}$ of Na$^{+}$ interaction with APC(S) to 6.2 ± 1 μM as compared with 24 μM (Fig. 1) when Ca$^{2+}$ is absent. Thus, Ca$^{2+}$ site is thermodynamically linked to the Na$^{+}$ site.

**Ca$^{2+}$ Potentiation of S-2288 Hydrolysis by APC in the Presence of Na$^{+}$**—These data are presented in Fig. 4. As noted previously (Fig. 2), Ca$^{2+}$ does not change the $K_{m \, app}$ but increases the $k_{cat}$ slightly (1.2 ± 0.1-fold) in the presence of Na$^{+}$. Because the change in $V_{max}$ is very small, these data could not be used to obtain the $K_{d \, app}$ of Ca$^{2+}$ interaction with APC(S). However, we later show that in the presence of Na$^{+}$ this $K_{d \, app}$ is ~105 μM as compared with ~438 μM (Fig. 2) when Na$^{+}$ is absent. Thus, as noted above, the Na$^{+}$ site is linked to the Ca$^{2+}$ site.

The above experiments were repeated using Gla domainless APC. Potentiation of S-2288 hydrolytic activity at several sub-saturating concentrations of Na$^{+}$ (5 mM Ca$^{2+}$) or Ca$^{2+}$ (200 mM Na$^{+}$) was indistinguishable from that obtained with APC. These data are consistent with extensive studies conducted by Hill and Castellino (17) using bovine APC and Gla domainless APC. Thus, the effects of Na$^{+}$ and/or Ca$^{2+}$ in potentiating the activity of both human and bovine APC and Gla domainless APC are comparable. Importantly, our results for the first time reveal that the substrate binding is allosterically linked to the binding of Na$^{+}$.

**Binding of Ca$^{2+}$ to Human Decarboxylated FPRck-APC and Gla Domainless FPRck-APC**—Bovine Gla domainless APC has
strength was kept constant in each reaction mixture by adding 185 mM Ca$^{2+}$ to the protease domain of human APC has also been implicated (12). Here, we have performed direct Ca$^{2+}$ binding studies with human decarboxylated FPRck-APC and Glu domainless FPRck-APC. These data are presented in Fig. 5 and reveal that the Glu domainless FPRck-APC contains a single Ca$^{2+}$-binding site with a $K_{d}$ value of 105 ± 11 μM, whereas the decarboxylated FPRck-APC contains two Ca$^{2+}$-binding sites with $K_{d}$ values of 120 ± 19 μM. Because Glu domainless FPRck-APC has only one high affinity Ca$^{2+}$-binding site compared with the two in the decarboxylated FPRck-APC, it is likely that the EGF1 domain residues 42–49 containing Asp-46 and Gln-49 that are implicated in binding to Ca$^{2+}$ (39) are flexibly disordered in the Glu domainless FPRck-APC and cannot participate in forming the high affinity site in this domain. Thus, the Ca$^{2+}$ effects observed on the S-2288 amidolytic activity of APC are due to the Ca$^{2+}$-binding site in the protease domain and not in the EGF1 domain.

Table II

| Conditions | $K_{m}$ (μM) | $k_{cat}$ (min⁻¹) | $V_{max}$ (μM/min) |
|------------|--------------|-------------------|-------------------|
| Na⁺ | Ca⁺ | $K_{app}$ | $k_{cat}$ app | $V_{max}$ app |
| − | − | − | 1723 ± 208 | 37 ± 2 | 21 (1) * |
| + | − | + | 433 ± 51 | 905 ± 44 | 2090 (100) |
| + | + | − | 1705 ± 187 | 385 ± 24 | 2296 (11) |
| + | + | + | 466 ± 53 | 1070 ± 35 | 2296 (109) |

* The fold change in specificity constant ($k_{cat}/K_{m}$) is given in parentheses.

been reported to contain one high affinity Ca$^{2+}$ site in the presence of Na$^+$ (11). By mutagenesis experiments, a Ca$^{2+}$-binding site in the protease domain of human APC has also been implicated (12). Here, we have performed direct Ca$^{2+}$ binding studies with human decarboxylated FPRck-APC and Glu domainless FPRck-APC. These data are presented in Fig. 5 and reveal that the Glu domainless FPRck-APC contains a single Ca$^{2+}$-binding site with a $K_{d}$ value of 105 ± 11 μM, whereas the decarboxylated FPRck-APC contains two Ca$^{2+}$-binding sites with $K_{d}$ values of 120 ± 19 μM. Because Glu domainless FPRck-APC has only one high affinity Ca$^{2+}$-binding site compared with the two in the decarboxylated FPRck-APC, it is likely that the EGF1 domain residues 42–49 containing Asp-46 and Gln-49 that are implicated in binding to Ca$^{2+}$ (39) are flexibly disordered in the Glu domainless FPRck-APC and cannot participate in forming the high affinity site in this domain. Thus, the Ca$^{2+}$ effects observed on the S-2288 amidolytic activity of APC are due to the Ca$^{2+}$-binding site in the protease domain and not in the EGF1 domain.

The data presented thus far establish the interdependence of the binding of Na$^+$ and Ca$^{2+}$ in the protease domain of APC. APC(S) can be converted to the state with both ions bound (sodium-APC(S)-calcium) by acquiring either Na$^+$ (via APC(S)-calcium) or Ca$^{2+}$ (via sodium-APC(S)). The ratio of the apparent $K_{C_{Na}}$ in the presence and absence of Ca$^{2+}$ is 3.9 and that of apparent $K_{C_{Ca}}$ in the presence and absence of Na$^+$ is 4.2. Therefore, within experimental error, the net sum of binding energy over the cycle appears to be 0. This establishes an accurate thermodynamic linkage between the Na$^+$ site and the protease domain Ca$^{2+}$ site of APC as opposed to the 16–20-fold ratio proposed by He and Rezaie (19). Furthermore, because Na$^+$ affects the $K_{m}$ of S-2288 hydrolysis, there appears to be a link between the substrate-binding and the Na$^+$-binding site.
Both in the absence and presence of Ca2+, Na+ decreases the $K_m$ of S-2288 by ~3.8-fold. Using $K_m$ as an approximation of substrate affinity, one can complete this part of the linkage using the thermodynamic principles (40). Therefore, apparent $K_{d, Na}$ and apparent $K_{d, Ca}$ to the free form of APC in the absence of substrate or the other cation can be calculated; these values are ~95.1 mM and ~443 mM, respectively.

Effects of Na+ and Ca2+ on the Interaction of PABA with APC—We next investigated whether Na+ and/or Ca2+ affect the S1 site of the active site in APC. We used the S1 site probes PABA for these studies. The interaction of PABA with APC was determined under each of the four salt conditions. These data are presented in Fig. 6 and summarized in Table III. Under a saturating concentration of Na+, the affinity of PABA for APC was increased ~4.2 to 4.4-fold as compared with that in its absence. Further, Ca2+ had essentially no effect on the apparent $K_{d, PABA}$ in the absence or presence of Na+. These $K_{d, PABA}$ data agree well with the $K_m$ data presented in the previous sections where Na+ decreased the $K_m$ ~4-fold and Ca2+ had no effect. Our PABA data are similar to the PABA binding to bovine APC, where the $K_{d, PABA}$ is 13 mM in the presence and 95 mM in the absence of Na+ (17). However, the absolute apparent $K_{d, PABA}$ value for human APC is ~79 mM in the presence and ~333 mM in the absence of Na+ (Table III). Thus, it would appear that compared with human APC, bovine APC binds PABA with ~6- and ~3.5-fold higher affinity in the presence and absence of Na+, respectively. Importantly, our data establish that Na+ binding is linked to the substrate binding through its S1 site.

**TABLE III**

| Conditions | Na+ | Ca2+ | $K_{d, PABA}$ | Fold change of $K_d$ |
|------------|-----|------|----------------|---------------------|
| -          | $\mu M$ | - | 333 ± 42 | 1.0 |
| +          | + | 79 ± 9 | 4.2 |
| -          | + | 259 ± 32 | 1.3 |
| +          | + | 76 ± 7 | 4.4 |

* The apparent $K_{d, PABA}$ values were calculated from the $IC_{50}$ data of Fig. 6 using Equation 4.

A similar apparent $K_{d, PABA}$ value was obtained when the Na+ concentration was increased to 485 mM (Fig. 6).

A similar apparent $K_{d, PABA}$ value was obtained when the Na+ concentration was 485 mM and the Ca2+ concentration was 5 mM (Fig. 6).

Glu-c80, carboxyl oxygens of c72 and c75, and two water molecules. The Na+-binding site is similar to that in factor Xa (36) and involves carboxyl oxygens of c184A, c185, c221A, and c224 and two water molecules.

**DISCUSSION**

The purpose of the present study was to structurally define the protease domain Ca2+ site as well as the Na+ site and their roles in regulating the catalytic activity of human APC. The spatial relationship of the Ca2+ site, the Na+ site, the autolysis loop, the Asp-c189 S1 site, and the catalytic triad is provided in Fig. 7A. Dang and Di Cera (13) recently reported that several serine proteases, including those in coagulation, possess a func-

3 In APC (25), the residues in the c183 loop are numbered as c184, c184A, c185, c186, c186A, and c187. In factor Xa (36), the equivalent residues in this loop are numbered as c184, c185, c185A, c185B, c186, and c187. The residues in the c221 loop of APC are numbered as c221, c221A, c222, c223, c224, and c225. The equivalent residues in this loop of factor Xa are numbered as c221, c222, c222A, c223, c224, and c225. The residues involved in binding to Na+ in APC and factor Xa are given in italics in this footnote.
tional Na\textsuperscript{+} site. X-ray crystal structures of thrombin (35, 42) and factor Xa (35, 36) are reported where the Na\textsuperscript{+} site in these molecules is defined. The Na\textsuperscript{+} site in thrombin uses a single loop involving the carbonyl oxygen atoms of residues c221A and c224 as well as four water molecules, whereas the Na\textsuperscript{+} site in factor Xa uses two loops involving the carbonyl oxygen atoms of...
residues c185, c185A, c222, and c224 as well as two water molecules. The nature of the residue c225 plays an important role in orienting the carbonyl oxygen atom of c224 toward the Na^+ coordination shell (35, 36, 42). As shown in Fig. 7C, the Na^+ site in APC resembles that of factor Xa but not thrombin. This may be due to the insertion of three residues in the c183 loop of thrombin. As a result of this insertion, the carbonyl oxygens of this loop in thrombin are spatially distant and unable to coordinate with Na^+. Instead, the cavity in thrombin is filled with water molecules, two of which are optimally situated to coordinate with Na^+. In the presence or absence of Na^+, Ca^{2+} increased the affinity of S-2288 to APC (Table II) by \(-4\)-fold and binding of PABA (Table III) by a similar fold. Because Ca^{2+} does not influence the \(K_{m,app}\) value of S-2288 and has essentially no effect on the PABA binding, it would appear that these effects are primarily mediated through the S1 site in APC. These results can be readily rationalized, because the Na^+ site in APC is directly linked to the S1 specificity pocket residue Asp-c189 (Fig. 7C). Occupancy of the Na^+ site could rigidify the c189 side chain as well as the c183–c189 loop for optimal interaction with the basic P1 residue (Arg) of the substrate or the basic amino groups of the S1 probe, PABA.

In the absence of Na^+, Ca^{2+} increased the \(k_{cat}\) of S-2288 hydrolysis \(-10\)-fold (Table II). In the presence of Na^+, however, Ca^{2+} had a minimal effect (\(-1.2\)-fold) on the hydrolysis of S-2288. Of interest, Ca^{2+} in the presence or absence of Na^+ had no effect on the \(K_{m,app}\) of S-2288 hydrolysis as well as on the PABA binding. Thus, Ca^{2+} does not affect the ground state binding of substrates/inhibitors to APC. The \(-10\)-fold increase in \(k_{cat}\) for S-2288 hydrolysis thus appears to be due to a decrease in the transition state energy. All of the catalytic effects of Ca^{2+} on APC are remarkably similar to those observed earlier for factor Xa (43). This is not surprising because both

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\begin{align*}
\text{Na-APC-Ca} & \leftrightarrow \text{Na-APC(S)-Ca} \\
K_{d,Na} & = 22.7 \text{mM} \\
K_{d,Na} & = 6.2 \text{mM} \\
\text{Km} & = 466 \mu\text{M} \\
\text{Km} & = 438 \mu\text{M} \\
\text{Kd,Na} & = 95.1 \text{mM} \\
\text{Kd,Na} & = 23.9 \text{mM} \\
\text{APC-Ca} & \leftrightarrow \text{APC(S)-Ca} \\
K_{d,Na} & = 443 \mu\text{M} \\
K_{d,Na} & = 438 \mu\text{M} \\
\text{Km} & = 1705 \mu\text{M} \\
\text{Km} & = 1723 \mu\text{M} \\
\text{APC} & \leftrightarrow \text{APC(S)} \\
K_{d,Na} & = 97.6 \mu\text{M} \\
K_{d,Na} & = 105 \mu\text{M} \\
\text{Na-APC} & \leftrightarrow \text{Na-APC(S)} \\
K_{d,Na} & = 97.6 \mu\text{M} \\
K_{d,Na} & = 105 \mu\text{M} \\
\text{Km} & = 433 \mu\text{M} \\
\text{Km} & = 466 \mu\text{M} \\
\text{Na-APC-Ca} & \leftrightarrow \text{Na-APC(S)-Ca} \\
K_{d,Na} & = 97.6 \mu\text{M} \\
K_{d,Na} & = 105 \mu\text{M} \\
\text{Km} & = 466 \mu\text{M} \\
\text{Km} & = 438 \mu\text{M}
\end{align*}
\]

**Fig. 8.** Linkage relations in binding of S-2288, Na^+, and Ca^{2+} to the protease domain of APC. In addition to the substrate-binding site, the protease domain of APC possesses a monovalent (Na^+) as well as a divalent (Ca^{2+}) cation-binding site. Thus, the protease domain can exist in eight forms: APC, APC(S), sodium-APC, APC-calcium, sodium-APC(S), APC(S)-calcium, sodium-APC-calcium, and sodium-APC(S)-calcium. The dissociation constant listed for each equilibrium was calculated using the data of Figs. 1, 3, 5, and 6. Note that the apparent \(K_{d,Na}\) and apparent \(K_{d,Ca}\) depicted on the left of the figure are not experimentally determined values but rather are calculated values based upon thermodynamic principles (40).
proteases are structurally similar.

Of interest is the fact that the Na⁺ site and the protease domain Ca²⁺ site are thermodynamically linked. Thus, Ca²⁺ increases the affinity of Na⁺ by ~4.0-fold, and Na⁺ increases the affinity of protease domain Ca²⁺ binding by a similar fold. The thermodynamic linkage between the Na⁺ site and the protease domain Ca²⁺ site of APC is depicted in Fig. 8. This part of the linkage is supported by the data presented in Figs. 1, 2, 3, and 5. Thus, although the effects of Na⁺ and Ca²⁺ at individual steps of the thermodynamic cycle depicted in Fig. 8 are different, the overall change in apparent affinity for Na⁺ or Ca²⁺ is the same regardless of the pathway followed in going from APC to the sodium-APC(S)-calcium state or the pathway followed in going from APC to the sodium-APC-calcium state.

A major observation of significance in this paper is that the S1 site in APC is linked to the Na⁺ site. Hence, occupancy of the S1 site increases the affinity of Na⁺ ~ 4-fold, and Na⁺ increases the affinity (Kₘ) of the substrate by a similar fold. This part of the thermodynamic linkage between the S1 site and the Na⁺ site in APC is also depicted in Fig. 8 and is supported by the data presented in Figs. 1, 3, and 6. Consequently, although the effects of Na⁺ and substrate at individual steps of this part of the thermodynamic cycle are different, the overall change in apparent affinity for Na⁺ or substrate S-2288 is the same regardless of the pathway followed in going from APC to the sodium-APC(S)-calcium state or the pathway followed in going from APC-calcium to the sodium-APC(S)-calcium state. Note that Ca²⁺ is not thermodynamically linked to the S1 site. Thus, in going from APC to the S1 site, the Ca²⁺ site in the protease domain (Fig. 7B) is typical of that found in trypsin-like proteases (41). It is of note that the c70–c80 calcium loop in APC contains three basic and two hydrophobic Trp residues (25). The serine proteases that bind Ca²⁺ contain primarily acidic and polar residues in this loop, which facilitate attraction of a positively charged divalent cation. Thus, trypsin contains four acidic and three polar residues (41), and elastase contains two acidic and seven polar residues (44). Similarly, human factor IXa contains five acidic and five polar residues and is thought to bind Ca²⁺ in this loop (45). Human factor VIIa contains six acidic and three polar residues and contains a Ca²⁺-binding site in this loop (46). Furthermore, the Gla domainless PC or APC was reported to contain only a single Ca²⁺-binding site (11), which was thought to be located in the EGF1 domain (39). Based upon the above considerations, Bajaj et al. (45) had initially proposed that PC or APC might not contain a Ca²⁺-binding site in the protease domain. However, recent mutagenesis experiments (12), direct binding studies (Fig. 5), and crystal structure data (Fig. 7B) clearly reveal the presence of a Ca²⁺-binding site in the c70–c80 loop of APC. Thus, the Ca²⁺ site in the Gla domainless PC or APC previously attributed to the EGF1 domain is most likely the Ca²⁺ site in the protease domain.

Structural features of the c70–c80 Ca²⁺-binding loop of APC are shown in Fig. 9. A unique feature of this loop appears to be that all three basic residues and the two hydrophobic Trp residues are exposed to the solvent. The other hydrophobic residue Leu-c73 is buried in a hydrophobic pocket involving spatially close neighbors. The Trp-c76 is stabilized by two hydrogen bonds and van der Waals’ interactions involving the side chain carbon atoms of Lys-c78. Similarly, Trp-c79 is held in place by a hydrogen bond involving the carbonyl oxygen of

FIG. 9. **Surface features of the APC protease domain Ca²⁺-binding loop.** Location of Leu-c73 in a hydrophobic pocket comprised of Val-c82, Leu-c40, and Trp-c141 is shown in white. Two hydrophobic residues and three basic residues, namely, Trp-c76, Trp-c79, Arg-c74, Arg-c75, and Lys-c78 are exposed to the solvent. These residues are stabilized by hydrogen bonds and van der Waals’ interactions and are discussed in the text. The dotted lines represent hydrogen bonds as well as coordination of Ca²⁺ with its ligands. Magenta sphere (Ca), calcium; red sphere (w), oxygen of water.
The Na\textsuperscript{+} site in APC is thermodynamically linked to the S1 site as well as to the protease domain Ca\textsuperscript{2+} site. Such information was critical in defining the accurate thermodynamic cycle for the linkage of the Na\textsuperscript{+} site to the Ca\textsuperscript{2+} site as well as to the S1 site (Fig. 8).

Our data also indicate that most of the APC under physiologic conditions of Na\textsuperscript{+} concentrations (1 mM) of Na\textsuperscript{+} Arg-c24. Thus, it is not unreasonable to conclude that the Na\textsuperscript{+} role in maintaining the fully active conformer of the protease domain in these vitamin K-dependent proteins.

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Addendum—Fig. 2 originally presented in Papers in Press has been deleted. In this figure the velocity curve as a function of substrate concentration showed weak sigmoidal behavior at subsaturating concentration (1 mM) of Na\textsuperscript{+}. Subsequently, when several simulation experiments were conducted each at different subsaturating concentrations of Na\textsuperscript{+}, linear curves were observed. Moreover, at each substrate concentration, the ratio of enzyme-substrate complex to the enzyme-Na\textsuperscript{+} substrate complex was also unchanged. Thus, Fig. 2 was removed, and the text has been adjusted appropriately.

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