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Effect of Monovalent Cations on the Pre-Steady-State Kinetic Parameters of the Plasma Protease Bovine Activated Protein C†

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Received June 29, 1984

ABSTRACT: Activated bovine plasma protein C (APC) was not reactive with the substrate *p*-nitrophenyl *p*-guanidinobenzoate (NPGB) in the absence of cations. In the presence of increasing concentrations of Na^+, the acylation rate constant, $k_{2,app}$, at 7 °C, progressively increased from 0.32 ± 0.03 s^{-1} at 12.5 mM Na^+ to 1.15 ± 0.10 s^{-1} at 62.5 mM Na^+. A linear dependence of the reciprocal of $k_{2,app}$ with $[Na^+]^{-2}$ was observed, indicating that at least two monovalent cation sites, or classes of sites, are necessary for the catalytic event to occur. From this latter plot, the $k_{2,max}$ for APC catalysis of NPGB hydrolysis, at saturating $[Na^+]$ and [NPGB], was calculated to be 1.21 ± 0.10 s^{-1}, and the K_m for Na^+ was found to be 21 ± 3 mM. The dissociation constant, K_s, for NPGB to APC, at 7 °C, was not altered as $[Na^+]$ was increased, yielding a range of values of 18.5 × 10^{-5} to 19.9 × 10^{-5} M as $[Na^+]$ was varied from 12.5 to 62.5 mM. The deacylation rate constant, k_3, for *p*-guanidinobenzoyl-APC hydrolysis was also independent of $[Na^+]$, with a value of (3.8 ± 1.0) × 10^{-3} s^{-1} in the absence of Na^+ or in the presence of concentrations of Na^+ up to 200 mM. Identical kinetic behavior was observed when Cs^+ was substituted for Na^+ in the above enzymic reaction. The pre-steady-state kinetic parameters were calculated according to the same methodology as described above. The $k_{2,max}$ for acylation by NPGB was found to be 1.36 ± 0.10 s^{-1}, the K_m for Cs^+ was 12 ± 2 mM, the K_s for NPGB was (1.8–2.2) × 10^{-5} M, and the k_3 for deacylation of *p*-guanidinobenzoyl-APC was determined to be (3.8 ± 1.0) × 10^{-3} s^{-1}.

Activated protein C (APC) is a serine protease which is formed by limited proteolytic hydrolysis of its precursor molecule, protein C (PC). Protein C has been purified from bovine (Stenflo, 1976) and human (Kisiel, 1979) plasmas and has been shown to be one of the vitamin K dependent plasma proteins (Stenflo, 1976). After purification, bovine PC has been found to consist of two polypeptide chains of total molecular weight of approximately 56000 per molecule. Its light chain contains 155 amino acid residues of known sequence and one site of glycosylation which exists at Asn_{97} (Fernlund & Stenflo, 1982). All 11 residues of Gla exist within the amino-terminal 35 residues of this chain (Fernlund & Stenflo, 1982), and a single residue of *erythro*-β-hydroxyaspartic acid has been identified herein at position 71 (Drakenberg et al., 1983). The heavy chain of bovine plasma PC contains 260 amino acid residues of known sequence, and three sites of glycosylation are present, at Asn residues 93, 154, and 170 (Stenflo & Fernlund, 1982). This chain contains the latent active-site serine residue of APC, at position 201, with the charge-relay system most likely comprising His_{56}, Asp_{102}, and Ser_{201} (Stenflo & Fernlund, 1982). The heavy and light chains of bovine PC and APC are linked by a single disulfide bond consisting of Cys_{122} in the heavy chain (Stenflo & Fernlund, 1982) bound to a yet nonidentified Cys residue in the light chain.

The activation of PC to APC is accompanied by release of a 14-residue polypeptide from the amino terminus of the heavy chain of bovine PC (Kisiel et al., 1976) and a 12-residue polypeptide from the same location in human PC (Kisiel, 1979). The enzymes capable of catalyzing cleavage of the requisite peptide bond include α-thrombin (Kisiel et al., 1977), trypsin (Kisiel et al., 1976), and the factor X activating enzyme from the venom of Russell's viper (Kisiel et al., 1976).

APC functions as an anticoagulant, most probably by nature of its ability to inactivate, by proteolysis, a cofactor, factor Va, necessary for prothrombin activation (Kisiel et al., 1977) and, similarly, a cofactor, factor VIIIa, necessary for factor X activation (Vehar & Davie, 1980). Bovine APC catalyzes the hydrolysis of a number of synthetic ester (Steiner et al., 1980) and amide (Kisiel et al., 1976; 1977; Steiner & Castellino, 1982) substrates. This enzyme is virtually unique as a protease in that monovalent and/or divalent cations are necessary in order for APC to exert activity toward small substrates (Steiner et al., 1980; Steiner & Castellino, 1982, 1985). We are greatly interested in identifying the enzymic properties of APC which are influenced by such cations and have examined in this report the pre-steady-state kinetics of APC toward a synthetic substrate and the influence of mo-

† This study was supported by Grant HL-19982 from the National Institutes of Health and Grant 81-659 from the American Heart Association.

0006-2960/85/0424-1136$01.50/0

novalent cations on these parameters.

Materials and Methods

Proteins. Bovine plasma protein C was prepared from fresh citrated plasma by modification of the procedure of Stenflo (1976). Our exact procedures have been described (Steiner & Castellino, 1982). Activated protein C (APC) was generated by treatment of PC with a preparation of the coagulant enzyme from the venom of Russel's viper (RVV-X), insolubilized to Sepharose 4B, according to the methodology of Steiner et al. (1980). The concentration of APC was determined by titration of a suitable stock solution of the enzyme with *p*-nitrophenyl *p*-guanidinobenzoate (NPGB) according to the procedure of Chase & Shaw (1967). RVV-X was purified from the crude venom (Miami Serpentarium) as described by Amphlett et al. (1982) and coupled to Sepharose 4B as described by Bryne & Castellino (1978). The molecular characteristics of each of these proteins can be found in the relevant publications referred to previously in this section.

Pre-Steady-State Kinetics. The rate of release of *p*-nitrophenolate from NPGB was monitored on a Durram stopped-flow spectrophotometer equipped with a digital storage oscilloscope. One of the syringes of the spectrophotometer was filled with the enzyme solution, consisting of 0.2 mg/mL (3.6 μM) APC, dissolved in a buffer composed of 75 mM *N*-(2-hydroxyethyl)piperazine-*N'*-2-ethanesulfonic acid–triethanolamine (HEPES–TEA), pH 8.3. The other syringe contained varying concentrations of NPGB (300–1500 μM) and cation (Na^+ or Cs^+ at 10–150 mM) in 25 mM HEPES–TEA, pH 8.3. The syringes, as well as the mixing chamber, were maintained at 7 °C in order to minimize the rate of background hydrolysis of the substrate in aqueous solution. The enzymic reaction was initiated with the rapid mixing of 0.2 mL of each reagent, and the absorbance of the solution at 410 nm, as a function of time, was recorded on the oscilloscope. The data, in the form of an apparent first-order curve, were plotted from the oscilloscope by employing an X–Y plotter and treated according to the analysis described below in order to calculate the pre-steady-state kinetic parameters.

Treatment of the Pre-Steady-State Rate Data. The basis for the measurement of the pre-steady-state rates of *p*-nitrophenolate release is the kinetic model for the mechanism of serine proteases proposed by Bender et al. (1966) in which the reaction is a three-step process:

$$E + S \underset{k_{-1}}{\overset{k_1}{\leftrightarrow}} ES \xrightarrow{k_2} EP' + P_1 \xrightarrow{k_3} E + P_2 \qquad (1)$$

The first step is the equilibrium binding of substrate (S) to enzyme (E), with the formation of the ES complex. The equilibrium dissociation constant (K_s) is governed by the association rate constant (k_1) and the dissociation rate constant (k_{-1}) where $k_{-1}/k_1 = K_S$. The second stage in the reaction mechanism is the acylation of the active-site seryl hydroxyl group on the enzyme by the substrate, with a rate constant (k_2) leading to the formation of the acyl-enzyme complex (EP') and concomitant liberation of the first product, P_1 (in this case, *p*-nitrophenolate). Deacylation of EP' then occurs, with the rate constant k_3, resulting in regeneration of E and liberation of the second product, P_2 (in this example, *p*-guanidinobenzoate).

The concentration of P_1 at any time (t) is given by

$$[P_1] = At + B(1 - e^{-bt}) \qquad (2)$$

where A and B are constants at given levels of E and S. The parameter b is the rate constant governing the process and is defined as

$$b = \frac{(k_2 + k_3)[S_0] + k_3K_S}{K_S + [S_0]} \qquad (3)$$

Here, $[S_0]$ is the initial concentration of NPGB, and all other terms are as above. The $K_{m,app}$ for the reaction can be expressed as

$$K_{m,app} = \frac{K_S k_3}{k_2 + k_3} \qquad (4)$$

Under conditions where $k_3 \ll k_2$ and $K_{m,app} \ll [S_0]$, eq 2 becomes

$$[P_1] = k_{cat}[E_0]t + [E_0](1 - e^{-bt}) \qquad (5)$$

where $[E_0]$ is the initial concentration of enzyme and

$$b = \frac{k_2[S_0]}{K_s + [S_0]} \qquad (6)$$

The first term of eq 5 describes the steady-state production of product, and the second term represents an expression for the pre-steady-state "burst". At longer times, the exponential term approaches zero, and this second term becomes constant. However, at short times, the burst is governed by the pseudo-first-order rate constant b.

NPGB is one of the synthetic ester substrates which form very stable acyl-enzyme complexes with several serine proteases, including APC, and which possess K_s values indicative of tight binding of S to E. These compounds effectively allow single turnover of the enzyme and are very suitable for determination of pre-steady-state rate parameters. In this case, eq 6 is rewritten as

$$\frac{1}{b} = \frac{1}{k_2} + \frac{K_S}{k_2[S_0]} \qquad (7)$$

A plot of $1/b$ vs. $1/[S_0]$ produces a line with an ordinate of $1/k_2$ and a slope of K_s/k_2, from which k_2 and K_S are readily calculated.

Determination of k_3. The deacylation rate constant k_3 for guanidinobenzoyl-APC was determined by a method similar to that published by Chibber et al. (1977). The guanidinobenzoyl-APC was formed by addition of NPGB (150 μM) to a solution of APC (1.6 μM) in a buffer consisting of 50 mM HEPS–TEA/200 mM NaCl, pH 8.3. The solution was allowed to incubate for 5 min at 7 °C, after which a quantity of 5 μL was added to 995 μL of an assay mixture of the steady-state substrate H-D-phenylalanylpipecolylarginine-*p*-nitroanilide (S-2238) present at a concentration of 1.0 mM in a buffer of 50 mM HEPES–TEA/200 mM NaCl, pH 8.3, containing various concentrations of Na^+ or Cs^+. The deacylation of APC was monitored by measurement of the rate of appearance of the product, *p*-nitroanilide, at 405 nm, which resulted from the hydrolytic activity of deacylated APC toward S-2238. The value for k_3 was determined from

$$-k_3t = \ln \frac{\text{rate}_\infty - \text{rate}_t}{\text{rate}_\infty} \qquad (8)$$

where rate_t is the rate of *p*-nitroanilide appearance at time t and rate_∞ is the same reaction rate at times at which the hydrolysis rate became linear (maximal rate).

Results

In an attempt to understand further which of the steps of synthetic substrate hydrolysis by APC are affected by monovalent cations, a pre-steady-state kinetic analysis of APC toward the substrate, NPGB, was performed. In these experiments, the effect of increasing concentrations of monovalent cations on the kinetic constants k_2, k_3, and K_S was

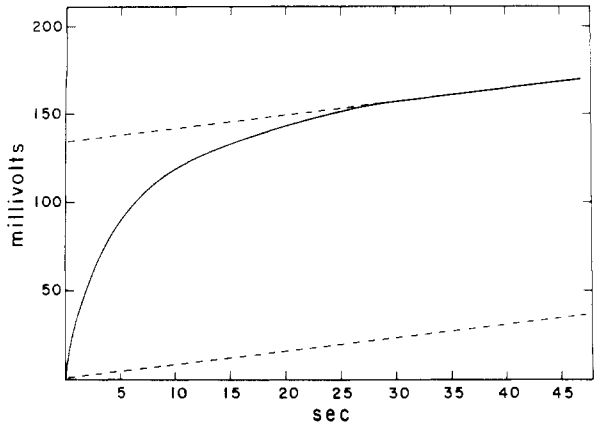

FIGURE 1: Reaction of *p*-nitrophenyl *p*-guanidinobenzoate (NPGB) with APC at 7 °C. The voltage reading on the oscilloscope is plotted against the time after which the reaction was initiated. The pre-steady-state phase of the reaction is represented by the curved section of the plot. The dashed lines show the steady-state formation of *p*-nitrophenolate. The bottom trace was obtained in the absence of APC. The reaction conditions are 3.6 μM APC, 200 mM NaCl, and 25 μM NPGB.

evaluated. Due to the very rapid rate of reaction of APC in this system, the experiments were performed with a stopped-flow spectrophotometer in order that the initial 10 s of the reaction could be observed readily. The reaction was conducted at 7 °C to minimize the rate of endogenous hydrolysis of this substrate. The reaction medium contained the buffer HEPES which was adjusted to pH 8.3 with the base triethylamine. Although the triethylammonium cation is itself able to activate the catalytic esterase activity of APC, it is a much poorer effector than any of the cations employed in this study. Therefore, the contribution of the triethylammonium cation, at all of the Na^+ and Cs^+ levels employed, was considered to be negligible, and no corrections to the rate data were employed. The present study was conducted by titrating the enzyme with various concentrations of NPGB at a number of different monovalent cation levels. Without addition of such cations, the reaction rate was very slow, as was the case when the substrates N^{α}-benzoyl-L-arginine-*p*-nitroanilide (Steiner & Castellino, 1985) and H-D-phenylalanylpipecolylarginine-*p*-nitroanilide (Steiner & Castellino, 1982) were used in steady-state analyses.

An example of the data obtained when the rate of *p*-nitrophenolate liberated upon incubation of NPGB with APC is measured is illustrated for a single representative case in Figure 1. Here, a rapid burst of *p*-nitrophenolate is seen followed by a slower steady-state production of this product, the latter being a combination of enzyme turnover and non-enzymatic hydrolysis of NPGB. This initial burst of *p*-nitrophenolate, followed by the virtual inactivation of the enzyme, indicates that a stable acyl-enzyme is produced, as is the case with many other serine protease–NPGB reactions. In other experiments, it has been found that when various levels of NPGB, all in excess of the concentration of APC, were added to APC, the amount of *p*-nitrophenolate released was stoichiometric with the enzyme level, although the rates of formation of this product may not have been equal. In addition, competitive inhibitors of APC, such as benzamidine and *p*-aminobenzamidine, were capable of inhibiting the rate of formation of *p*-nitrophenolate, but not its extent. Therefore, from all of the above information, it is clear that NPGB is a single turnover substrate for APC with an acylation rate constant, k_2, much greater than the deacylation rate constant, k_3.

Table I: Effect of Monovalent Cations on Pre-Steady-State Kinetic Parameters for Hydrolysis of NPGB by APC at 7 °C

cation	concn (mM)	$k_{2,app} \pm 10\%$ (s^{-1})	$K_S \pm 10\%$ ($\times 10^5$ M)
Na^+	12.5	0.32	18.5
	18.8	0.50	19.0
	37.5	0.93	19.9
	62.5	1.15	19.4
Cs^+	10.0	0.55	2.2
	20.0	1.02	1.8
	40.0	1.25	1.9
	60.0	1.30	2.1

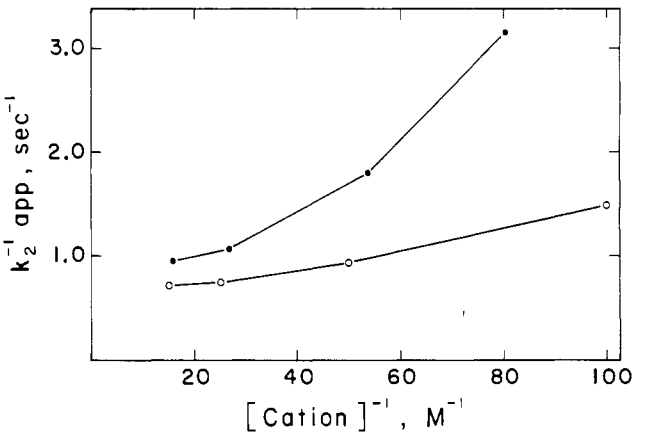

FIGURE 2: Double-reciprocal plot of the apparent acylation rate constant (k_2) at infinite [NPGB] against the concentration of cation: (●) Na^+; (○) Cs^+.

Under conditions of [NPGB] ≫ [APC], the pre-steady-state rate constants k_2 and k_3 for the reaction of NPGB with APC were obtained from analysis of the magnitude of the apparent pseudo-first-order rate constants for acylation, *b*, as a function of the concentration of NPGB. Double-reciprocal plots of the variation in *b* with the concentration of NPGB at various levels of Na^+ and Cs^+ were constructed (not shown). The values for $k_{2,app}$ and K_S for the reaction of NPGB and APC, at each concentration of monovalent cation examined, were obtained from the ordinates ($1/k_{2,app}$) and the slopes of ($K_S/k_{2,app}$) of such graphs and are summarized in Table I.

These results indicate that in the reaction between APC and NPGB, the presence of monovalent cations in the reaction medium has a substantial effect on the rate constant for acylation, k_2, but no appreciable effect on the dissociation constant, K_S, for this interaction. The maximal k_2 attainable ($k_{2,max}$) at saturation with cations and the apparent K_m for the cation ($K_{m,cation}$) have been determined by a double-reciprocal plot of the $k_{2,app}$ values against the cation concentration. These plots are presented in Figure 2 for Na^+ and Cs^+. Clearly, the effect of each cation on the $k_{2,app}$ for the acylation of APC by NPGB does not follow simple saturation kinetics, as was observed for steady-state kinetic analysis of the hydrolysis of amide substrates by APC (Steiner & Castellino, 1982, 1985). As was seen in the treatment of the steady-state data (Steiner & Castellino, 1982, 1985), a double-reciprocal plot of $k_{2,app}$ against [cation]$^{-2}$ is linear. These plots are shown in Figure 3 for Na^+ and Cs^+.

The K_S value for the interaction of NPGB with APC, which is independent of the cation concentration, has been evaluated by averaging the values obtained for this parameter as listed in Table I. For the Na^+–APC complex, the K_S for NPGB is 19.2×10^{-5} M at 7 °C. For the Cs^+–APC complex, the value for the K_S for NPGB, at this same temperature, is 2.0×10^{-5} M. The acylation rate constants for NPGB toward APC in

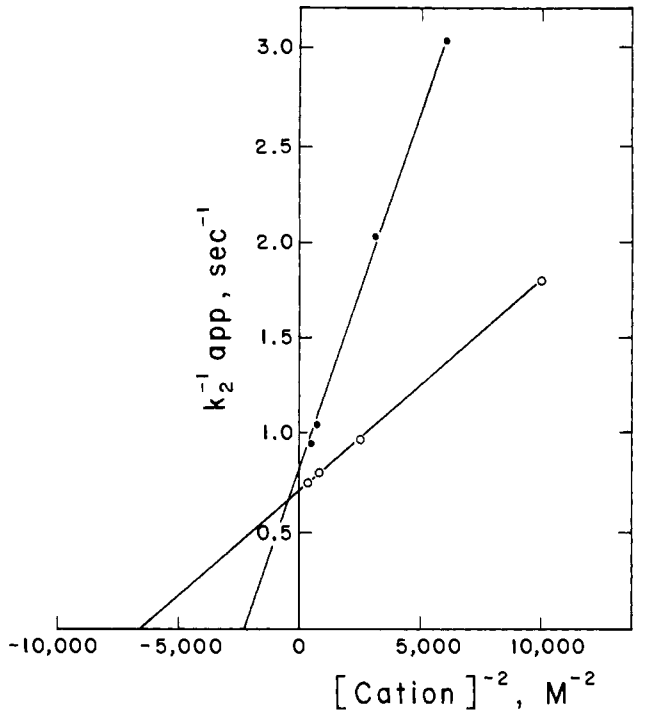

FIGURE 3: Same as in Figure 2, except that the abscissa is expressed in terms of the square of the cation concentration: (●) Na^+; (○) Cs^+.

Table II: Pre-Steady-State Kinetic Constants for APC toward NPGB in the Presence of Na^+ and Cs^+ at 7 °C

cation	$k_{2,max}$ (s^{-1})	K_S (M)	$K_{m,cation}$ (M)	k_3 (s^{-1})
Na^+	1.21 ± 0.10	19.2×10^{-5}	21 ± 3	$(3.8 \pm 1.0) \times 10^{-3}$
Cs^+	1.36 ± 0.10	2.0×10^{-5}	12 ± 2	$(3.8 \pm 1.0) \times 10^{-3}$

the presence of saturating concentrations of Na^+ and Cs^+ have been calculated from the ordinates of Figure 3. For the Na^+–APC complex at 7 °C, the $k_{2,max}$ is 1.21 ± 0.10 s^{-1}. In the case of the Cs^+–APC complex, at the same temperature, the $k_{2,max}$ has been calculated to be 1.36 ± 0.10 s^{-1}. The K_m for Na^+ [$K_m(Na^+)$] has been calculated as −(abscissa^{-2}) from the plots represented in Figure 3 (Steiner & Castellino, 1985). At 7 °C, the $K_m(Na^+)$ for the NPGB–APC complex is 21 ± 3 mM. This same value for Cs^+ to the NPGB–APC complex is 12 ± 2 mM. A summary of these pre-steady-state kinetic parameters is provided in Table II.

The deacylation rate constant, k_3, for guanidinobenzoyl-APC was determined by monitoring the rate of return of the amidolytic activity of APC toward the substrate, S-2238, following effective removal of excess NPGB by diluting the APC/NPGB mixture after acylation had occurred to a level at which [NPGB] $\ll K_S$. The diluent contained Na^+ and Cs^+ at their desired final concentrations. Under these experimental conditions, as the guanidinobenzoyl moiety is cleaved from APC, only a negligible portion of APC will rebind to NPGB, while the great majority of the free APC will hydrolyze S-2238. The amount of APC present will be proportional to the rate of hydrolysis of S-2238. Under our continuous assay procedure, the rate of deacylation can be determined by measuring the rate of acceleration of hydrolysis of S-2238. The experiment was performed in the presence of 50, 100, and 200 mM concentrations of Na^+ and Cs^+ in order to determine the effect of the cation concentrations on the value obtained for k_3. An example of the data obtained at 50 mM Na^+ is shown in Figure 4, wherein the rate of hydrolysis of S-2238 by free APC increases with time and reaches a constant rate when all of the enzyme has been deacylated. A first-order plot of

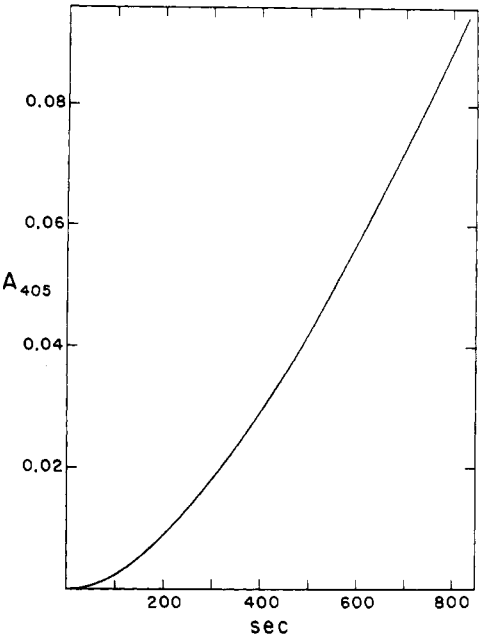

FIGURE 4: Deacylation of guanidinobenzoyl-APC at 7 °C. The rate of deacylation of guanidinobenzoyl-APC was determined as described under Materials and Methods by dilution of the preformed acyl-enzyme into a buffer containing the chromogenic substrate H-D-phenylalanylpipecolylarginine-*p*-nitroanilide (S-2238) and monitoring the return of activity. The appearance of *p*-nitroaniline from S-2238 was measured by measuring the absorbance at 405 nm (A_{405}) as a function of time.

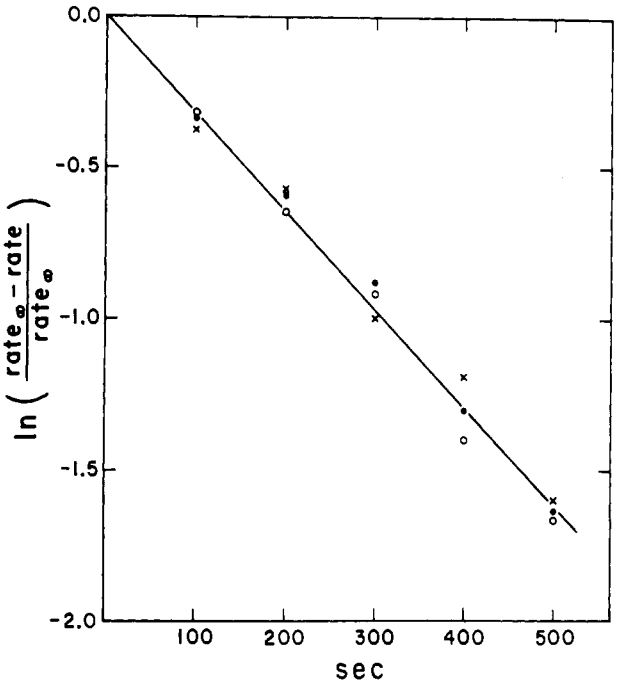

FIGURE 5: Determination of the deacylation rate constant k_3. A first-order kinetic plot of the data of Figure 4 was constructed in the presence of Na^+ concentrations of (●) 50, (○) 100, and (×) 200 mM.

this reaction, at three concentrations of Na^+, is provided in Figure 5. The k_3 determined from this graph, for all Na^+ concentrations, which is also listed in Table II, is (3.8 ± 1.0) × 10^{-3} s^{-1}. Similar data have been obtained when the cation was changed to Cs^+. During all deacylation rate assays, the quantity of S-2238 consumed at their conclusion did not exceed 5% of the initial amount added. This, coupled to the fact that the initial concentration of S-2238 present was $5K_m$ for this substrate, demonstrates that the concentration of S-2238 re-

mains virtually constant, in terms of recognition by APC, during the time course of each experiment.

The $K_{m,app}$ for this reaction has been calculated by employing the relevant K_S, $k_{2,max}$, and k_3 values for APC hydrolysis of NPGB and substitution of these parameters in eq 4. The $K_{m,app}$ for APC hydrolysis of NPGB at saturating levels of Na^+ is approximately 6×10^{-7} M, whereas the same value at saturating levels of Cs^+ is approximately 5.6×10^{-8} M.

Discussion

In a previous investigation (Steiner & Castellino, 1982), we have shown that the hydrolysis by APC of the multiple turnover substrate, H-D-phenylalanylpipecolylarginine-*p*-nitroanilide (S-2238), occurred with a V_{max} that was dependent upon the concentration of monovalent cations and a K_m that was independent of the cation concentration. In a later study (Steiner & Castellino, 1985), we forwarded a mechanism for this effect of monovalent cations on APC activity which involved random binding to APC of a pair of monovalent cations and substrate to form the catalytically active complex APC–$cation_2$–substrate. In this report, we have turned our attention to the effect of monovalent cations on the pre-steady-state kinetic properties of APC in order to examine the influence of such cations on the discrete steps involved in synthetic substrate hydrolysis by APC.

For such a substrate, we have selected NPGB. As is seen from the data listed in Table II, this ester appears to be essentially a single turnover substrate for APC. The $k_2 \gg k_3$, such that at early times of reaction only the acylation rate is being observed. Also, since the $K_{m,app}$ for the reaction, in the presence of both Na^+ and Cs^+, is much larger than the range of NPGB concentrations employed in the kinetic experiments, it would appear as though NPGB is an effective titrant for APC over this range of NPGB levels. It is significant to note that in all experiments wherein the initial concentration of NPGB is varied, the extent of *p*-nitrophenolate released at the conclusion of the reaction is always the same, again showing that the pre-steady-state portion of the hydrolysis of NPGB is being monitored under all experimental conditions.

The results of the pre-steady-state analysis indicate that the concentration of monovalent cations mainly affects k_2, the acylation rate constant, whereas the binding constant of substrate to enzyme, K_S, and the deacylation rate constant, k_3, are not greatly influenced by the level of monovalent cation present in the reaction mixture. In view of earlier results that monovalent cation concentrations affect the V_{max} in steady-state amide substrate hydrolysis (Steiner & Castellino, 1982, 1985), it would appear as though k_2 makes a significant contribution to the V_{max} of the reactions of APC with amides, whereas K_S and k_3 are of lesser importance in this respect. Similarly, since the steady-state K_m values for these same amide substrates are not greatly influenced by the concentration of monovalent cations (Steiner & Castellino, 1982, 1985), it can be concluded that the k_2 values for amide substrates do not play a great role in the determination of their K_m values.

The double-reciprocal plots of k_2 against cation concentration (Figure 2) are not linear, indicating that the dependence of k_2 on cation concentration is complex. As is also the case with the variation of the steady-state V_{max} of amide substrates with cation concentration (Steiner & Castellino, 1982, 1985), linear double-reciprocal plots are obtained when the value of k_2 is plotted against the square of the cation concentration (Figure 3). The result again indicates that since a similar dependency of k_2 and V_{max} on cation concentration is obtained, the V_{max} for amide substrates for APC is dominated by the acylation rate constant k_2. Also, for this acylation to occur, it appears as if at least two monovalent cation sites, or classes of sites, on APC must be filled. However, the binding of substrate to enzyme does not depend upon the presence of monovalent cations since the K_S values for NPGB binding to APC are not affected by the concentrations of monovalent cations.

Table III: Pre-Steady-State Kinetic Parameters for Selected Bovine Proteases toward NPGB

enzyme	k_2 (s^{-1})	K_S ($\times 10^6$ M)	k_3 ($\times 10^5$ s^{-1})	$K_{m,app}$ (M)
trypsin[a]	1.95	0.61	3.4	1.16×10^{-11}
factor Xa[b]	0.19	420	4.2	9.3×10^{-8}
plasmin[c]	0.54	22.2	0.53	2.2×10^{-10}
factor IX$\alpha\beta$[d]	0.10	440	0.30	1.3×10^{-8}
APC[e]	1.21	192[f]	380	6.0×10^{-7}
	1.36	20[g]	380	5.6×10^{-8}

[a] Chase & Shaw (1969). The temperature was ambient. [b] Lindhout et al. (1978). The temperature was 25 °C. These values differ substantially from those reported in Smith (1973). [c] Sodetz & Castellino (1972). The temperature was 22 °C. [d] Byrne et al. (1980). The temperature was 30 °C. [e] This report. The temperature was 7 °C. [f] Value determined at saturating levels of Na^+. [g] Value determined at saturating levels of Cs^+.

The K_m(cation) to APC at 7 °C has been determined from pre-steady-state analysis to be approximately 21 mM for Na^+ and approximately 12 mM for Cs^+. These values should represent the K_{diss} values for these cations to APC. Similar values can also be calculated from steady-state kinetic analysis at this same temperature. As a result of examination of the variation in initial hydrolytic rates of the multiple turnover substrate S-2238 at this same temperature, the K_m values for these same cations have been calculated by employing a procedure similar to that described by Steiner & Castellino (1982). From this analysis, the $K_m(Na^+)$ has been calculated to be approximately 18 mM and that for Cs^+ to be approximately 10 mM, at 7 °C. Thus, the necessary agreement has been reached, again showing that the effect of monovalent cations on the k_2 of the reactions of APC with synthetic substrates is of major influence on their ability to activate the hydrolytic properties of APC.

The pre-steady-state kinetic parameters for APC toward NPGB are compared to those for other proteases isolated from bovine sources in Table III. The acylation rate constant (k_2) for NPGB binding to APC most closely resembles that for trypsin than for any of the other proteases. The K_S values for NPGB binding to APC are similar to those for factor IX$a\beta$ and factor Xa and are much higher than those for trypsin or plasmin. However, the deacylation rate constant, k_3, for APC is substantially higher than those for other proteases listed. The $K_{m,app}$ for trypsin binding to NPGB is lowest for all the proteases compared, due mainly to the tight binding of NPGB, as reflected by the K_S value. Similarly, the $K_{m,app}$ for plasmin is comparable to that for trypsin. In this case, however, NPGB binds to plasmin much more weakly than to trypsin, but the deacylation rate of *p*-guanidinobenzoylplasmin is considerably slower than that for *p*-guanidinobenzoyltrypsin, resulting in the low $K_{m,app}$ value seen. The $K_{m,app}$ for NPGB toward APC is quite similar to that for factor IX$a\beta$ and factor Xa but for different reasons. The binding of NPGB to APC is tighter than that for factor IX$a\beta$, and the acylation rate of NPGB toward APC is much faster than that for factor IX$a\beta$. However, the deacylation rate of *p*-guanidinobenzoyl-APC is approximately 1000-fold faster than that for *p*-guanidinobenzoyl-factor IX$a\beta$, a consideration which raises the $K_{m,app}$ for NPGB binding to APC to a level nearly the same as that

for factor IXaβ. A similar set of facts applies to a comparison of the $K_{m,app}$ of NPGB binding to factor Xa and APC, which leads to similarity in the $K_{m,app}$ for NPGB binding to each enzyme.

Registry No. APC, 42617-41-4; NPGB, 21658-26-4; Na, 7440-23-5; Cs, 7440-46-2.

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Effect of High pN_2 and High pD_2 on NH_3 Production, H_2 Evolution, and HD Formation by Nitrogenases†

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Received July 30, 1984

ABSTRACT: We have investigated the effect of the partial pressure of N_2 and D_2 on HD formation, H_2 evolution, and NH_3 production by nitrogenases from *Klebsiella pneumoniae* and *Clostridium pasteurianum*. By using pressures up to 4 atm, we have been able to extend the concentration range of N_2 and D_2 in our investigations beyond that used in previous studies. The pN_2 dependence of HD formation with constant pD_2 ideally shows no HD formation under zero pN_2, reaches a peak which depends on the pD_2, and then decreases to zero at very high pN_2. *K. pneumoniae* and *C. pasteurianum* nitrogenases differ in their $K_i(D_2)$ for nitrogen fixation. *C. pasteurianum* nitrogenase had the lower activity for formation of HD. With *K. pneumoniae* nitrogenase, D_2 enhanced H_2 evolution from 31% of the electron flux partitioned to H_2 in the absence of D_2 to 51% of the electron flux partitioned to H_2 at 400 kPa of D_2. With *C. pasteurianum* nitrogenase, the equivalent values were 33% and 48% of the total electron flux. Our results support the mechanism for nitrogenase-catalyzed reductions proposed by W. W. Cleland [Guth, J., & Burris, R. H. (1983) *Biochemistry 22*, 5111–5122].

Since Hoch et al. (1960) showed that soybean nodules produced HD when they were incubated with D_2 and N_2, HD formation has been used as a tool to provide information about the mechanism of nitrogen fixation. Hoch et al. proposed that HD is formed when D_2 reacts with an enzyme intermediate in N_2 reduction.

In the absence of other substrates, nitrogenases reduce protons to H_2 (Bulen et al., 1965); this H_2 evolution cannot be completely suppressed by N_2 although substrates such as C_2H_2 and HCN can completely suppress H_2 evolution at high substrate concentrations (Rivera-Ortiz & Burris, 1975; Simpson & Burris, 1984). Not only is H_2 a product of nitrogenase but it also is a specific inhibitor of N_2 reduction by nitrogenase (Wilson & Umbreit, 1937). Bulen (1976) combined these observations and proposed that inhibition of NH_3 formation by H_2 (D_2) and N_2-dependent HD formation from D_2 and H_2O are different manifestations of the same molecular process. As pointed out by Guth & Burris (1983) there is

† This work was supported by the College of Agricultural and Life Sciences, University of Wisconsin—Madison, by the Science and Education Administration of the U.S. Department of Agriculture under Grant 5901-7020-9-0202-0 from the Competitive Research Grants Office, by National Science Foundation Grant PCM-8115077, by Public Health Service Grant AI-00848 from the National Institute of Allergy and Infectious Diseases, by the Danish Natural Science Research Council, and by the Danish Agricultural and Veterinary Research Council.

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0006-2960/85/0424-1141$01.50/0