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STIMULATION OF THE AMIDASE AND ESTERASE ACTIVITY OF ACTIVATED BOVINE PLASMA PROTEIN C BY MONOVALENT CATIONS

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Summary: The rate of hydrolysis of suboptimal concentrations of N-benzoyl-L-phenylanalyl-L-valyl-L-arginine-p-nitroanilide hydrochloride (S-2160) by activated bovine plasma protein C is greatly enhanced by the presence of monovalent cations. Of the cations tested, at their maximal effective concentrations, the order of the ability to enhance the S-2160 amidase activity of activated protein C is $Cs^+ > Rb^+ > K^+ > NH_4^+ > Na^+ > Li^+$. Progressive substitution of methyl groups for hydrogen in NH_4^+ leads to a concomitant decrease in the ability of these compounds to enhance the amidase activity of activated protein C. Divalent cations, at low concentrations, weakly enhance the amidase activity of activated protein C and show a decreased tendency in this regard at high concentrations. The above observations also apply to hydrolysis of the substrate α-N-tosyl-L-arginine methyl ester.

INTRODUCTION

Protein C is a recently discovered Vitamin K-dependent protein, which possesses amino terminal amino acid sequence homology with other well-known Vitamin K-dependent blood coagulation proteins (1). Especially notable in this regard is the presence of γ-carboxyglutamic acid residues in bovine plasma protein C at sequence positions corresponding to location of this unusual amino acid in bovine prothrombin, Factor VII, Factor IX, and Factor X (1).

Protein C exists in plasma in the form of a zymogen (2, 3). This proenzyme possesses a molecular weight of approximately 56,000 (1, 3) distributed in a γ-carboxyglutamic acid containing light chain of molecular weight approximately 21,000, disulfide-linked to a heavy chain of molecular weight approximately 41,000 (3). Protein C can be activated to a serine amidase (3, 4) by a protease (RVVx) from Russell's Viper Venom (3), and

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by α-thrombin (4). Activation is accomplished upon cleavage of an Arg-Ile bond in the heavy chain, and concomitant release of a tetradecapeptide (3). As a consequence of this peptide bond cleavage, a diisopropylfluorophosphate reactive active site is expressed in the heavy chain, which is homologous in sequence to other plasma serine proteases (4).

Activated protein C has been shown to possess amidolytic activity toward synthetic peptide substrates (3, 4). Also, activated protein C has been shown to be capable of marked prolongation of the partial thromboplastin time of bovine plasma (4). A basis for the anticoagulant activity of activated protein C may reside in its capacity to inactivate previously activated Factor V (Factor Va), in the presence of Ca^{2+} (5). This latter process is accelerated by phospholipid (4, 5). The amidolytic, anticoagulant, and Factor Va inhibitory activity of activated protein C is dependent on the presence of its active site serine residue (4).

Since the presence of γ-carboxyglutamic acid residues in protein C and activated protein C serves as a strong indicator of the ability of these proteins to interact with metal ions, we decided to examine the effect of various cations on the amidolytic and esterolytic (vide infra) activity of this enzyme. This communication was stimulated by our surprising discovery of the great enhancement of activity of activated protein C by monovalent cations.

MATERIALS AND METHODS

Proteins. The protein C used in these studies was either generously donated by Dr. Walter Kisiel, University of Washington, or prepared from fresh bovine plasma according to minor operational modifications of the procedures of Stenflo (1) and Kisiel et al. (3).

Activated protein C was prepared by incubation of 5.0 ml of protein C (1.8 mg/ml), dissolved in 50 mM Tris·HCl/100 mM NaCl, pH 7.4 with 2.5 ml of settled Sepharose-RVVx (6) and 0.5 ml of 100 mM $CaCl_2$. The

mixture was gently stirred for 30 min., at 37°. At the conclusion of the reaction, the mixture was percolated over a 0.6 cm x 6 cm column of Chelex-100, equilibrated and washed with 75 mM Tris·HCl, pH 7.4, in order to remove Ca^{2+}. The enzyme solution was equilibrated against 75 mM Tris·HCl, pH 7.4, by gel filtration, using a 2 cm x 40 cm column of Sephadex G-25, equilibrated with this buffer. The protein pool was concentrated by ultrafiltration to an absorbancy at 280 nm of approximately 1.0, and stored frozen in small aliquots for future use. The enzyme retained full activity for at least one month, when stored in this fashion. The concentration of activated protein C was determined from the absorbance at 280 nm, using an $\epsilon^{1\%}_{1\ cm} = 13.7$ (3).

Assay of activated protein C. The amidolytic activity of activated protein C was assayed utilizing the substrate N-benzoyl-L-phenylalanyl-L-valyl-L-arginine-p-nitroanilide hydrochloride (S-2160), purchased from AB Kabi. A quantity (0.25 ml) of 250 mM Tris·HCl, pH 7.4 was mixed in a cuvette with 0.05 ml of a 1.0 mM solution of S-2160, dissolved in H_2O. Following this, 0.1 ml of the desired cation salt was added (in 10 mM Tris·HCl, pH 7.4), and the final volume adjusted to 1.0 ml with H_2O. The cuvette was placed in a Cary 219 Spectrophotometer, equipped with a thermostatted cell holder, and allowed to equilibrate at 30°. Subsequent to this, 0.01 ml of activated protein C (0.53 mg/ml) was added to initiate the assay. The p-nitroanilide released was continuously monitored at 405 nm, on a full scale output of 0.1 absorbancy unit. For calculation of the final activity of activated protein C, a molar extinction coefficient of 9620, at 405 nm, (in a cell of 1 cm path length) was utilized for p-nitroanilide (7).

Esterase assays of activated protein C, utilizing N-α-tosyl-L-arginine [^{3}H]-methyl ester ([^{3}H]-TAME) were also employed. The procedure utilized is based upon that of Roffman et al. (8), except that a fixed time assay was employed. In a typical experiment, 0.01 ml of activated protein C (53 μg/ml) was preincubated at 30° with 0.005 ml of 1 M Tris·HCl, pH 7.4, the desired quantity of 1 M NaCl, and H_2O, to yield a final volume of 0.29 ml. After 2 min., 0.01 ml of 1.2 mM [^{3}H]-TAME (12,000 dpm/nmol) was added, and incubation continued at 30°. At desired time intervals, an aliquot (0.025 ml) was withdrawn and added to 0.005 ml of glacial acetic acid, in order to inhibit further enzymatic hydrolysis. A quantity of 0.025 ml of this solution was then added to 10 ml of scintillant (prepared by dissolving 15.2 g of diphenyloxazole and 0.19 g of 1,4-bis-[2-(5-phenyloxazolyl]-benzene in 1 l of toluene), in a scintillation vial. The mixture was thoroughly stirred for 1 min. This served to fully extract the [^{3}H]-methanol which was released in the assay. The amount of [^{3}H]-methanol was then determined by liquid scintillation counting in a Beckman LS-100C Liquid Scintillation Counter.

RESULTS AND DISCUSSION

The effect of progressive increase in the concentration of Na^+ and Cs^+ on the activity of activated protein C, toward the substrate S-2160, is illustrated in Figure 1. The concentration of S-2160 for these experiments,

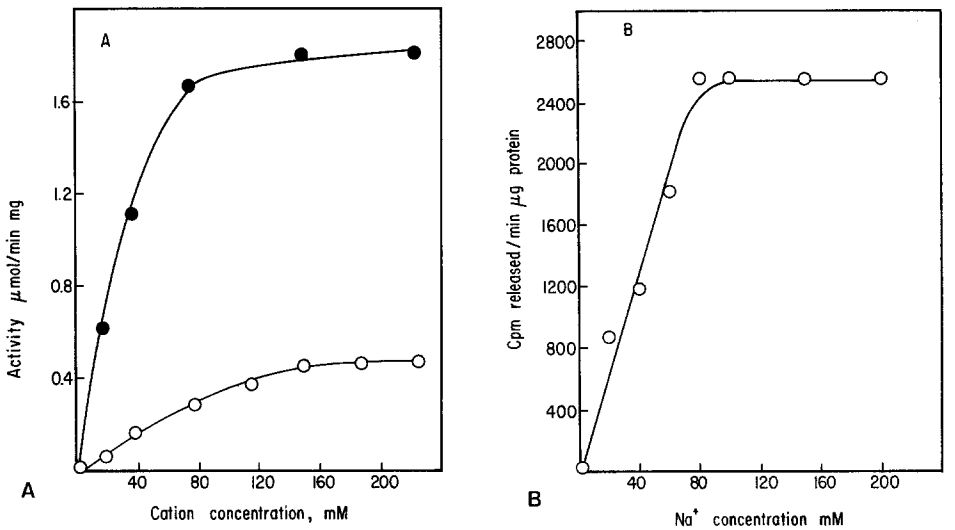

Fig. 1. The effect of varying concentrations of monovalent cations on the activity of activated protein C toward two synthetic substrates. (A) Effect of varying Na^+ (o) and Cs^+ (•) on the activity toward S-2160; (B) effect of varying Na^+ on the activity toward [^{3}H]-TAME.

For complete experimental details in both cases see Materials and Methods.

of 50 μM, was purposely chosen to be sub-saturating so that rate differences due to alterations in both Km and Vmax would be present. As seen in Figure 1A, a slow, but finite, rate of hydrolysis of the substrate by activated protein C is noted in the absence of added metal ions, perhaps due to the presence of $Tris^+$. Greatly increased hydrolysis rates are observed as the concentration of Na^+ and Cs^+ is progressively increased, with Cs^+ showing a much greater enhancement than Na^+. Maximal rates of hydrolysis occur at metal ion concentrations > 100 mM.

Since the ionic strength of the medium constantly changes through the titration, we examined the nonspecific effect of ionic strength on this reaction. At a constant concentration of NaCl of 37.5 mM, the ionic strength of the medium was varied from 0.075 to 0.216, as a consequence of addition of Tris·HCl. Only a 10% increase in the rate of S-2160 hydro-

lysis was noted, under the exact assay conditions described above, over the entire range of buffer conditions employed. Thus, we conclude that specific metal monovalent cation stimulation of the amidolytic activity of activated protein C has occurred.

In order to show that the specific metal ion stimulatory effect on this enzyme was not a unique property of the peptide substrate employed, we have shown that Na^+ does not enhance the amidolytic activity of trypsin toward S-2160. Further, we have examined the effect of cations on the activity of activated protein C toward an ester substrate. In this regard, we find that L-TAME is an excellent substrate for activated protein C. The effect of a progressive increase in the Na^+ concentration on the enzymatic hydrolysis rate of sub-saturating levels of [^{3}H]-L-TAME is shown in Figure 1B. Again, an extremely large enhancement of esterolytic activity is observed, which is maximal at a Na^+ concentration equivalent to that required to saturate the effect on S-2160. This shows the generality of the monovalent cation effect on the activity of activated protein C.

We have examined several other monovalent and divalent cations, regarding their potential enhancement of the activity of activated protein C toward S-2160. The results are listed in Table 1 and reflect the maximal stimulatory capacity of the cations employed (150 mM for monovalent cations - 0.5 mM for divalent cations). In the case of monovalent cations, the stimulatory capacity increases, through the series $Li^+ < Na^+ < NH_4^+ < K^+ < Rb^+ < Cs^+$. The radius of these cations increase through $Li^+ < Na^+ < K^+ < NH_4^+ = Rb^+ < Cs^+$. Thus, for protein C enhancement by monovalent cations, the efficacy of the process appears to correlate with the size of the cation, with the exception of the reversal in trend of NH_4^+ and K^+. A similar qualitative progression has been observed for the K_D of these

monovalent cations to pyruvate kinase (9), as well as with activation of other classes of enzymes (10). As the size of the NH_4^+ cation is progressively increased as a consequence of substitution of methyl groups, a decreased tendency toward enhancement of the amidolytic activity of activated protein C is noted (see Table 1). This effect is likely due to the increased shielding of the charge on the nitrogen, as methyl groups are substituted for hydrogen in these derivatives. In the case of pyruvate kinase, $CH_3NH_3^+$ activates the enzyme much less effectively than NH_4^+, whereas $(CH_3)_2NH_2^+$, $(CH_3)_3NH^+$, and $(CH_3)_4N^+$ do not activate the enzyme (11). Divalent cations, such as Ca^{2+} and Mn^{2+}, are much less effective stimulators of the amidolytic activity of activated protein C (Table 1), at their maximal effective concentrations (0.5 mM). Further,

TABLE 1

ENHANCEMENT OF THE ACTIVITY OF ACTIVATED PROTEIN C BY VARIOUS METAL IONS AT 30°[a]

Cation Present (150 mM)	Initial Rate (μ mole min^{-1} mg^{-1} of enzyme)
None	0.008
Li^+	0.328
Na^+	0.439
K^+	0.984
Rb^+	1.680
Cs^+	1.749
NH_4^+	0.599
$CH_3NH_3^+$	0.146
$(CH_3)_2NH_2^+$	0.083
$(CH_3)_3NH^+$	0.079
$(CH_3)_4N^+$	0.040
Ca^{2+}(0.5 mM)[b]	0.134
Mn^{2+}(0.5 mM)[b]	0.101

[a] In all cases the concentration of S-2160 was 50 μM.

[b] A decreased enhancement was noted at higher concentrations of divalent cations.

the enhancement of enzymatic activity with these latter cations significantly falls off from the maximum, at high levels (Fig. 2), in contrast to the effect of monovalent cations.

Several extremely interesting and unique features of cation stimulation of activated protein C have surfaced from this study. To our knowledge the only protease previously shown to be stimulated by monovalent cations was bovine plasma Factor Xa (12). However, in this case, only Na^+ was effective in this regard. Further, in a survey published by Evans and Sorger (13), it was observed that a wide variety of enzymes activated by K^+ were not effectively activated by Na^+ and Li^+, and some enzymes activated by Na^+ were not activated to a large extent by K^+, NH_4^+, or Rb^+. In the case of activated protein C, all of the above cations are effective activators. Thus, it is likely that the ions present in plasma have a significant role in the modulation of activity of this enzyme.

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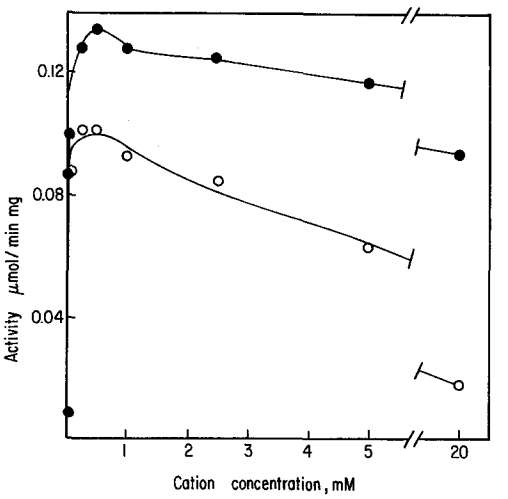

Fig. 2. The effect of varying concentrations of Mn^{2+} (o) and Ca^{2+} (•) on the activity of activated protein C toward S-2160. Assays were conducted as described in Materials and Methods in the absence of additional monovalent cation.

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