Polgár, L. (1974) *FEBS Lett. 47*, 15–18.
Polgár, L. (1981) *Biochim. Biophys. Acta 658*, 262–269.
Polgár, L., & Bender, M. L. (1970) *Adv. Enzymol. Relat. Areas Mol. Biol. 33*, 381–400.
Polgár, L., & Halász, P. (1982) *Biochem. J. 207*, 1–10.
Robertus, J. D., Kraut, J., Alden, R. A., & Birktoft, J. J. (1972) *Biochemistry 11*, 4293–4303.
Sluyterman, L. A. E., & Wijdenes, J. (1970) *Biochim. Biophys. Acta 200*, 593–595.
Whitaker, J. R., & Bender, M. L. (1965) *J. Am. Chem. Soc. 87*, 2728–2737.
Wolthers, B. G., Drenth, J., Jansonius, J. N., Koekoek, R., & Swen, H. M. (1970) *Struct.—Funct. Relat. Proteolytic Enzymes, Proc. Int. Symp.*, 272–288.

# Kinetic Mechanism for Stimulation by Monovalent Cations of the Amidase Activity of the Plasma Protease Bovine Activated Protein C†

Stephen A. Steiner and Francis J. Castellino*

*Department of Chemistry, The University of Notre Dame, Notre Dame, Indiana 46556*
*Received May 22, 1984*

ABSTRACT: A study of the effect of monovalent cations on the steady-state kinetic parameters for the hydrolysis of the synthetic substrate $N^{\alpha}$-benzoyl-L-arginine-*p*-nitroanilide by activated bovine plasma protein C (APC) has been undertaken. The enzyme displayed a strict requirement for monovalent cations in its expression of amidolytic activity toward this substrate. Analysis of the variation in initial hydrolytic reaction rates, as a function of metal ion concentrations, suggested that at least two cation sites, or classes of sites, were necessary for catalysis to occur. After examination of the rate equations consequential to many different enzymic mechanisms that could account for these kinetic data, a mechanism was developed that fit the great majority of the experimental observations. In this mechanism it is postulated that cations bind to the enzyme in pairs, with a kinetically observable single binding constant, either preceded by or followed by binding of substrate. Catalysis occurs only after the enzyme–(metal cation)$_2$–substrate complex is assembled. Some physical support for this mechanism was obtained upon the discovery that the binding (dissociation) constant for a competitive inhibitor of APC, *p*-aminobenzamidine, as determined by kinetic methodology, was independent of the concentration of $Na^+$ and $Cs^+$.

Protein C (PC) is a vitamin K dependent plasma glycoprotein (Stenflo, 1976), which serves as the zymogen of the serine protease activated protein C (APC) (Kisiel et al., 1976). Bovine plasma PC contains two disulfide-linked polypeptide chains of known amino acid sequence. Its light chain consists of 155 amino acid residues and possesses all 11 of the Gla residues of the protein, which have been placed within the first 35 residues from the amino terminus (Fernlund & Stenflo, 1982). A single glycosylation site in this chain is present at residue $Asn_{97}$. The heavy chain of PC comprises 260 amino acids and contains the latent active site residues $His_{56}$, $Asp_{102}$, and $Ser_{201}$, as well as three points of glycosylation at residuess $Asn_{93}$, $Asn_{154}$, and $Asn_{170}$ (Stenflo & Fernlund, 1982). It is believed that $Cys_{122}$ represents the heavy chain residue that covalently binds the two chains of PC (Stenflo & Fernlund, 1982). The sequence position of its companion residue in the light chain is not known.

PC is converted to APC as a consequence of cleavage of the $Arg_{14}$–$Ile_{15}$ peptide bond in the heavy chain of PC (Kisiel et al., 1976), and cations play a significant role in this process. The coagulant protein from the venom of Russell's viper (RVV-X) is able to catalyze the cleavage required for activation (Kisiel et al., 1976), in a step accelerated by $Ca^{2+}$ (Amphlett et al., 1981). Thrombin also catalyzes this same reaction (Kisiel et al., 1977), but this process is inhibited by $Ca^{2+}$ (Amphlett et al., 1981). The thrombin-catalyzed activation of PC is greatly accelerated by a protein cofactor, thrombomodulin, present in endothelial cells (Esmon & Owen, 1981; Owen & Esmon, 1981). The resultant protease, APC, possesses esterolytic (Steiner et al., 1980) and amidolytic (Kisiel et al., 1976, 1977) activities toward synthetic substrates.

The physiological role of APC centers around its anticoagulant activity (Kisiel et al., 1977). This activity may be explained by the observations that APC inactivates an important cofactor for prothrombin activation, factor Va (Kisiel et al., 1977; Walker et al., 1979), and an important cofactor for factor X activation, factor VIIIa (Vehar & Davie, 1980). APC is also believed to function in fibrinolysis, since increased levels of activators of plasminogen are found in plasma upon infusion of APC (Comp & Esmon, 1981). The resultant effect of all of the above activities of APC is to maintain the fluid state of blood. The importance of APC in this regard is punctuated by the finding that members of a family with a history of recurrent thrombotic episodes possessed abnormally low levels of plasma PC (Griffin et al., 1981).

Our laboratory had originally demonstrated that the amidolytic and esterolytic activities of APC were dependent upon the presence of cations (Steiner et al., 1980; Steiner & Castellino, 1982). While monovalent cations exerted the greatest influence in this regard, divalent cations, such as $Ca^{2+}$, also served this function (Steiner et al., 1980). Although certain monovalent cations have been shown to enhance, to a small extent, the amidolytic activities of $\alpha$-thrombin (Orthner & Kosow, 1980) and factor Xa (Orthner & Kosow, 1978), our observations (Steiner et al., 1980) that the amidolytic acitvity of APC was absolutely dependent upon monovalent (and to a lesser degree, divalent) cations and was progressively enhanced by monovalent cations through the Hofmeister series

† These studies were supported by Grant HL-19982 from the National Institutes of Health and Grant 81-609 from the American Heart Association.

0006-2960/85/0424-0609$01.50/0 

rendered APC unique as a serine protease in this regard. Further, our finding that the dependence of the amidolytic activity of APC on monovalent cations was a function of multiple sites for cations on APC (Steiner & Castellino, 1982) greatly stimulated our interest in discovering a mechanistic basis for this unprecedented behavior of monovalent cations in functioning of a serine protease. This paper represents our efforts to uncover such a mechanism.

## Materials and Methods

*Proteins.* Bovine plasma PC was isolated from plasma obtained fresh from a local slaughterhouse. The purification procedure employed was essentially that described by Stenflo (1976), with minor operational modifications. These have been described in detail by Steiner & Castellino (1982). APC was generated from PC by activation of PC with insolubilized RVV-X (Steiner et al., 1980). Dodecyl sulfate ($DodSO_4$)–polyacrylamide gel electrophoresis, under reducing conditions, showed that the requisite molecular weight conversion of the heavy chain had taken place under the activation conditions described. Titration of the APC produced with *p*-(nitrophenyl)-*p'*-guanidobenzoate (NPGB) (Chase & Shaw, 1969) demonstrated, in many different experiments, that at least 90% of the total protein present after activation was APC.

Bovine plasma factor VIII was prepared by the procedure of Legaz & Davie (1976), as modified by Link & Castellino (1983). The bovine thrombin employed to activate factor VIII was generated as described by Link & Castellino (1983) and purified by the methodology of Lundblad et al. (1976).

*Steady-State Kinetics.* The steady-state kinetic parameters of APC toward the synthetic substrate $N^{\alpha}$-benzoyl-L-arginine-*p*-nitroanilide (BAPA) were determined under a variety of experimental conditions. All assays described were performed at 30 °C on a Cary 219 spectrophotometer equipped with a thermostated cell holder. The reactions were initiated by addition of enzyme to a final concentration of approximately 260 nM. The progress of the reaction was monitored by continued recording of the absorbance at 405 nm, which resulted from the liberation of *p*-nitroaniline.

For all assays, the reaction mixture consisted of 50 nM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.4, and various amounts of substrate (50–600 $\mu$M) and cation (0–500 mM). The final volume was 1.0 mL. In each case, the chloride salt of the cation was used. An extinction coefficient of 9620 $M^{-1}$ $cm^{-1}$ (Pfleiderer, 1970) was employed in order to calculate the amount of substrate hydrolyzed at any time. When $K_I$ values for *p*-aminobenzamidine (pAB) were desired, these same assays were conducted in the presence of various concentrations of this inhibitor and with D-phenylalanylpipecolylarginine-*p*-nitroanilide (S-2238) as the substrate. Prior to use in the assay, APC was subjected to chromatography on Chelex-100, followed by exhaustive dialysis against a buffer of 50 mM Tris-HCl, pH 7.4.

*Inactivation of Factor VIIIa by APC.* The effect of APC on the coagulant activity of factor VIIIa was investigated. First, factor VIII was activated to factor VIIIa by incubation with thrombin at 37 °C for 2 min, as described by Link & Castellino (1983). The incubation mixture contained 45 units $mL^{-1}$ factor VIII and 0.05 unit $mL^{-1}$ thrombin in 15 $\mu$L of a buffer consisting of 20 mM imidazole–HCl/150 mM NaCl, pH 7.0. After 2 min, the mixture ws diluted with a solution of 60 mM Tris-HCl, pH 7.4, to a final volume of 140 $\mu$L and adjusted to 250 mM with $CaCl_2$, in order to stabilize the coagulant activity. This procedure was found to produce at least a 90-fold increase in factor VIII activity. APC was added to a final concentration of 3.3 $\mu$M, and this mixture was allowed to incubate at room temperature. Aliquots were removed at various time periods and assayed for factor VIIIa coagulant activity. The first sample was taken immediately following the addition of the APC and represents the zero time point. The control experiment was performed by adding buffer (60 mM Tris-HCl, pH 7.4) instead of APC in the inactivation mixture. The effect of $Na^+$ on the activity of APC toward factor VIIIa inactivation was investigated by including a concentration of 210 mM NaCl in the inactivation mixture.

*Coagulant Activity of Factor VIIIa.* Factor VIIIa coagulant activity was measured by a slight modification of the method of Legaz & Davie (1976). In this assay, 0.1 mL of human factor VIII deficient plasma was incubated with 0.1 mL of actin (activated cephaloplastin reagent) for 5 min at 37 °C in a glass culture tube. At this time, a 0.1-mL aliquot of the sample solution was added to the mixture, followed immediately by 0.1 mL of 25 mM $CaCl_2$. A stopwatch was started upon addition of the $CaCl_2$, and the time for formation of fibrin strands in the solution was determined. A standard calibration curve was produced by obtaining similar clot times for serial dilutions of fresh citrated pooled bovine plasma as the factor VIII source. One unit of factor VIII activity is defined as that amount present in 1.0 mL of the plasma reference. The standard curve was generated by plotting the logarithm of the plasma dilution against the logarithm of the clotting time. The resultant graph was linear through the region of dilutions of 0.1–0.001 (v/v) of the standard plasma, with clotting times ranging from 30–60 s.

*Intrinsic Fluorescence of APC.* The effect of $Na^+$ on the intrinsic fluorescence of APC was investigated in a series of experiments carried out on a Perkin-Elmer MPF-44 fluorescence spectrophotometer. A solution of 0.1 mg/mL APC, subjected to Chelex-100 chromatography and exhaustively dialyzed against 20 mM Tris-HCl, pH 7.4, was placed in a 3-mL quartz cuvette and scanned in the spectrophotometer in order to reveal the wavelengths for maximum emission and excitation. It was found that maximum excitation occurred at 283 nm and maximum emission was at 332 nm. The wavelengths of these maxima were unchanged upon increasing the concentration of Tris-HCl to 860 mM, upon addition of NaCl to 800 mM, and upon increasing the protein concentration to 1.0 mg/mL.

All fluorescence measurements were performed at 30 °C. Two identical cuvettes were filled with 2.5 mL of 0.1 mg/mL APC in the above buffer and were found to give identical fluorescence intensities with the spectrophotometer, which was set for an excitation wavelength of 283 nm and an emission wavelength of 332 nm. Small volumes of a cation solution were added to one cuvette, while equal volumes of the same Tris-HCl solution were added to the reference cuvette, so that the protein concentration and the ionic strength were maintained at equal levels in both. After each addition, the fluorescence was measured and recorded on a chart recorder. The ratio of the fluorescence of the sample to the fluorescence of the reference ($F/F_0$) was calculated and plotted against the concentration of cation in the sample solution. A control experiment was performed by repeating the procedure with the addition of the Tris-HCl solution to the sample cuvette and adding water to the reference, such that the ionic strength was allowed to change at constant protein concentration. No difference in fluorescence was observed, indicating that the concentration of Tris-HCl and the general ionic strength of the solution had no effect on the intrinsic fluorescence of APC.

## Results

Previous work from this laboratory has demonstrated that

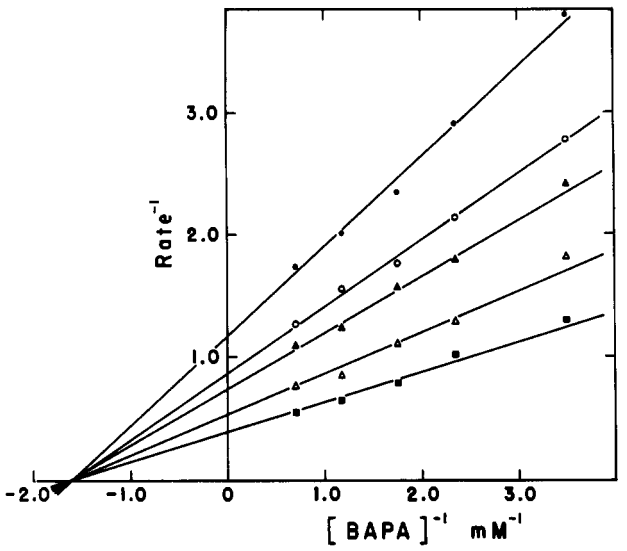



FIGURE 1: Lineweaver–Burk plots of initial rates of BAPA hydrolysis of APC at varying substrate levels at a series of fixed concentrations of $Na^+$. The initial rates are expressed as nanomoles of BAPA cleaved per minute. $Na^+$ concentrations are (●) 40, (○) 50, (▲) 60, (△) 75, and (■) 100 mM.

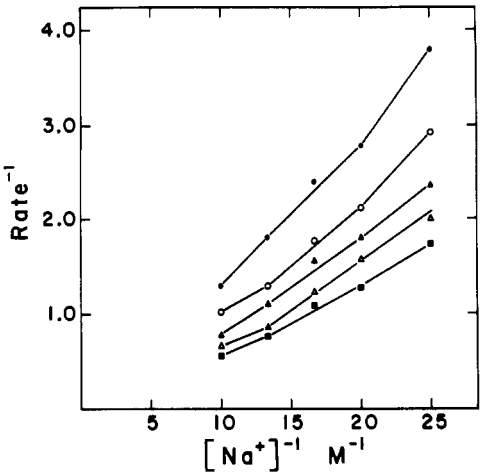



FIGURE 2: Lineweaver–Burk plots of the initial rates of BAPA hydrolysis by APC at varying $Na^+$ levels at a series of fixed concentrations of BAPA. The initial rates are expressed as nanomoles of BAPA cleaved per minute. BAPA concentrations are (●) 285, (○) 428, (▲) 570, (△) 855, and (■) 1425 μM.

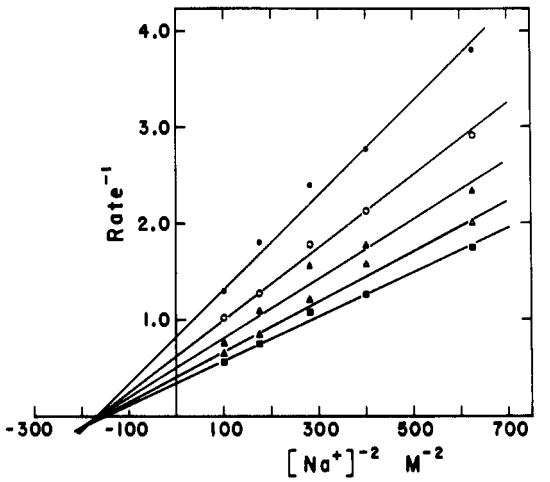



FIGURE 3: Lineweaver–Burk plots of the initial rates of BAPA hydrolysis by APC at varying $Na^+$ levels at a series of fixed concentrations of BAPA, plotted as $[Na^+]^{-2}$. The initial rates are expressed as nanomoles of BAPA cleaved per minute. BAPA concentration are (●) 285, (○) 428, (▲) 570, (△) 855, and (■) 1425 μM.

the hydrolysis by APC of two tripeptide substrates (Steiner et al., 1980; Steiner & Castellino, 1982) and a synthetic ester substrate (Steiner et al., 1980) was strictly dependent upon the presence of cations. Monovalent cations allowed substantially higher final activities to be reached for this enzyme, when compared to divalent cations. In the present investigations we sought a mechanism for this effect and have employed for kinetic measurements a simple amino acid amide substrate, BAPA, in order to reduce concerns regarding possible binding, with resultant kinetic influence, of cations to peptide bonds of the substrate.

As with the tripeptide substrate, D-phenylalanylpipecolylarginine-*p*-nitroanilide (S-2238), the rate of hydrolysis, by APC, of BAPA was strictly dependent upon the presence of cations in the reaction medium. This was demonstrated by results of an experiment in which the concentration of a poor activating cation, $Tris^+$, was varied from 25 to 160 mM, at pH 7.4. A plot of the initial hydrolytic rates against the Tris-HCl concentration yielded a straight line that could be extrapolated through the origin.

Initial rates of hydrolysis by APC of BAPA, in the presence of varying BAPA concentrations and in the presence of two activating cations, $Na^+$ and $Cs^+$, have been determined. Figure 1 illustrates the results of experiments for $Na^+$, plotted in double-reciprocal form, in which initial BAPA hydrolytic rates were determined at various concentrations of BAPA, at a series of concentrations of $Na^+$. As is evident from the graph, a family of straight lines was obtained in each case, which essentially intersect at the abscissa, indicating that the effect of the cation is on the $V_{max}$(app) of APC and not on the $K_m$ for BAPA. The behavior of $Cs^+$ in this regard is qualitatively similar to that of $Na^+$.

Figure 2 depicts the results of replots of the data of Figure 1 in which the reciprocals of the initial rates of hydrolysis of BAPA by APC are plotted against the reciprocals of the $Na^+$ concentrations, at various fixed levels of BAPA. Here, a series of curved lines are obtained, showing that the dependency of the initial BAPA hydrolytic rates on cation concentration is a complex function of cation levels. A similar family of graphs was obtained from experiments with $Cs^+$. As can be seen from the data of Figure 3, however, straight lines are produced when these same rate data are plotted against the reciprocal of the square of the $Na^+$ concentration. The same situation exists with $Cs^+$ (not shown). Hill plots of the same data were constructed, and Hill coefficients were calculated to be 1.9 for $Na^+$ and 2.0 for $Cs^+$. This indicates that the activity of APC toward BAPA is cooperative with regard to cation binding, with a minimum of two cation sites, or classes of sites, of importance to the kinetic mechanism of the cation effects.

A double-reciprocal replot of the values for the $V_{max}$(app) for various BAPA concentrations, taken from the ordinates of Figure 3 and from similar plots for $Cs^+$, against the substrate concentrations, for $Na^+$ and $Cs^+$, respectively, is illustrated in Figure 4. Similarly, a replot of the reciprocal values for the $V_{max}$(app) for various cation concentrations, taken from the ordinates of Figure 1 and from similar plots with $Cs^+$, against the reciprocal of the square of the cation concentrations for $Na^+$ and $Cs^+$, respectively, is shown in Figure 5.

The values for the $K_m$ for BAPA to APC with $Na^+$ and $Cs^+$ have been obtained from the abscissas of Figure 4. These values are 0.90 ± 0.10 mM with saturating $Na^+$ and 0.43 ± 0.05 mM with saturating $Cs^+$. For each cation, the $V_{max}$ of APC toward BAPA, at saturating substrate and cation concentrations, has been calculated from the ordinate of Figure 4 to be 0.32 ± 0.05 $s^{-1}$. The $K_m$ for the metal cation(s) that

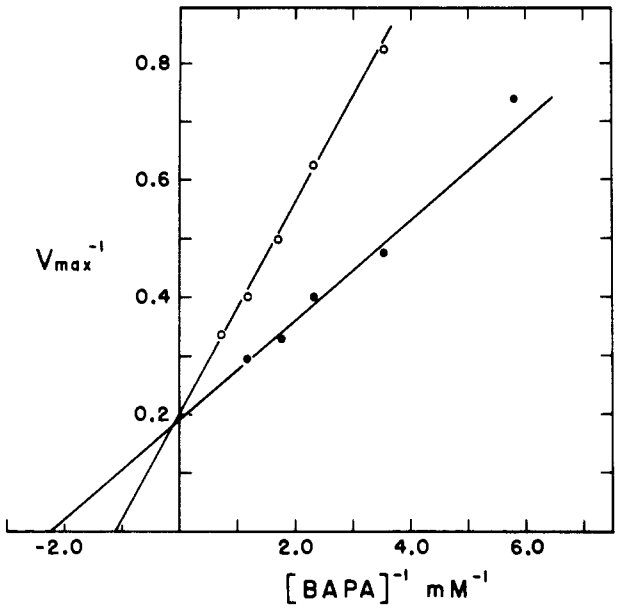



FIGURE 4: Replot of $V_{max}$(app) for various BAPA concentrations, taken from the $y$ intercepts of Figure 3 for $Na^+$ (○) and from similar data with $Cs^+$ (●), against the concentrations of BAPA.

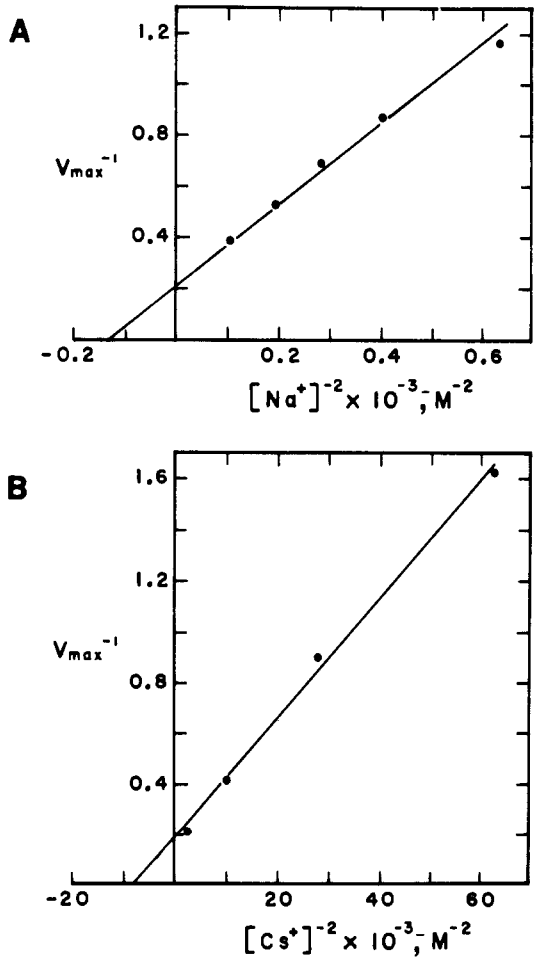



FIGURE 5: Replot of $V_{max}$(app) for various cation concentrations, taken from the $y$ intercepts of Figure 1 for $Na^+$ (A) and from similar data for $Cs^+$ (B), against the $[cation]^{-2}$.

is (are) relevant to the catalytic event has been calculated from Figure 5 as $-(abscissa)^{-1/2}$ (this approach will be justified under Discussion). The value of $Na^+$, at saturating BAPA, is 87 ± 8 mM, and this same parameter for $Cs^+$, at saturating BAPA, has been calculated to be 11 ± 1 mM. Similarly, the $V_{max}$ of APC toward BAPA, at saturating substrate and cation concentrations, has been calculated from the reciprocals of either of the ordinates of Figure 5. This value is 0.32 ± 0.05 $s^{-1}$ in each case, which is the same as the $V_{max}$ obtained by the same method from the data of Figure 4, showing the consistency of the data. A summary of these kinetic constants is provided in Table I.

Table I: Steady-State Kinetic Constants at 30 °C for APC toward BAPA with $Na^+$ and $Cs^+$ as Activating Cations

| cation | $K_m$(cation) (mM) | $K_m$(substrate) (mM) | $k_{cat}$ ($s^{-1}$) |
|---|---|---|---|
| $Na^+$ | 87 ± 8 | 0.90 ± 0.10 | 0.32 ± 0.05 |
| $Cs^+$ | 11 ± 1 | 0.43 ± 0.05 | 0.32 ± 0.05 |

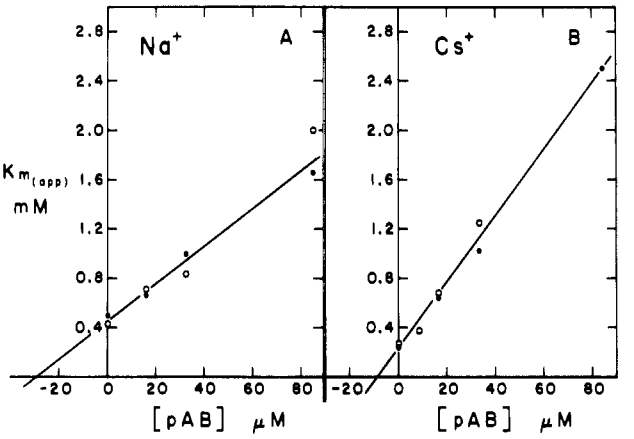



FIGURE 6: Determination of the $K_I$ for $p$-aminobenzamidine (pAB) at different concentrations of $Na^+$ and $Cs^+$. The apparent $K_m$ values for the substrate, D-phenylalanylpipecolylarginine-$p$-nitroanilide (S-2238), to APC, at various levels of pAB, are plotted against (pAB). The values for $K_m$(app) were obtained from Lineweaver–Burk plots of the variation of initial hydrolysis rates of S-2238 by APC with [S-2238], at different [pAB] and [cation]. (A) The concentrations of $Na^+$ were (○) 50 and (●) 240 mM. (B) As in (A) except that the cation was $Cs^+$.

We wished next to determine whether the direct binding of substrate to APC was influenced by the concentration and/or the nature of the metal cation. In order to do so, we measured the $K_I$ values for a competitive inhibitor of APC, $p$-aminobenzamidine (pAB), to APC at concentrations of $Na^+$ and $Cs^+$ at which the activity of APC was differentially affected. Here, the substrate employed was S-2238, due to its ease of handling in the assay. Lineweaver–Burk plots of the initial rates of substrate hydrolysis measured as a function of substrate concentration, at different levels of inhibitor, were constructed. The cation concentrations were 50 and 240 mM for $Na^+$ and $Cs^+$. The apparent $K_m$ values at each inhibitor level, calculated from the abscissas of such graphs, have been plotted against the concentration of inhibitor, in order to obtain the $K_I$ value of pAB to APC. The graphs for each concentration of $Na^+$ and $Cs^+$ are presented in panels A and B of Figure 6, respectively. Although the enzymic activity of APC is greatly different at each level of $Na^+$, the $K_I$ values for $p$-aminobezamidine are virtually identical, with values of approximately 33 μM. In the case of $Cs^+$, the $K_I$ values for this inhibitor, at 50 and 240 mM $Cs^+$, are very similar and of the magnitude of approximately 9 μM.

During the course of these studies, it was discovered that monovalent cations produced an alteration in the intrinsic fluorescence of APC. This finding was utilized as a direct means to measure the binding of $Na^+$ to APC, such that comparisons to the same binding parameters as determined by kinetic means could be made. The intrinsic fluorescence

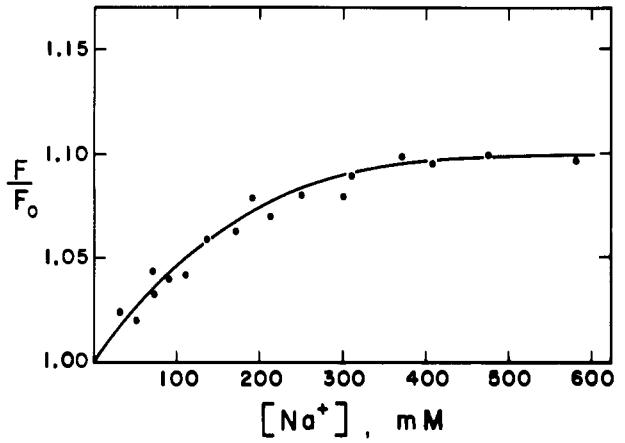



FIGURE 7: Effect of NaCl on the intrinsic fluorescence of APC. In this experiment, a solution of 0.1 mg/mL of APC was titrated with NaCl and the intrinsic fluorescence of each sample determined. The excitation wavelength was 283 nm, and emission was measured at 332 nm. The data are plotted as the ratio of the observed fluorescence ($F$) to the fluorescence of a reference sample ($F_0$) containing an equal concentration of protein, against [NaCl].

of a solution of APC, containing increasing concentrations of $Na^+$, was measured and compared to a reference solution containing an identical protein concentration and ionic strength. The ratio of fluorescence of the sample solution to that of the reference ($F/F_0$) was plotted against the concentration of $Na^+$ in the solution. The results, illustrated in Figure 7, demonstrate that there is an increase in the intrinsic fluorescence of APC, with the addition of $Na^+$. This effect of $Na^+$ is saturable, with a maximal fluorescence increase of approximately 10%. The magnitude of this change indicates the occurrence of a significant conformational alteration, which results in translocation of at least one tryptophan residue, upon cation binding. The concentration of $Na^+$ that produces half of the maximum fluorescence enhancement is approximately 120 mM. This value can be compared to the $K_m$ for $Na^+$ in hydrolysis of S-2238 (Steiner & Castellino, 1982) and BAPA (Table I) of approximately 129 mM and 87 mM, respectively. The close correlation of these values suggest that the effect of the conformational alteration produced by $Na^+$ is responsible for the activation of APC toward synthetic substrates.

A Hill plot of the fluorescence data of Figure 7 was constructed in order to determine whether the cooperativity seen in the kinetic analysis was also present in the conformational alteration. The Hill coefficient was calculated to be 1.09, a value that indicates that such cooperativity is not present. This implies that a single monovalent cation site, or class of sites, produces the conformational alteration that is monitored by the fluorescence changes.

The effect of $Na^+$ on the activity of APC toward a natural substrate, factor VIIIa, was evaluated next. Here, APC, in the absence and presence of several concentrations of NaCl, was incubated with factor VIIIa. Aliquots of this mixture were removed at various time periods and assayed for factor VIIIa activity by the coagulant assay described under Materials and Methods. Prior to such evaluation, the samples were diluted approximately 10000-fold with the assay buffer. The results of this study are depicted in Figure 8 and demonstrate that virtually the same rate of inactivation of factor VIIIa occurs in the absence and presence of $Na^+$. In evaluating the effect of $Na^+$ on this process, it is important to note that the concentration of $Ca^{2+}$ in the inactivation mixture is 250 mM. Since divalent cations were shown to substitute to a limited extent for monovalent cations in activation of APC toward synthetic amide substrates (Steiner et al., 1980), it is possible

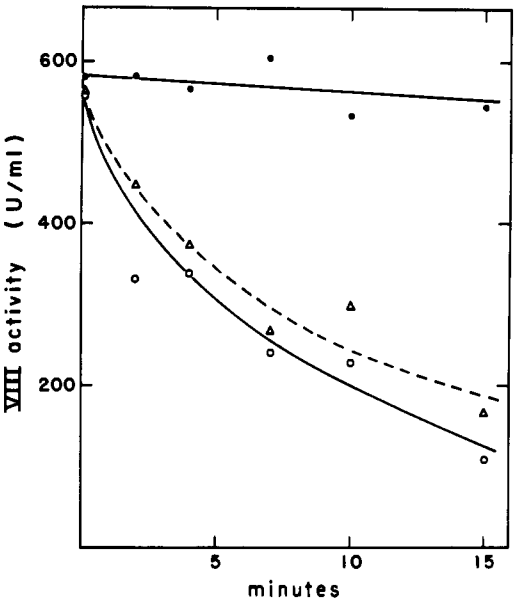



FIGURE 8: Inactivation of factor VIIIa by APC. The activity of factor VIIIa, expressed as units/mL, following incubation with APC in the absence (○) of NaCl and in the presence (△) of 210 mM NaCl was measured by a factor VIII dependent coagulation assay. A control experiment (●) was simultaneously performed in the absence of APC.

that the high level of $Ca^{2+}$ masks the effect of $Na^+$ in this system. However, the instability of factor VIIIa activity in the absence of high levels of $Ca^{2+}$ creates a highly unfavorable situation in attempting to perform the experiment in the absence of $Ca^{2+}$. Importantly, since $Na^+$ further enhances the effect of $Ca^{2+}$ in stimulation of the amidolytic activity of APC toward synthetic substrates but does not do so when factor VIIIa is the substrate, the data strongly suggest that the activity of APC toward its natural substrate does not depend upon the presence of monovalent cations.

## Discussion

Considerable interest has been shown toward PC and APC since their discovery, due to the important control influence that APC exerts on the coagulation of blood and due to the direct stimulation by APC of a fibrinolytic state in animals. Further, since our discovery (Steiner et al., 1980) that the amidolytic and esterolytic activities of APC are expressed only in the presence of cations and since monovalent cations played a particularly relevant role in this regard, this enzyme has gained a new basic significance. Monovalent cations have been known for some time to modulate the activity of certain enzymes. Suelter (1970) has categorized monovalent cation dependent enzymes into two main classes, according to the general type of reaction catalyzed by those enzymes: phosphoryl-transfer reactions and elimination reactions, where, in the latter, a keto–enol tautomer is either the product or a logical intermediate of the reaction. Pyruvate kinase is a classic example of the first category. In this case, no enzymic activity is detectable in the absence of monovalent cations, and $K^+$, $NH_4^+$, $Rb^+$, and $Tl^+$ are the best activators. It has been suggested that the cation serves as a bridge between the enzyme and substrate in this process (Mildvan et al., 1967). The second class of enzymes, elimination enzymes, which displays a dependence on monovalent cations, can be represented by tryptophanase (Hogberg-Raibaud et al., 1975). As in the case of pyruvate kinase, tryptophanase possesses no detectable enzymic activity in the absence of monovalent cations and is activated best by $NH_4^+$ and $K^+$ and to a much lesser extent by $Na^+$ and $Li^+$ (Suelter & Snell, 1977). While the mecha-

Table II: Summary of Possible Mechanistically Derived Rate Equations for Hydrolysis by APC of BAPA in the Presence of Monovalent Cations[a]

| mechanism | rate eq |
|---|---|
| (1) random binding of A, S, A | $\frac{1}{v}=\frac{1}{[S]}\frac{K_s}{V_{max}}\left(\frac{K_a^2}{[A]^2}+2\frac{K_a}{[A]}+1\right)+\frac{1}{V_{max}}\left(\frac{K_a^2}{[A]^2}+2\frac{K_a}{[A]}+1\right)$ |
| (2) S binding first, A and A random | $\frac{1}{v}=\frac{1}{[S]}\frac{K_s}{V_{max}}\frac{K_a^2}{[A]^2}+\frac{1}{V_{max}}\left(\frac{K_a}{[A]^2}+\frac{K_a}{[A]}+1\right)$ |
| (3) A binding first, S and A random | $\frac{1}{v}=\frac{1}{[S]}\frac{K_s}{V_{max}}\left(\frac{K_a^2}{[A]^2}+\frac{K_a}{[A]}+1\right)+\frac{1}{V_{max}}\left(\frac{K_a}{[A]}+1\right)$ |
| (4) A and A random, S binding second | $\frac{1}{v}=\frac{1}{[S]}\frac{K_s}{V_{max}}\left(\frac{K_a^2}{[A]^2}+2\frac{K_a}{[A]}\right)+\frac{1}{V_{max}}\left(2\frac{K_a}{[A]}+1\right)$ |
| (5) S and A random, A binding second | $\frac{1}{v}=\frac{1}{[S]}\frac{K_s}{V_{max}}\left(\frac{K_a^2}{[A]^2}+\frac{K_a}{[A]}+1\right)+\frac{1}{V_{max}}\left(\frac{K_a^2}{[A]^2}+\frac{K_a}{[A]}+1\right)$ |
| (6) A and A random, S binding third | $\frac{1}{v}=\frac{1}{[S]}\frac{K_s}{V_{max}}\left(\frac{K_a^2}{[A]^2}+2\frac{K_a}{[A]}+1\right)+\frac{1}{V_{max}}$ |
| (7) A binding third, S and A random | $\frac{1}{v}=\frac{1}{[S]}\frac{K_s}{V_{max}}\left(\frac{K_a^2}{[A]^2}+\frac{K_a}{[A]}\right)+\frac{1}{V_{max}}\left(\frac{K_a^2}{[A]^2}+\frac{K_a}{[A]}+1\right)$ |
| (8) ordered A, A, S | $\frac{1}{v}=\frac{1}{[S]}\frac{K_s}{V_{max}}\left(\frac{K_a^2}{[A]^2}+\frac{K_a}{[A]}+1\right)+\frac{1}{V_{max}}$ |
| (9) ordered A, S, A | $\frac{1}{v}=\frac{1}{[S]}\frac{K_s}{V_{max}}\left(\frac{K_a}{[A]^2}+\frac{K_a}{[A]}\right)+\frac{1}{V_{max}}\left(\frac{K_a}{[A]}+1\right)$ |
| (10) ordered S, A, A | $\frac{1}{v}=\frac{1}{[S]}\frac{K_s}{V_{max}}\frac{K_a^2}{[A]^2}+\frac{1}{V_{max}}\left(\frac{K_a^2}{[A]^2}+\frac{K_a}{[A]}+1\right)$ |

[a] Equations were derived from the rate expressions described in Segel (1975). Abbreviations: A, activating cation; S, substrate. All others are standard.

nism for monovalent cation participation in tryptophanase activity is not known, it appears as though cations somehow are involved in the catalytic step, as the substrate will bind to the enzyme in the absence of such cations (Suelter & Snell, 1977).

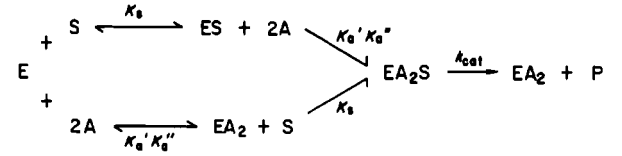
A small number of enzymes that have been found to be sensitive to monovalent cations cannot be placed in either of the two classifications described above. These include several peptidases, such as the ribosomal peptidase from *Escherichia coli* (Tsai & Matheson, 1965), as well as thrombin (Orthner & Kosow, 1980) and factor Xa (Orthner & Kosow, 1978) from human plasma. In the case of the bacterial peptidase, the presence of monovalent cations was not a strict requirement for the enzyme, and only relatively small stimulation occurred upon their addition (Tsai & Matheson, 1965). Similarly, neither thrombin nor factor Xa showed an absolute requirement for monovalent cations. Further, of those cations studied, only $Na^+$ and $K^+$ stimulated thrombin, whereas $Na^+$ was the sole activator for factor Xa.

In contrast to these other proteolytic enzymes, the activities of which are enhanced by monovalent cations, APC activity toward small ester and amide substrates has been found to be strictly dependent upon monovalent cations (Steiner et al., 1980). In addition, a wide variety of monovalent cations possessed this property, and their relative effectiveness in this regard (except for $Tl^+$) was in parallel with their ionic radii, a classic Hofmeister phenomenon. In a previous study of the kinetic behavior of monovalent cations toward APC hydrolysis of a tripeptide substrate (Steiner & Castellino, 1982), we found that the effect shown by each cation, except $Tl^+$ (unpublished observation), was cooperative with a minimum of two sites, or classes of sites, of kinetic importance. In this paper, we have evaluated possible kinetic mechanisms for participation of monovalent cations in APC activity toward small substrates. Prior to performing this analysis, we evaluated the kinetic properties of monovalent cations in APC activity by using a much simpler amino acid substrate, which was less likely to bind monovalent cations than the previously employed tripeptide substrates.

From the collective data presented in Figure 1–5, it is concluded that APC is inactive toward BAPA in the absence of monovalent cations, that these cations cause an increase in the $k_{cat}$ for this hydrolytic reaction, without altering the $K_m$ for BAPA, that the $k_{cat}$ for the reaction is independent of the identity of the cation employed, whereas the $K_m$ shows a slight dependency on the nature of the cation, and that at least two cation sites, or classes of sites, are required for expression of APC amidolytic activity. These results are similar to those obtained with the tripeptide substrate and are of considerable further importance since they demonstrate that these effects are not dependent upon the nature of the small substrate. Additional evidence for this latter point has been obtained as a result of our observations (unpublished) that similar kinetic behavior is exhibited with the ester substrate $N^{\alpha}$-tosyl-L-arginine methyl ester and $N^{\alpha}$-(carboxybenzyl)tryptophanyl-arginine *S*-benzyl ester.

Assuming that the reaction of APC with BAPA involves a single site for substrate and two sites for activating (A) monovalent cations, we have attempted to fit the experimental initial rate data of Figures 1–5 to double-reciprocal equations obtained from a wide variety of possible mechanisms, for terreactant systems, as summarized in Table II. Comparisons of the rate equation for each mechanism with the experimental data of figures 1–5 allow several to be eliminated. Since the rate equation must account for a parabolic curve in the replot of $1/V_{max}$(app) against $1/[A]$ (Figure 2), mechanisms 3, 4, 6, 8, and 9 from Table II, wherein the cation must bind to the enzyme prior to the substrate, can be eliminated. Some of the

Scheme I




remaining mechanisms can be discarded on the basis of other evidence. Mechanisms 2, 7, and 10 of Table II do not account for the experimental data in Figure 1, since the derived rate equations would predict the lines of Figure 1 to intersect in the second quadrant, not on the abscissa, as in the case. Thus, the only mechanisms remaining from those of Table II predict either completely random binding of metal ions and substrate (mechanism 1) and/or random binding of substrate and metal ion as the first component, with obligatory binding of the metal ion as the second component (Mechanism 5). However, even these do not precisely fit the experimental data. Both predict that the initial rates of hydrolysis of BAPA in the presence of APC would vary in a linear fashion as a function of [A] + [A], which, as shown in Figure 3, is not the case.

An alternative general mechanism would be that only one of the cations binds to APC and the other to the substrate. Thus, the SA complex would be the true substrate. In this scheme, a plot of the (initial hydrolytic rates)$^{-1}$ against [S]$^{-1}$ would form lines that intersect in the second quadrant. This is not consistent with the observed data of Figure 1.

A rate equation for the terreactant system can be written that is most predictive of the experimental data. This is based upon the requirement for two cations to bind to the protein in order to form an active enzyme. The $EA_2S$ complex is the species required for catalysis to occur. The order of the binding is completely random; however, the cations must bind as a pair. The model that allows this is shown in Scheme I. Here, $K_s$ is the dissociation constant for S and $K_a'$ and $K_a''$ are similar constants for binding of the first and second cations, respectively. All other terms have been defined previously. In this model, the free enzyme (E) possesses a single catalytically important cation binding site. The binding of a cation to this site allows an alteration in APC, which creates a second cation site. The affinity of this site is tighter than the first such that this site is occupied at cation levels sufficient for binding to the first site, rendering the two cation sites kinetically indistinguishable. Therefore, the EA and EAS complexes do not exist at any concentration of cation and substrate. The overall effect is that cations bind in pairs, with a single observable binding constant of the first cation. A rate equation can be derived from these considerations and is expressed in double-recripocal form as

$$\frac{1}{v} = \frac{1}{[S]}\frac{K_s}{V_{max}}\left(\frac{K_a'K_a''}{[A]^2} + 1\right) + \frac{1}{V_{max}}\left(\frac{K_a'K_a''}{[A]^2} + 1\right)$$

This derivation is detailed in the Appendix. From this equation, it can be seen that a plot of $1/v$ vs. $1/[S]$ would be linear with slopes that decrease in value as a linear function of the square of the cation concentration and that intersect on the abscissa. These predictions are obeyed by the data in Figures 1–3.

Given this rate equation, the $K_m$ for the cation can be calculated from the experimental data of Figure 5. Here, the intercepts on the abscissa would be equal to $-1/(K_a'K_a'')$ for each cation. As shown in the Appendix, $K_a'K_a'' = [E][A]^2/[EA_2]$. When $[E] = [EA_2]$, $K_a'K_a'' = [A]^2$. Assuming that $EA_2$ is required for catalysis and that EA does not exist, then under conditions where $[A]^2 = K_a'K_a''$ ($x$ intercepts) half of E would exist as $EA_2$, and the reaction rate would be at half-maximal velocity (in the presence of S). Thus, the negative of the square roots of the $x$ intercepts of the data of Figure 5 would equal the $K_m$ for each cation. In order to evaluate this point further, initial reaction rates obtained with $Cs^+$ and $Na^+$ were plotted against the cation concentrations, and the values for the $K_{m,app}$(cation) were obtained from the cation levels required for half-maximal velocity. Close correlation was obtained in all cases with the $K_m$(cation) values calculated in the manner described above, a situation that lends support to the assumptions of the proposed rate equation.

The prediction of an initial cation binding site that induces a conformational alteration in APC is borne out by the data of Figure 7. Here, the intrinsic fluorescence of APC is altered by $Na^+$, suggestive of a conformational alteration in the protein. A Hill plot of the data indicates that a single cation is responsible for this effect. From titration with $Na^+$ of the $Na^+$-induced fluorescence increase in APC, the concentration of $Na^+$ that produces half of the conformational alteration (120 mM) has been determined. The value is not much different from the kinetically determined $K_m$ for $Na^+$ of approximately 129 mM, with S-2238 as the substrate (Steiner & Castellino, 1982), and of approximately 87 mM, with BAPA as the substrate (Table I). The close agreement of these values allows the conclusion to the reached that the effect of monovalent cations on the conformation of APC is the same as that which ultimately is responsible for the activation of the enzyme toward these substrates. This is also predicted by the mechanism proposed.

The kinetic mechanism forwarded in this paper requires that the substrate binds to the enzyme equally well at all cation concentrations, as well as to the free enzyme. This latter point cannot be established by kinetic means, since APC is inactive toward synthetic substrates in the absence of cations. However, we have attempted to evaluate whether the binding of a competitive inhibitor of APC, pAB, is altered at cation concentrations at which the activity of APC varies. The data of Figure 6 clearly show that this inhibitor possesses the same $K_I$ value for APC at differing levels of $Na^+$ and $Cs^+$. Therefore, although APC amidase activity is quite different at each of the cation concentrations employed, the binding of a competitive inhibitor is not affected. This observation is also consistent with the proposed mechanism.

While much of the experimental kinetic data is accurately predicted by the mechanism described, at least two aspects are not as clear. With BAPA as the substrate, the kinetic $K_m$ values for $Na^+$ and $Cs^+$ are approximately 87 mM and 11 mM, respectively (Table I). However, with a tripeptide substrate, S-2238, these values have been calculated to be approximately 130 mM and 41 mM, respectively (Steiner & Castellino, 1982). The mechanism predicts that the $K_m$ for the cation does not depend upon the nature of the substrate. While the observed differences are not large, and may not be meaningful, it is possible that the binding of the different substrates may induce different conformational alterations in APC, leading to slightly different binding constants for the cation. In addition, the mechanism does not treat the concept as to whether that the $K_m$ for the substrate should be dependent upon the nature of the monovalent cation. The data of Table I show that there is approximately a 2-fold difference in the $K_m$ for BAPA, with $Na^+$ and $Cs^+$ as the metal cations. A similar situation has been found with another substrate, S-2238 (Steiner & Castellino, 1982). While the differences in these parameters are not large, the results suggest that some variations in APC exist when bound to the various cations that

slightly affect its kinetic properties. This could be due to differences in the nature of the binding sites for the various cations on APC and/or slightly dissimilar conformational alterations produced by each cation, each with disparate effects on APC kinetic parameters. Further, some level of interaction of the substrate with the metal cation on the surface of APC, not otherwise kinetically detectable, which varies with the nature of the cation, is possible. While these aspects are not necessarily counter to the proposed mechanism, they are not treated by it. In any event, when a single cation is considered, the mechanism proposed for the role of the cation in APC hydrolysis of a synthetic substrates appears to agree with essentially all of the experimental data, and the mechanism proposed appears to be the most feasible one.

The question remains as to the molecular basis for the effect of monovalent cations on APC hydrolytic activity. We have shown that these cations function by altering the catalytic rate constant for the reaction. Hydrolysis by serine proteases, such as APC, is thought to occur by nucleophilic attack on the carbonyl carbon of the sensitive bond in the substrate by an active seryl hydroxyl group in the enzyme. The resultant carbonyl intermediate adopts a tetrahedral conformational, which is unstable and readily collapses, releasing the amine (or alcohol, if the substrate is an ester). Two manners in which the metal cation can influence this process are by (a) altering the enzyme such that the seryl hydroxyl group is a more efficient nucleophile, perhaps by influencing the charge-relay system, and/or (b) stabilizing the tetrahedral intermediate, effectively reducing the energy of the transition state. Since the carbonyl oxygen of the substrate possesses considerable negative charge in the tetrahedral intermediate in the transition state, a mechanism by which the monovalent cations can act to neutralize this charge would increase the stability of the intermediate. It can be imagined that the monovalent cation would be bound by the protein in such a fashion as to coordinate this oxygen and, thereby, stabilize the intermediate. A precedent for this exists in the role of $Zn^{2+}$ in stimulating carboxypeptidase A activity (Matthews et al., 1975). However, since monovalent cations do not coordinate oxygen very strongly, except in specific cases, such a mechanism should not be readily invoked. Another means for stabilization of the transition state may be a conformational change induced by the binding of a cation, allowing the carbonyl oxygen to form hydrogen bonds with protons of backbone nitrogen. Such hydrogen bonding has been suggested by crystallographic analysis of subtilisin (Pressman, 1968). It is possible that binding of monovalent cations to the APC is required to produce the proper conformation in the enzyme to allow similar hydrogen bonds to form. Since $Na^+$ has been shown to produce a conformational alteration in APC, such a mechanism has a high degree of plausibility.

Finally, we have investigated whether or not monovalent cations influence the activity of APC toward a physiological substrate, factor VIIIa. Due to constraints on the assay, by nature of the inherent properties of factor VIIIa, this study could only be performed in the presence of another cation stimulator, $Ca^{2+}$. Whereas the enzymic activity of APC toward small synthetic substrates, in the presence of $Ca^{2+}$, is still greatly stimulated by $Na^+$, no such additional stimulation was observed when factor VIIIa was the substrate (Figure 8). Thus, it would appear as though the active site of APC can accommodate large protein substrates but needs to be altered, likely conformationally, by metal cations in order for this enzyme to hydrolyze small substrates. This does not appear to be the case for any other protease thus far examined.

This investigation of the effect of monovalent cations on the activity of APC toward synthetic substrates has allowed a model for the effect of these cations to be proposed. The existence of at least two allosteric cation sites is an important feature of this mechanism. Such a mechanism for activation of a protease is unique, since no other such enzyme has been shown to be as strictly regulated by monovalent cations as is APC. Of all enzymes that have been shown to require monovalent cations in expression of activity, few have demonstrated such a wide specificity with regard to the cations. Due to these considerations, APC provides as excellent system for the study of the role of monovalent cations in enzymatic reactions.

ACKNOWLEDGMENTS

We thank Dr. Thomas L. Nowak for his helpful comments on the content of the manuscript.

## APPENDIX

*Derivation of a Rate Equation for the Role of Monovalent Cations in the Hydrolysis of Synthetic Substrates by APC.* The proposed mechanism involves reaction of the enzyme with two cations (A) and substrate (S) in order to form an active enzyme. The $EA_2S$ complex is the species that undergoes catalysis. The order of binding of the components is random, but the cations must bind pairwise. Such a model is depicted in Scheme I. Importantly, EA and EAS do not exist at any [S] and [A]. Derivation of a rate expression from this mechanism proceeds as follows:

$$\text{rate} = v = k_{cat}[EA_2S] \tag{1}$$

Since $[E]_T = [E]_f + [ES] + [EA_2] + [EA_2S]$, where $[E]_T$ and $[E]_f$ are the total and free enzyme concentrations, respectively, and [ES], $[EA_2]$, and $[EA_2S]$ are the concentrations of enzyme–substrate, enzyme–cation, and enzyme–cation–substrate complexes, respectively, then

$$\frac{v}{[E]_T} = \frac{k_{cat}[EA_2S]}{[E]_f + [ES] + [EA_2] + [EA_2S]} \tag{2}$$

The appropriate equilibrium constants of importance can be summarized as

$$K_s = \frac{[E]_f[S]}{[ES]} \text{ or } \frac{[EA_2][S]}{[EA_2S]} \tag{3}$$

$$K_a' = \frac{[E]_f[A]}{[EA]} \text{ or } \frac{[ES][A]}{[EAS]} \tag{4}$$

$$K_a'' = \frac{[EA][A]}{[EA_2]} \text{ or } \frac{[ESA][A]}{[EA_2S]} \tag{5}$$

$$K_a'K_a'' = \frac{[E]_f[A]^2}{[EA_2]} \text{ or } \frac{[ES][A]^2}{[EA_2S]} \tag{6}$$

Rearranging the above equations in order to solve for $[EA_2S]$, [ES], and $[EA_2]$ and placing the resulting equations in eq 2, eq 7 is obtained. Dividing by $[E]_T$ and designating $k_{cat}[E]_T = V_{max}$, the following is obtained:

$$\frac{v}{[E]_T} = \frac{k_{cat}\dfrac{[E][A]^2[S]}{K_a'K_a''K_s}}{[E]_T + \dfrac{[E]_f[S]}{K_s} + \dfrac{[E]_f[A]^2}{K_a'K_a''} + \dfrac{[E]_f[A]^2[S]}{K_a'K_a''K_s}} \tag{7}$$

$$\frac{v}{V_{max}} = \frac{\dfrac{[A]^2[S]}{K_a'K_a''K_s}}{1 + \dfrac{[S]}{K_s} + \dfrac{[A]^2}{K_a'K_a''} + \dfrac{[A]^2[S]}{K_a'K_a''K_s}} \tag{8}$$

Rearranging eq 8 to provide double-reciprocal rate equations, yields

$$\frac{1}{v} = \frac{1}{[A]^2}\frac{K_a'K_a''}{V_{max}}\left(\frac{K_s}{[S]} + 1\right) + \frac{1}{V_{max}}\left(\frac{K_s}{[S]} + 1\right) \quad (9)$$

and

$$\frac{1}{v} + \frac{1}{[S]}\frac{K_s}{V_{max}}\left(\frac{K_a'K_a''}{[A]^2} + 1\right) + \frac{1}{V_{max}}\left(\frac{K_a'K_a''}{[A]^2} + 1\right) \quad (10)$$

In all cases, above, the total [S] and [A] are presumed to be equal to their free concentrations. The initial concentrations of those components are, therefore, used for all calculations.

**Registry No.** APC, 42617-41-4; BAPA, 6208-93-1; S-2238, 62354-65-8; pAB, 3858-83-1; Na, 7440-23-5; Cs, 7440-46-2; factor VIIIa, 72175-66-7.

## REFERENCES


Amphlett, G. W., Kisiel, W., & Castellino, F. J. (1981) *Biochemistry 20*, 2156–2161.

Chase, T., & Shaw, E. (1969) *Biochemistry 8*, 2212–2214.

Comp, P. C., & Esmon, C. T. (1981) *J. Clin. Invest. 68*, 1221–1228.

Esmon, C. T., & Owen, W. G. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 2249–2252.

Fernlund, P., & Stenflo, J. (1982) *J. Biol. Chem. 257*, 12170–12179.

Griffin, J. H., Evett, B., Zimmerman, T. S., Kleiss, A. J., & Wideman, C. (1981) *J. Clin. Invest. 68*, 1370–1373.

Hogberg-Raibaud, A., Raibaud, O., & Goldberg, M. E. (1975) *J. Biol. Chem. 250*, 3352–3358.

Kisiel, W., Ericsson, L. H., & Davie, E. W. (1976) *Biochemistry 15*, 4893–4900.

Kisiel, W., Canfield, W. M., Ericsson, L. H., & Davie, E. W. (1977) *Biochemistry 16*, 5834–5831.

Legaz, M. E., & Davie, E. W. (1976) *Methods Enzymol. 45*, 83–89.

Link, R. L., & Castellino, F. J. (1983) *Biochemistry 22*, 4033–4041.

Lundblad, R. L., Kingdon, H. S., & Mann, K. G. (1976) *Methods Enzymol. 45* 156–176.

Matthews, D. A., Alden, R. A., Birktoft, J. J., Freer, S. T., & Kroft, J. (1975) *J. Biol. Chem. 250*, 7120–7126.

Mildvan, A. S., Leigh, J. S., & Cohn, M. (1967) *Biochemistry 6*, 1805–1818.

Orthner, C. L., & Kosow, D. P. (1978) *Arch. Biochem. Biophys. 185*, 400–406.

Orthner, C. L., & Kosow, D. P. (1980) *Arch. Biochem. Biophys. 202*, 63–75.

Owen, W. G., & Esmon, C. T. (1981) *J. Biol. Chem. 256*, 5532–5535.

Pfleiderer, G. (1970) *Methods Enzymol. 19*, 514–521.

Pressman, B. C. (1968) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 27*, 1283–1288.

Segel, I. H. (1975) *Enzyme Kinetics*, pp 338–339, Wiley, New York.

Steiner, S. A., & Castellino, F. J. (1982) *Biochemistry 21*, 4609–4614.

Steiner, S. A., Amphlett, G. W., & Castellino, F. J. (1980) *Biochem. Biophys. Res. Commun. 94*, 340–347.

Stenflo, J. (1976) *J. Biol. Chem. 251*, 355–363.

Stenflo, J., & Fernlund, P. (1982) *J. Biol. Chem. 257*, 12180–12190.

Suelter, C. H., & Snell, E. E. (1977) *J. Biol. Chem. 252*, 1852–1857.

Tsai, C. S., & Matheson, A. T. (1965) *Can. J. Biochem. 43*, 1643–1652.

Vehar, G. A., & Davie, E. W. (1980) *Biochemistry 19*, 401–410.

Walker, F. J., Sexton, P. W., & Esmon, C. T. (1979) *Biochim. Biophys. Acta 571*, 333–342.