Kinetic Studies of the Role of Monovalent Cations in the Amidolytic Activity of Activated Bovine Plasma Protein C†

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ABSTRACT: The interaction of monovalent cations with activated bovine plasma protein C (APC) has been examined by kinetic methods, with H-D-phenylalanylpipecolylarginine-*p*-nitroanilide (S-2238) being employed as the substrate. By use of a kinetic model in which it is assumed that two monovalent cation sites (or classes of sites) need to be occupied for the catalytic event to occur, it has been shown that the $K_{m,app}$ ± 10% of S-2238, at saturating cation concentrations, is dependent upon the nature of the cation and decreases in parallel with increasing alkali cation radius, according to the following series ($K_{m,app}$ for S-2238 in parentheses): Li^+ (630 μM) > Na^+ (220 μM) > K^+ (189 μM) > Cs^+ (70 μM). For the cation NH_4^+ at saturating cation levels, the $K_{m,app}$ for S-2238 is 270 μM. The $K_{m,app}$ ± 10% for the cation has been determined at saturating S-2238 levels and a similar trend is noted. On the basis of the values obtained, the cation order ($K_{m,app}$ for cation at saturating S-2238 levels in parentheses) is as follows: Li^+ (182 mM) > Na^+ (129 mM) > K^+ (55 mM) > Cs^+ (41 mM). The $K_{m,app}$ for NH_4^+ at saturating S-2238 concentrations is 70 mM. These data indicate that an active participation of the cation in the amidolytic activity of APC exists, which is correlated with the ionic radius of the cation.

Protein C (PC), a protease zymogen that requires vitamin K for its functional biosynthesis, has been purified from bovine (Stenflo, 1976) and human (Kisiel, 1979) plasmas. This glycoprotein consists of two polypeptide chains, linked by disulfide bonds (Stenflo, 1976). From analysis of the amino acid sequences (Fernlund et al., 1978; Fernlund & Stenflo, 1979) and carbohydrate compositions [listed in Kisiel & Davie (1981)], the molecular weight of bovine PC has been calculated to be approximately 54 300, the component heavy chain possessing a molecular weight of approximately 33 800 and the corresponding light chain possessing a molecular weight of 20 500 (Kisiel & Davie, 1981). Approximately 14% of the total mass of PC consists of carbohydrate, which is distributed among the component heavy and light chains (Kisiel et al., 1976). The γ-carboxyglutamic acid residues, a necessary feature of vitamin K dependent blood coagulation proteins, originate from the first 11 glutamic acid residues of the light chain of bovine plasma PC (Fernlund et al., 1978). This same light chain has been shown to bind Ca^{2+} (Stenflo, 1976), again in common with other γ-carboxyglutamic acid containing proteins. The Ca^{2+} binding isotherms of bovine PC, at 25 °C, have been determined previously (Amphlett et al., 1981). This protein contains approximately 16 equivalent Ca^{2+} binding sites, of average K_D of 8.7×10^{-4} M.

PC is converted into a serine protease, activated protein C (APC), as a result of proteolytic cleavage of a peptide bond between Arg-14 and Ile-15, with concomitant release of a tetradecapeptide (Kisiel et al., 1976). At least three enzymes, i.e., α-thrombin (Kisiel et al., 1977), the factor X activating enzyme from Russell's viper venom, RVV-X (Kisiel et al., 1976), and trypsin (Kisiel et al., 1976), have been shown to catalyze this cleavage. The dependence of the activation rate of PC, by α-thrombin on divalent metal ions, is complex. Calcium and other divalent cations stimulate this rate in the presence of a protein cofactor isolated from rabbit lung endothelial cells (Esmon et al., 1982). In the absence of this protein cofactor, these same divalent metal ions inhibit the activation of PC by α-thrombin (Amphlett et al., 1981). In contrast, the activation of PC by RVV-X is augmented by Ca^{2+} and other divalent metals (Amphlett et al., 1981).

APC contains a diisopropyl fluorophosphate (iPr_2PF) reactive serine residue in its heavy polypeptide chain (Kisiel et al., 1976). This enzyme possesses amidolytic activity (Kisiel et al., 1976, 1977) and esterolytic activity (Steiner et al., 1980) toward synthetic substrates. Interestingly, we have originally shown that these activities have an absolute requirement for monovalent cations and are further stimulated by divalent cations (Steiner et al., 1980). These features were recently reproduced for another synthetic amide substrate (Ohno et al., 1981). In whole plasma, APC possesses anticoagulation activity (Kisiel et al., 1977). The basis for this effect likely resides in the ability of APC to inactivate coagulation cofactors Va (Kisiel et al., 1977; Walker et al., 1979) and VIIIa (Vehar & Davie, 1980). In the case of factor Va inactivation, it has been shown that this process is the result of proteolysis of factor Va, catalyzed by APC, and that both Ca^{2+} and phospholipid are required in this reaction (Walker et al., 1979). Recently, it has been demonstrated that factor Xa exhibits a protective effect on factor Va toward inactivation by APC (Nesheim et al., 1982). In addition to the anticoagulant activity of APC, it has been found that this enzyme induces a profibrinolytic state, when administered intravenously in dogs. It is believed that this property is due to elevation of the plasma levels of plasminogen activator (Comp & Esmon, 1981). Since APC appears so important in control of coagulation and fibrinolysis, it is expected that congenital abnormalities in this protein would lead to thrombotic states, as has been discovered (Griffin et al., 1981).

Due to our finding (Steiner et al., 1980) that both monovalent cations were critical to expression of activity of APC toward synthetic substrates, we have extended our kinetic analysis of this effect in the present paper. We believed that a more thorough understanding of this phenomena was necessary prior to application of this knowledge to possible cation control mechanisms with natural substrates.

Materials and Methods

Materials. All materials utilized were obtained from usual commercial sources. Heparin was purchased from Sigma

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Chemical Co. For preparation of the Sepharose–heparin affinity chromatography column, approximately 1 g of heparin was added to 50 mL of Sepharose 4B, activated with CNBr, as described by Sodetz & Castellino (1975). All monovalent cation salts were purchased as ultrapure products from Ventron.

Proteins. Bovine plasma PC was isolated by slight modifications of the procedure of Stenflo (1976). Bovine blood was collected in 10-L buckets, containing 1 L of an anticoagulant solution, consisting of 0.1 M trisodium citrate/0.1 M benzamidine hydrochloride, and 50 mg of crude soybean trypsin inhibitor. The plasma was then separated from the blood cells with a continuous-flow separator (Westfalia Model LG-205) at room temperature. All subsequent purification steps were carried out at 4 °C, with approximately 20-L of plasma being employed.

PC was adsorbed to barium citrate by addition of 80 mL of 1 M $BaCl_2$/L of plasma, dropwise, with continuous stirring, over a 30-min period, as described by Stenflo (1976). The precipitate was collected by continuous-flow centrifugation, as above. The precipitate was then washed twice with a cold solution of 0.1 M NaCl/5 mM benzamidine hydrochloride, pH 7.5 (~200 mL/L of starting plasma), and the precipitate was collected. PC was eluted from the barium cake upon addition of a solution consisting of 0.25 M EDTA[1]/5 mM benzamidine, pH 7.4 (~150 mL/L of starting plasma). To the supernate was added solid $(NH_4)_2SO_4$ to achieve 40% saturation (243 g/L), and the precipitate was discarded. Solid $(NH_4)_2SO_4$ was again added to the supernate, to a final concentration of 70% saturation (additional 205 g/L), after which the precipitate was retained. The precipitate was dissolved in a buffer consisting of 0.1 M sodium phosphate/1 mM benzamidine hydrochloride, pH 6.0, and the solution was equilibrated with this buffer by dialysis at 4 °C. Insoluble material was removed from the solution by centrifugation.

DEAE-Sephadex A50 ion-exchange chromatography was performed on the solution, according to the procedure described by Stenflo (1976). Fractions containing PC were identified by nonreduced $DodSO_4$ gel electrophoresis (Weber & Osborn, 1969). In our hands, PC was not separated from prothrombin by this procedure, as it was in the procedure described by Stenflo (1976), and PC always appeared on the trailing edge of the prothrombin peak. Thus, further purification was necessary.

Fractions containing PC were pooled, and the final solution contained approximately an equal mixture of prothrombin and PC. The solution was equilibrated against a buffer containing 50 mM Bis-Tris/10 mM $CaCl_2$/1 mM benzamidine hydrochloride, pH 6.0, by extensive dialysis and was applied to a column (1 cm × 35 cm) of Sepharose–heparin previously equilibrated with the dialysis buffer, at 4 °C. Protein was eluted from the column by a linear NaCl gradient. The starting buffer consisted of 200 mL of column equilibration buffer, and the limit buffer was 200 mL of the equilibration buffer, to which was added NaCl to a final concentration of 0.6 M. Fractions (5 mL) were collected at a flow rate of 20 mL/h. Two protein peaks were obtained and separately pooled. The first was clearly identified as PC, by both reduced and nonreduced $DodSO_4$ gel electrophoresis, and the second was found to contain prothrombin. The PC obtained was judged to be homogeneous to an extent >99%. This protein was concentrated to approximately 2 mg/mL by ultrafiltration and stored frozen in aliquots at −80 °C. The protein concentration was determined from the absorbance at 280 nm, an $\epsilon^{1\%}_{1cm}$ of 13.7 (Kisiel et al., 1976) being employed.

APC was prepared by activation of PC with insolubilized RVV-X, as described by Steiner et al. (1980). The concentration of APC was determined by titration of the active enzyme with *p*-nitrophenyl *p*-guanidinobenzoate (Chase & Shaw, 1967). In all cases, this procedure resulted in activation of PC to an extent of 90–95%.

RVV-X was purified from the crude venom, purchased from the Miami Serpentarium, according to the procedure of Amphlett et al. (1982). The purified enzyme was coupled to Sepharose 4B, as described by Byrne & Castellino (1978).

Kinetic Assays. Prior to these studies, APC and all buffer solutions were deionized by passage over a column of Chelex 100 (Bio-Rad), in order to remove trace contamination by divalent cations. Atomic absorption analysis (Amphlett et al., 1981) demonstrated that our procedures (Amphlett et al., 1981) were effective in this regard and that no detectable Ca^{2+} or Mn^{2+} was present in APC preparations.

All assays of APC employed the use of the chromogenic peptide substrate H-D-phenylalanylpipecolylarginine-*p*-nitroanilide hydrochloride (S-2238) purchased from AB Kabi. Assays were carried out by addition to a cuvette of 0.1 mL of a 0.5 M solution of Tris-HCl, pH 7.4, various quantities (0.025–0.2 mL) of a 2.32 mM solution of S-2238, in H_2O, and measured amounts (0.05–0.6 mL) of the desired cation (chloride salt) solution, also in H_2O. The final volume of the solution was adjusted to 1 mL with H_2O. The cuvette was then placed in a thermostated cell holder of a Cary 219 spectrophotometer and allowed to equilibrate at 30 °C. An identical cell was placed in the reference chamber, also equipped with a thermostatically controlled cell holder, at 30 °C. To the sample chamber was added 0.01 mL of a 1.9 μM solution of APC, in 0.05 M Tris-HCl, pH 7.4, in order to initiate the assay. The rate of reaction was determined by continuous monitoring of the release of *p*-nitroanilide, at 405 nm. For calculation of the activity of APC, an E = 9620 M^{-1} cm^{-1}, at 405 nm, for *p*-nitroanilide was employed (Pfleiderer, 1970).

Results

In a previous study (Steiner et al., 1980), we have shown that the rate of hydrolysis, by APC, of two synthetic substrates, viz., *N*-benzoyl-L-phenylalanyl-L-valyl-L-arginine-*p*-nitroanilide hydrochloride (S-2160) and N^{α}-tosyl-L-arginine methyl ester, is dependent upon the presence of monovalent cations. Prior to a detailed analysis of this effect by kinetic approaches, we first found it necessary to evaluate whether the monovalent cation effect was due to stimulation of an existing activity or whether the presence of monovalent cations was an absolute requirement for this enzyme. Any experiments designed to test this point must allow a means of providing ionic strength to the solution. Difficulties arise here, since a wide variety of monovalent cations influence the activity of APC, since divalent cations also stimulate the enzymatic activity of APC, and since it is highly likely that trivalent cations will also bind to APC, as is the case with all other γ-carboxyglutamic acid containing blood coagulation enzymes that have been examined in this regard. We have employed highly substituted amines in the assay (such as trimethyl- and tetramethylammonium) in the hope that one could be found that would not exhibit activation properties, but in the presence of those tested, APC did show very slight, but measurable, activity toward S-2238. In studies with Tris-HCl as a supporting electrolyte, we have measured the activity of APC toward S-2238 (150 μM), at

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; DEAE, diethylaminoethyl; $DodSO_4$, dodecyl sulfate; Bis-Tris, [bis(2-hydroxyethyl)amino]tris(hydroxymethyl)methane.

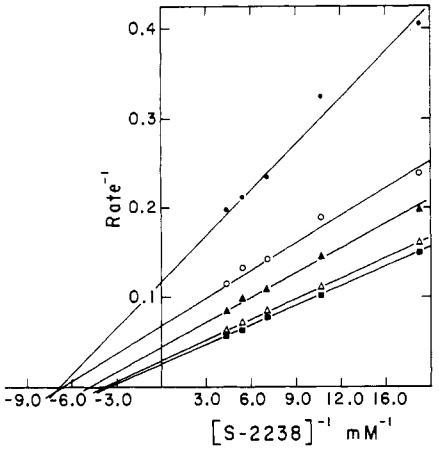

FIGURE 1: Steady-state kinetics of APC with varying substrate at several fixed Na^+ concentrations. Rates are expressed as nanomoles of S-2238 cleaved per minute. Na^+ concentrations are (●) 50, (○) 75, (▲) 100, (△) 150, and (■) 200 mM. Reaction conditions are as given under Materials and Methods.

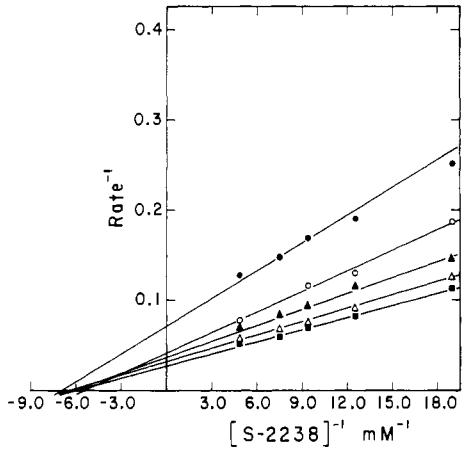

FIGURE 2: Steady-state kinetics of APC with varying substrate concentrations and various fixed K^+ concentrations. Rates are expressed as nanomoles of S-2238 cleaved per minute. K^+ concentrations are (●) 50, (○) 75, (▲) 100, (△) 150, and (■) 200 mM. Reaction conditions are as given under Materials and Methods.

various levels of Tris-HCl, up to an ionic strength of 0.25. In all cases, the activity was never greater from 2 to 3% of the activity found with the poorest cation, Li^+. When the data obtained were extrapolated to zero [$Tris^+$], the activity of APC was essentially zero, suggesting that the activity seen in the presence of $Tris^+$ was due to low-level activation of the enzyme by $Tris^+$. Addition of 2 mM EDTA did not affect these results or the results obtained with any monovalent cation, showing that contamination of the solutions with divalent cations was not a factor. In order to determine whether the small enhancement of APC by $Tris^+$ was a specific effect and not merely due to a general ionic strength effect, we have shown that the suboptimal enhancement of APC by 37.5 mM Na^+ toward S-2238 is not substantially affected by addition of $Tris^+$, up to a total ionic strength of 0.25. From experiments of this type, we conclude that APC has an essential requirement for monovalent cations. This conclusion would be fortified by the discovery of a monovalent cation that does not show stimulatory activity toward APC. However, we have found this effect to be a general one, and such a cation may, indeed, not exist.

In the current study, we have examined the effect of various concentrations of several monovalent cations on the rate of hydrolysis of S-2238, as a function of the concentration of

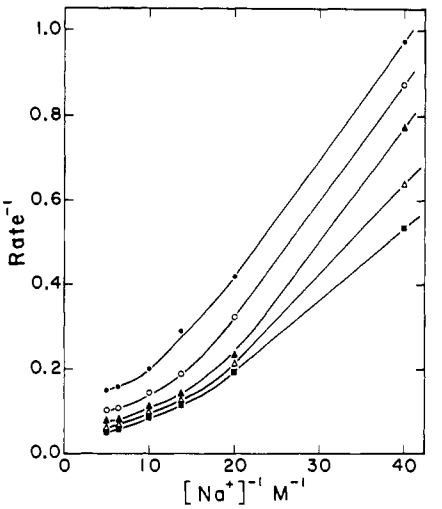

FIGURE 3: Steady-state kinetics of APC with varying Na^+ concentration at several fixed substrate concentrations, plotted as 1/rate vs. 1/[Na^+]. Rates are expressed as nanomoles of S-2238 cleaved per minute. Substrate concentrations are (●) 58, (○) 93, (▲) 139, (△) 186, and (■) 232 μM.

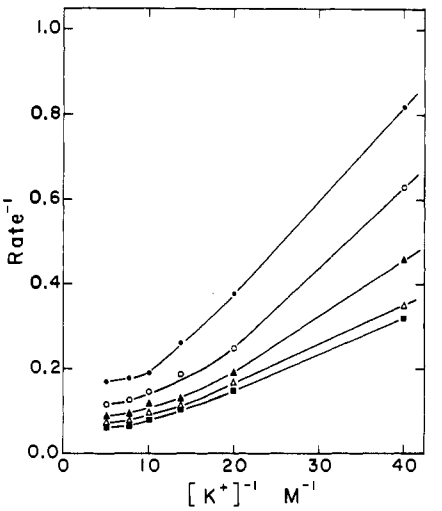

FIGURE 4: Steady-state kinetics of APC with varying K^+ concentration at several fixed substrate concentrations, plotted as 1/rate vs. 1/[K^+]. Rates are expressed as nanomoles of S-2238 cleaved per minute. Substrate concentrations are (●) 33, (○) 53, (▲) 80, (△) 133, and (■) 200 μM.

S-2238. Figures 1 and 2 illustrate the data obtained for two of these cations, Na^+ and K^+, respectively, when the concentration of S-2238 is varied at fixed concentrations of cations. Progressive increases in the maximal initial rate of S-2238 hydrolysis are seen as the cation concentrations are increased. This is expected since the cation concentrations shown are not saturating with respect to the steady-state kinetic constants. In addition, smaller comparative decreases in K_m for S-2238 are also noted, for each cation, as the cation concentration is increased. These same trends have been obtained when examining other cations, i.e., Li^+, NH_4^+, and Cs^+. It should be noted that, in these experiments, the total ionic strength of the medium, at various cation concentrations, has not been maintained at a constant value. As seen above, it was found that this procedure was not necessary, at least over the range of cation concentrations of importance to the data in Figure 1 and 2. Also, control experiments demonstrated that the extinction coefficient of *p*-nitroanilide varied less than 2% over all cation concentrations employed in Figures 1 and 2.

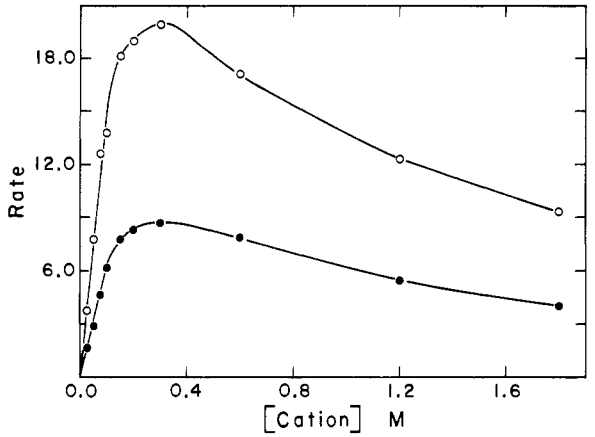

FIGURE 5: Inhibition of APC by high cation concentration. The cations shown are (●) Na^+ and (○) K^+. The substrate concentration is 150 μM. Rates are expressed as nanomoles of S-2238 cleaved per minute.

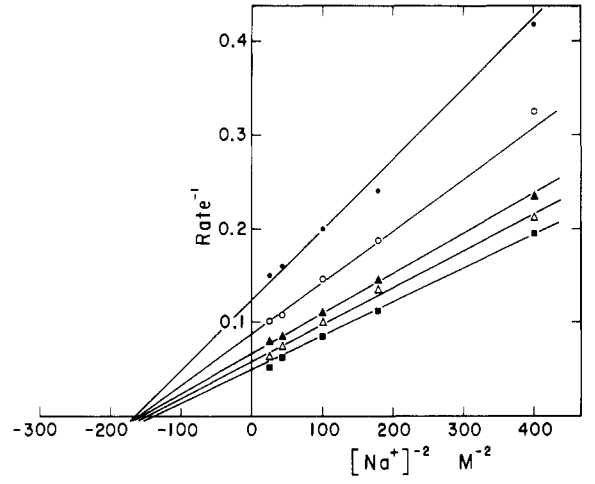

FIGURE 6: Steady-state kinetics of APC with varying Na^+ concentration and several fixed substrate concentrations, plotted as 1/rate vs. $1/[Na^+]^2$. Rates are expressed as nanomoles of S-2238 cleaved per minute. Substrate concentrations are as given in Figure 3.

Figures 3 and 4 illustrate the data of Figures 1 and 2 replotted as the variation in reaction rate as a function of the concentration of Na^+ (Figure 3) and K^+ (Figure 4), at several fixed levels of S-2238. The plots are not linear, indicating a complex dependency of APC-catalyzed hydrolysis of S-2238 on the concentration of monovalent cation. The stimulatory capacity of metal cations toward this reaction appears not to follow normal simple saturation kinetics. This same behavior, exemplified in Figures 3 and 4, for Na^+ and K^+, respectively, is present for all cations tested in this report. At much higher cation concentrations (Figure 5), the initial reaction rate, at 150 μM S-2238, becomes progressively slower as the concentration of metal ion is increased. This effect, shown here for Na^+ and K^+, is present at all levels of S-2238 and for all cations examined. It should be pointed out that the inhibition noted at a cation concentration greater than 0.3 M does not affect the curves shown in Figures 3 and 4 since the latter data are plotted only to cation concentrations of 0–0.2 M, over which range significant inhibition does not occur.

The plots shown in Figures 3 and 4 are linearized when the rate data are replotted against the reciprocal of the square of the metal ion concentration. This point is illustrated for Na^+ and K^+, in Figures 6 and 7, respectively. In both cases, it appears as though the level of S-2238 present does not greatly affect the K_m of the cation but obviously does affect the maximal rate obtainable in the presence of "infinite" cation

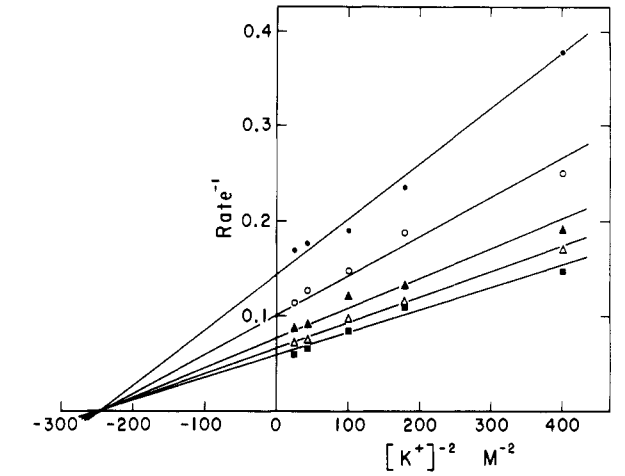

FIGURE 7: Steady-state kinetics of APC with varying K^+ concentration at several substrate concentrations, plotted as 1/rate vs. $1/[K^+]^2$. Rates are expressed as nanomoles of S-2238 per minute. Substrate concentrations are as given in Figure 4.

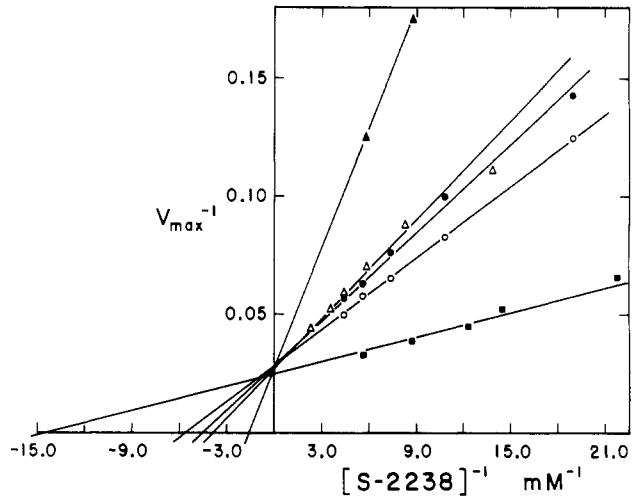

FIGURE 8: Replot of $1/V_{max}$ values for various substrate concentrations, taken as the y intercepts of data such as those presented in Figures 6 and 7 for Na^+ and K^+, respectively, vs. 1/[substrate]. The cations are (▲) Li^+, (△) NH_4^+, (●) Na^+, (○) K^+, and (■) Cs^+. The V_{max} values are expressed in units of nanomoles of S-2238 cleaved per minute. The line drawn for Li^+ was based on five data points, only two of which could be shown on the scale chosen.

concentrations. All cations tested in this study behave similarly in this regard.

The value of the $K_{m,app}$ of S-2238, at infinite concentrations of each cation employed, can be determined from replots of the data of Figures 6 and 7. Here, the reciprocals of the maximal initial rate of hydrolysis of various levels of S-2238, at infinite concentration of cation, are obtained from the y intercepts of Figures 6 and 7. These are then plotted against the reciprocal of the appropriate concentration of S-2238 and shown in Figure 8. $K_{m,app}$ values obtained with each cation are shown in Table I. Here, the $k_{cat,app}$ of S-2238 is independent of the cation used at infinite cation and S-2238 concentration and is equal to 35 ± 5 s^{-1}. However, the $K_{m,app}$ for S-2238 is dependent upon the cation and ranges from 70 μM for the largest cation employed, Cs^+, to 630 μM, for the smallest cation employed, Li^+.

The $K_{m,app}$ for the cation, at infinite substrate concentration, can be evaluated in a similar fashion, by replots of the data of Figures 1 and 2. In this case, the reciprocal of the maximal initial rate of hydrolysis of S-2238, at infinite levels of S-2238, obtained from the y intercepts of Figures 1 and 2, is plotted

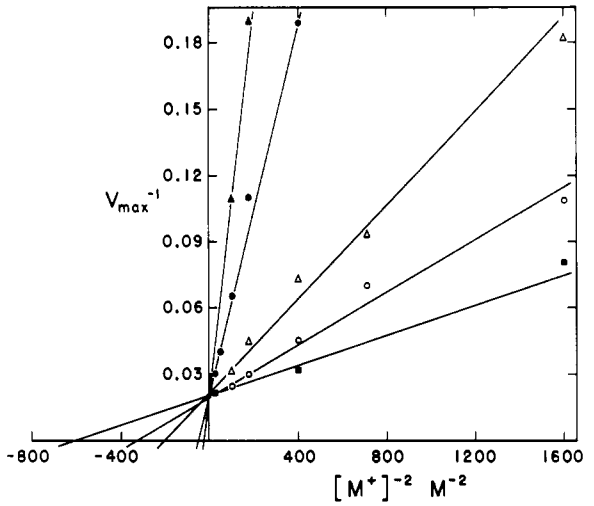

FIGURE 9: Replot of $1/V_{max}$ values for various cation concentrations, taken as the y intercepts of data such as those presented in Figures 1 and 2 for Na^+ and K^+, respectively, vs. $1/[\text{cation}]^2$. The cations are (▲) Li^+, (●) Na^+, (△) NH_4^+, (○) K^+, and (■) Cs^+. The V_{max} values are in units of nanomoles of S-2238 cleaved per minute. The line drawn for Li^+ was based on five data points, only two of which could be shown on the scale chosen.

Table I: Steady-State Kinetic Constants for APC toward S-2238 with Various Monovalent Cations

cation	$K_{m,app}$ ± 10% for cation (mM)	$K_{m,app}$ ± 10% for substrate (μM)
Li^+	182	630
Na^+	129	220
NH_4^+	70	270
K^+	55	180
Cs^+	41	70

against the reciprocal of the square of appropriate metal ion concentration and shown in Figure 9. $K_{m,app}$ values for each cation, thus obtained, are listed in Table I. Again, these values appear related to the size of the cation. The smallest $K_{m,app}$ is found to be 41 mM, for Cs^+, and the largest $K_{m,app}$ is obtained for Li^+, at 132 mM. The $k_{cat,app}$ at infinite substrate and cation levels, from the data in Figure 9 is, again, 35 ± 5 s^{-1} and is virtually identical to the $k_{cat,app}$ obtained from the data of Figure 8. This should indeed be the case for a valid kinetic argument to be forwarded.

Discussion

In a previous report (Steiner et al., 1980) we have found that monovalent cations are necessary for optimal amidolytic and esterolytic activity of APC. The effect was a very general one, in that a variety of cations including highly substituted amines exhibited this property. Divalent cations also exhibited a stimulatory effect, however, not to the same extent as monovalent cations (Steiner et al., 1980). The stimulatory effect of divalent cations on APC is not surprising since APC is a γ-carboxyglutamic acid containing enzyme (Fernlund et al., 1978) and, as all other proteins of this class, binds Ca^{2+}, which is displaced by other divalent cations (Amphlett et al., 1981). Other vitamin K dependent coagulation enzymes, such as bovine factor IXaα (Byrne & Castellino, 1978), bovine factor IXaβ (Byrne et al., 1980), and human factor Xa (Orthner & Kosow, 1978), which also bind Ca^{2+}, catalyze reactions that are stimulated by divalent cations.

The extent of the monovalent cation influence on the activity of APC is a unique discovery in serine proteases. Previously, amidolytic activity of human α-thrombin has been found to be stimulated by Na^+ and K^+. In each case, the $K_{m,app}$ was decreased by 0.2–0.7-fold, and the $k_{cat,app}$ was increased 3.1–4.5-fold, when the enzyme was saturated with these cations (Orthner & Kosow, 1980). Monovalent cations have also been previously found to affect the amidolytic activity of human factor Xa (Orthner & Kosow, 1978). Here, saturating levels of Na^+ resulted in a 2-fold increase in the affinity for the substrate to factor Xa, without affecting the V_{max} of the reaction. No other monovalent cation tested enhanced the amidolytic activity of factor Xa. The cations Li^+, NH_4^+, and K^+ inhibited the Na^+-induced activation of this enzyme. With regards to APC, we have shown (Steiner et al., 1980) that monovalent cations influence the activity of this enzyme in a specific fashion that is not a general effect of ionic strength, since different cation solutions, of the same ionic strength, result in disparate effects on the activity of the enzyme.

The differences in the action of monovalent cations between APC and human α-thrombin and human factor Xa are profound. In the case of APC, we have shown that a wide variety of monovalent cations are involved, that monovalent cations are more effective than divalent cations, and that monovalent cations appear not to be simple activators but are required for expression of the amidolytic activity of APC. While this latter point has not been conclusively proven, it is more than a reasonable interpretation of available data. The small activating activity produced by $Tris^+$ and other substituted amines extrapolates approximately to zero when the concentrations of those cations are extrapolated to zero. Since a monovalent cation is yet to be found that does not display some propensity for activation, as evidenced by the increase in APC amidolytic activity as a function of increases in the cation concentration, only logical inferences on the point of whether monovalent cations are essential requirements or very substantial activators of APC amidolytic activity can be forwarded at this time.

Steady-state kinetic constants relating to the effect of monovalent cations on the $K_{m,app}$ and $k_{cat,app}$ for S-2238, at infinite cation concentration can be most unambiguously obtained from the graphs of Figure 8, which have been generated from plots of 1/(initial rate) data vs. 1/(square of the metal ion concentration) (Figures 6 and 7). By utilization of this procedure, the assumption is made that activity of APC does not exist until two metal ion sites, or two functionally different classes of cation sites, are occupied. The data obtained from Figure 8 are listed in Table I and show that the $K_{m,app}$ of S-2238, at saturating cation concentrations, is dependent upon the nature of the cation employed, whereas the $k_{cat,app}$ at saturating cation concentrations is independent of the nature of the cation. The smaller cations (Li^+ and Na^+) do not result in as effective a stimulation of APC as do the larger cations (K^+ and Cs^+), at subsaturating levels, since the latter cations possess much smaller $K_{m,app}$ values for S-2238. This argues for a significant interplay of the cation and substrate in determining the rate constant that dominate the overall $K_{m,app}$ for S-2238. The data also show that the turnover ($k_{cat,app}$) of this substrate, at saturating cation and S-2238 levels, is independent of the nature of the cation.

The $K_{m,app}$ for the cation, determined from the data of Figures 1, 2, and 9, at saturating S-2238 levels, is also dependent upon the nature of the cation, being lowest for the largest cation and increasing in parallel with cations of decreasing ionic radius. This again argues strongly for active participation of cation and substrate in rate constants that determine the value for the $K_{m,app}$ of the cation. Again, however, it can be noted, from Figure 9, that the turnover

($k_{cat,app}$) of substrate on the enzyme, when the enzyme surface is saturated with monovalent cation and S-2238, is not greatly influenced by the nature of the cation, an interpretation also obvious from the data of Figure 8.

Another aspect worthy of discussion is the data of Figure 5, showing that activation of APC, by monovalent cations, appears to slightly decrease at very high levels of cations. Several interpretations of these data are possible. Among these are the possibilities that it is a general effect of high ionic strength on the structure of the enzyme or that a separate weaker class of cation binding sites exists, which, when occupied, inhibit the stimulatory effect of the stronger binding sites. Further, the possibility exists that the cation, at high levels, displaces the substrate, leading to a decreased enzyme activity.

Many types of diverse enzymes have been shown to be activated by monovalent cations (Suelter, 1970). In the case of vertebrate tissue pyruvate kinases, which have been studied in this regard in some detail and for which monovalent cations are essential requirements, it appears as though the monovalent cation plays a role in the catalytic processes, as a result of its ultimate interaction with both the enzyme and substrate in the ternary complex. The size of the cation was found to be an important consideration in its ability to activate this enzyme [for a review, see Nowak & Suelter (1981)], since only cations of certain size were capable of producing the metal-cation-induced conformational alteration in the enzyme necessary for activation to occur (Nowak, 1976). On the other hand, studies of the activation of a vertebrate fructose-1,6-bisphosphatase by monovalent cations suggest that monovalent cations function by altering the conformation of the enzyme, producing a more efficient catalytic state (Marcus, 1975). Concerning bacterial tryptophanase, several monovalent cations were found to be effective activators, resulting from their respective abilities to labilize the α proton on the substrate, a step necessary for the catalytic event to occur (Suelter & Snell, 1977). In this case, it was found that the activation by monovalent cations was related to their respective abilities to interact with the enzyme. With regard to APC, the activation by monovalent cations is related to the size of the cation, being more effective for alkali cations of larger ionic radius. NH_4^+ behaves anomalously in this regard, as has been shown for other systems (Suelter & Snell, 1977). It is not possible to state whether the dependency of activation on the size of the monovalent cation, in the case of APC, is due to differences in binding of cations of various size to the enzyme since we do not presently possess information as to whether monovalent cations are indeed even capable of binding to APC in the absence of substrate and we are not certain of the relationship between the $K_{m,app}$ for the cation (which we have obtained only in the presence of S-2238) and the binding of the cation. We have provided evidence, however, that the $K_{m,app}$ of S-2238 is dependent on the nature of the monovalent cation, and this argues for an active role of the monovalent cation in the steady-state amidolytic process of APC. This finding is very novel for proteases and suggests that monovalent cations, in this particular case, may be involved in modulation of the activity of an enzyme that is itself involved in a regulatory capacity in the blood coagulation cascade.

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