The effect of γ-carboxyglutamate residues on the enzymatic properties of the activated blood clotting factor X

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I. ACTIVITY TOWARDS SYNTHETIC SUBSTRATES

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Summary

The esterolytic and amidolytic properties of activated blood coagulation factor X (factor X_a) and the analogous decarboxy species were compared in order to find out if the γ-carboxyglutamic acid residues influence the function of the active centre. It was found that the two proteins (1) showed similar kinetic parameters when titrated with *p*-nitrophenyl-*p'*-guanidinobenzoate hydrochloride (2) had a similar K_m and k_{cat} for various synthetic chromogenic tri- and tetrapeptides and (3) were inhibited in the same way by benzamidine. Further it was observed that (4) Ca^{2+} inactivates factor X_a, but has no influence on the amidase activity of decarboxyfactor X_a (5) factor V prevents Ca^{2+}-induced inactivation of factor X_a but does not influence the amidase activity of both factor X_a and decarboxyfactor X_a.

We conclude that the interaction of the γ-carboxyglutamic acid residues with Ca^{2+} in factor X has no measurable influence on the properties of the active site per se.

Introduction

Factor X_a is a protease belonging to the chymotrypsin family. With thrombin, plasmin, trypsin and chymotrypsin it shares many structural and functional features. It can be safely assumed that a similar catalytic mechanism, involving the "charge relay system" Asp-His-Ser and common features in the three dimensional structure are shared by these mammalian serine proteases [1—4]. Amino acid sequences in the region surrounding the reactive serines in the heavy chain of factor X_a are found to be homologous with the same regions of trypsin, chymotrypsin-A, elastase and thrombin [4—9]. Like trypsin, acti-

vated factor X is inhibited by soybean trypsin inhibitor [10,11] diisopropylphosphofluoridate [11,12] and synthetic aromatic amidine and guanidino inhibitors [13].

Factor X_a catalyzes the hydrolysis of various synthetic substrates such as *N*-α-benzoyl-L-arginine ethylester [14], *N*-α-*p*-tolyene sulfonyl-L-arginine methylester [11,15], *p*-nitrophenyl-*p'*-guanidinobenzoate [16] and benzoxy-isoleucyl-glutamyl-glycyl-arginyl-*p*-nitroanilide [17]. Contrary to other known serine proteases (with the exception of clotting factor IX_a) factor X_a has unique structural feature in that it possesses several couples of γ-carboxyglutamic acid residues in the N-terminal part of its smaller chain. Activated factor X also can be considered a protease with trypsin-like specificity in that it selectively attacks amido bonds adjacent to arginine. Unlike trypsin, the *N*-acylamino acid esters containing lysine are less readily hydrolized by factor X_a than the arginine-containing esters [14].

When acting on its natural substrate (prothrombin) factor X_a cleaves only two peptide bonds adjacent to arginine, the $\overset{274}{\text{Arg}}-\overset{275}{\text{Thr}}$ and $\overset{323}{\text{Arg}}-\overset{324}{\text{Ile}}$ bonds [18—21], whereas there are 75 bonds which in principle, must be considered susceptible. The highly restricted substrate specificity of factor X_a is an interesting, but hardly investigated, feature. Experiments comparing factor X_a to trypsin with respect to their inhibition by various amidines and guanidines, carried out by Johnson et al. [13] suggest that factor X_a contains a primary substrate binding site equal to that of trypsin in potential binding energy and a secondary substrate binding site responsible for the specificity of factor X_a. It can be safely assumed that apart from the active site-vulnerable site interaction in a protease and its protein substrate "subsite" interaction are largely responsible for highly specific interactions like these of factor X_a and prothrombin [22—25].

It is the purpose of the experiments described in this paper to elucidate a possible role of these γ-carboxyglutamate residues in the catalytic activity of factor X_a by comparing it with the activity of decarboxyfactor X_a. This protein, isolated from dicoumarol-treated cattle is completely identical to factor X_a but for the presence of glutamyl instead of γ-carboxyglutamyl residues.

Materials and Methods

Materials and methods, except for those described below, are reported earlier [26].

p-Nitrophenyl-*p'*-guanidinobenzoate hydrochloride (NPGB) was purchased from Biochemical Nutrition Corp. Benzoxy-phenylalanyl-valyl-arginyl-*p*-nitroanilide (Bz-Phe-Val-Arg-*p*NA) and benzoxy-isoleucyl-glutamyl-glycyl-arginyl-*p*-nitroanilide (Bz-Ile-Glu-Gly-Arg-*p*NA) were products of A.B. Bofors, Nobel Pharma, Sweden.

Activated factor X and activated decarboxyfactor X were prepared with the method descrived in a preceding paper [26]. Factor V was prepared according to Smith and Hanahan [41]. A specific activity of 180 U/mg was obtained after activation with a trace of factor V activator from Russell's Viper venom. No other procoagulant activities could be found in this preparation.

Titration of factor X_a and decarboxyfactor X_a

Titration of activated factor X and activated decarboxyfactor X was performed according to Smith [16]. In a typical titration the sample cuvette contains 150 μg of factor X_a or 180 μg of decarboxyfactor X_a in 300 μl of 0.1 M sodium barbital buffer, pH 8.3. The reference cuvette contains the same volume of buffer solution alone. To each cuvette 3 μl of a 0.01 M solution of NPGB in dimethyl-formamide-acetonitrile (1 : 3, v/v) were simultaneously added. The reaction was followed at 410 nm in an Aminco DW-2 spectrophotometer at 25° C. The concentration of *p*-nitrophenol released during the reaction was calculated from the zero time intercept of the extrapolated steady state line. In the Aminco DW-2 spectrophotometer, ϵ_{410} for *p*-nitrophenol at pH 8.3 was calculated to be 17 500.

Determination of kinetic constants K_s and k_2

The presteady-state reaction constants of factor X_a and decarboxyfactor X_a with NPGB were determined according to Bender et al. [27]. Enzyme concentrations were $3.2 \cdot 10^{-6}$ M factor X_a and $2.2 \cdot 10^{-6}$ M decarboxyfactor X_a. The NPGB concentrations ranged from $3.3 \cdot 10^{-5}$ M to $13.3 \cdot 10^{-5}$ M. The operational first order rate constant for the presteady-state reaction (b) was determined at each concentration of NPGB by linear regression analysis. The rate constant of acylation (k_2) and the association constant K_s of the enzyme · substrate complex were determined from the intercept and slope of a plot of $1/b$ versus 1/[NPGB].

Determination of k_3 and K_{pb}

Acylation of factor X_a and decarboxyfactor X_a was performed by addition of 10 μl of 0.01 M NPGB to 1.2 ml of a solution of factor X_a or decarboxyfactor X_a in 0.1 M sodium barbital buffer, pH 8.3. The final concentration of NPGB was $8.3 \cdot 10^{-5}$ M. The final enzyme concentrations were $3.2 \cdot 10^{-6}$ M factor X_a and $2.2 \cdot 10^{-6}$ M decarboxyfactor X_a. The acylenzyme solutions with excess NPGB were applied to a column (0.9 × 30 cm) of Sephadex G-25 equilibrated with 0.1 sodium barbital buffer, pH 8.3. Enzyme-containing fractions were collected and incubated at 25° C. At several intervals aliquots were removed from the incubation mixture and assayed for amidase activity with Bz-Ile-Glu-Gly-Arg-*p*NA. The time by which the acylenzyme had sunk into the column was taken as zero time. The deacylation rate constant (k_3) was determined from the slope of $\ln [E_0]/([E_0] - [E_t])$ versus time, where $[E_0]$ is the concentration of acylenzyme at zero time and $[E_t]$ the concentration of deacylated enzyme at time t. Amidase activity is determined as described in this section. The rate constant for the postburst *p*-nitrophenol production (K_{pb}) is determined at $1.6 \cdot 10^{-4}$ M NPGB.

Amidase activity assay of factor X_a and decarboxyfactor X_a

The initial rates of hydrolysis of the amides *N*-benzoyl-L-phenylalanyl-L-valyl-L-arginine-paranitroanilide-HCl and *N*-benzoyl-L-isoleucyl-L-glutamyl-glycyl-L-arginine-paranitroanilide-HCl by factor X_a and decarboxyfactor X_a were measured in Tris-imidazole buffer, pH 8.2, and ionic strength of 0.15 at 37° C by determining the increase of *p*-nitroaniline absorbance in the Aminco

DW-2 spectrophotometer operating in the dual wavelength mode with λ_r = 344 nm and λ_s = 391 nm at 37° C. The $\epsilon_{391\text{-}344}$ of *p*-nitroaniline in the buffer solution of pH 8.2 was determined as 11 400 A/mol.

Results

Titration of factor X_a and decarboxyfactor X_a

Functional enzyme concentration. Factor X_a and decarboxyfactor X_a were titrated with NPGB as described under Materials and Methods. As shown in Fig. 1 the presteady-state *p*-nitrophenol production was complete after about 2 min for both factor X_a and decarboxyfactor X_a and was followed by a very low linear postburst production of *p*-nitrophenol. From this type of curve the presteady-state production of *p*-nitrophenol (π) was determined [16]. As can be seen from Table I, π does not vary with the NPGB concentration. This fact, and the low postburst production of *p*-nitrophenol indicate that $S_0 >> K_M$ and $k_2 >> k_3$. Therefore $[E_0] \approx \pi$ and the molarity of the enzyme solutions can be calculated as $(3.1 \pm 0.1) \cdot 10^{-6}$ M and $(2.1 \pm 0.1) \cdot 10^{-6}$ M for factor X_a and decarboxyfactor X_a, respectively.

Determination of kinetic constants. The kinetic constants of the hydrolysis of NPGB catalyzed by factor X_a and decarboxyfactor X_a are summarized in Table II. The linear relationship between $1/b$ versus 1/NPGB (Fig. 2) and $\ln[E_0/ES']$ versus time (Fig. 3) show that the kinetics of both factor X_a and decarboxyfactor X_a reacting with NPGB are adequately described by the theory of Bender et al. [27]. The rate constant for the postburst *p*-nitrophenol production, K_{pb}, determined at an NPGB concentration of $1.6 \cdot 10^{-4}$ M was about twice the deacylation constant k_3 for both factor X_a and decarboxyfac-

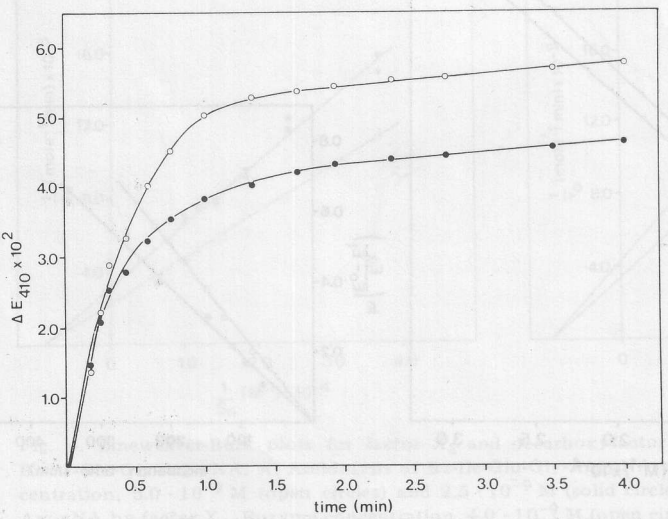

Fig. 1. Time courses of the reaction between *p'*-nitrophenyl-*p*-guanidinobenzoate (NPGB) and factor X_a (open circles) and decarboxyfactor X_a (solid circles). Procedure is as described under Materials and Methods. The actual protein concentration is $3.6 \cdot 10^{-6}$ and $3.1 \cdot 10^{-6}$ M for factor X_a and decarboxyfactor X_a, respectively. The NPGB concentration is $9.9 \cdot 10^{-5}$ M.

TABLE I

TITRATION OF FACTOR X_a AND DECARBOXYFACTOR X_a WITH p-NPGB: INDEPENDENCE OF π AND p-NPGB CONCENTRATION

Final p-NPGB concentration in cuvette (M)	Presteady-state production *p*-nitrophenol (π) ΔE_{410}		Functional enzyme concentration (μM)	
	Factor X_a	Decarboxy-factor X_a	Factor X_a	Decarboxy-factor X_a
$3.3 \cdot 10^{-5}$	$5.30 \cdot 10^{-2}$	$3.64 \cdot 10^{-2}$	3.03	2.08
$6.6 \cdot 10^{-5}$	$5.68 \cdot 10^{-2}$	$4.06 \cdot 10^{-2}$	3.25	2.32
$9.9 \cdot 10^{-5}$	$5.53 \cdot 10^{-2}$	$3.85 \cdot 10^{-2}$	3.16	2.20
$13.2 \cdot 10^{-5}$	$5.28 \cdot 10^{-2}$	$3.94 \cdot 10^{-2}$	3.02	2.25

TABLE II

KINETIC CONSTANTS OF REACTION OF FACTOR X_a AND DECARBOXYFACTOR X_a WITH p-NPGB

Constant	Factor X_a	Decarboxyfactor X_a
K_s	$4.2 \cdot 10^{-4}$ M	$4.0 \cdot 10^{-4}$ M
k_2	$0.19\ s^{-1}$	$0.15\ s^{-1}$
k_3	$4.2 \cdot 10^{-5} \cdot s^{-1}$	$3.0 \cdot 10^{-5} \cdot s^{-1}$
k_2/k_3	$4.5 \cdot 10^{-3}$	$5.0 \cdot 10^{-3}$
K_M	$9.3 \cdot 10^{-8}$ M	$8.0 \cdot 10^{-8}$ M
K_{pb}	$8.0 \cdot 10^{-5}$ M	$7.0 \cdot 10^{-5}$ M

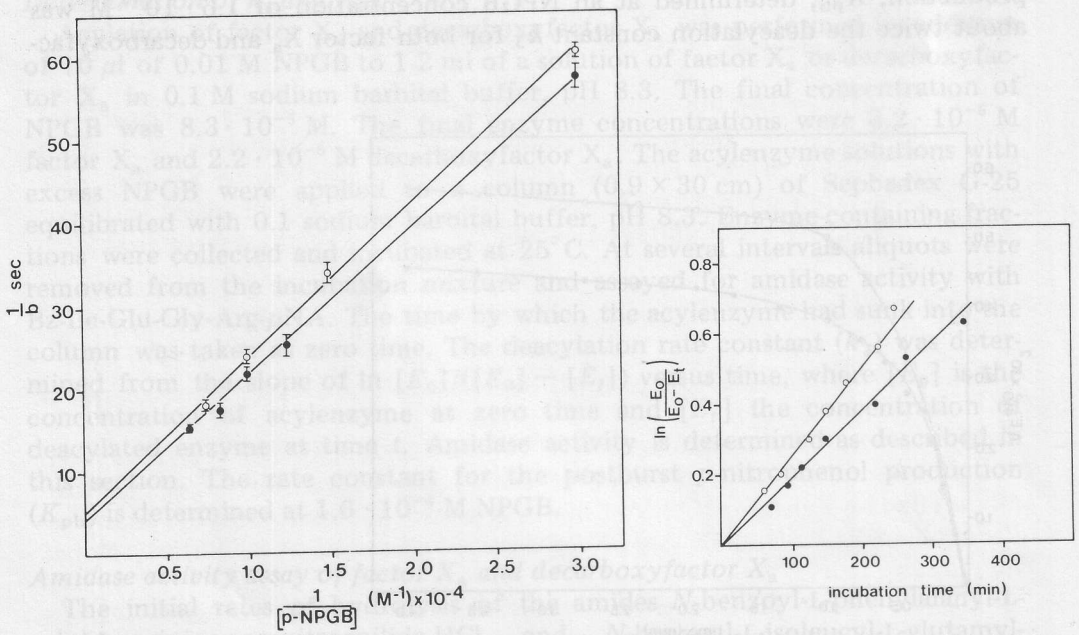

Fig. 2. Plot of the first order rate constant of the presteady state reaction of factor X_a (open circles) and decarboxyfactor X_a (solid circles) versus the reciprocal *p*-NPGB concentration.

Fig. 3. Deacylation of guanidinobenzoyl-factor X_a (open circles) and guanidinobenzoyl-decarboxyfactor X_a (solid circles). Experimental details are described under Materials and Methods.

tor X_a. However, the ratio k_2/k_3 is very large; therefore the postburst *p*-nitrophenol production had no serious effect on the accuracy of the factor X_a and decarboxyfactor X_a titration under the conditions used.

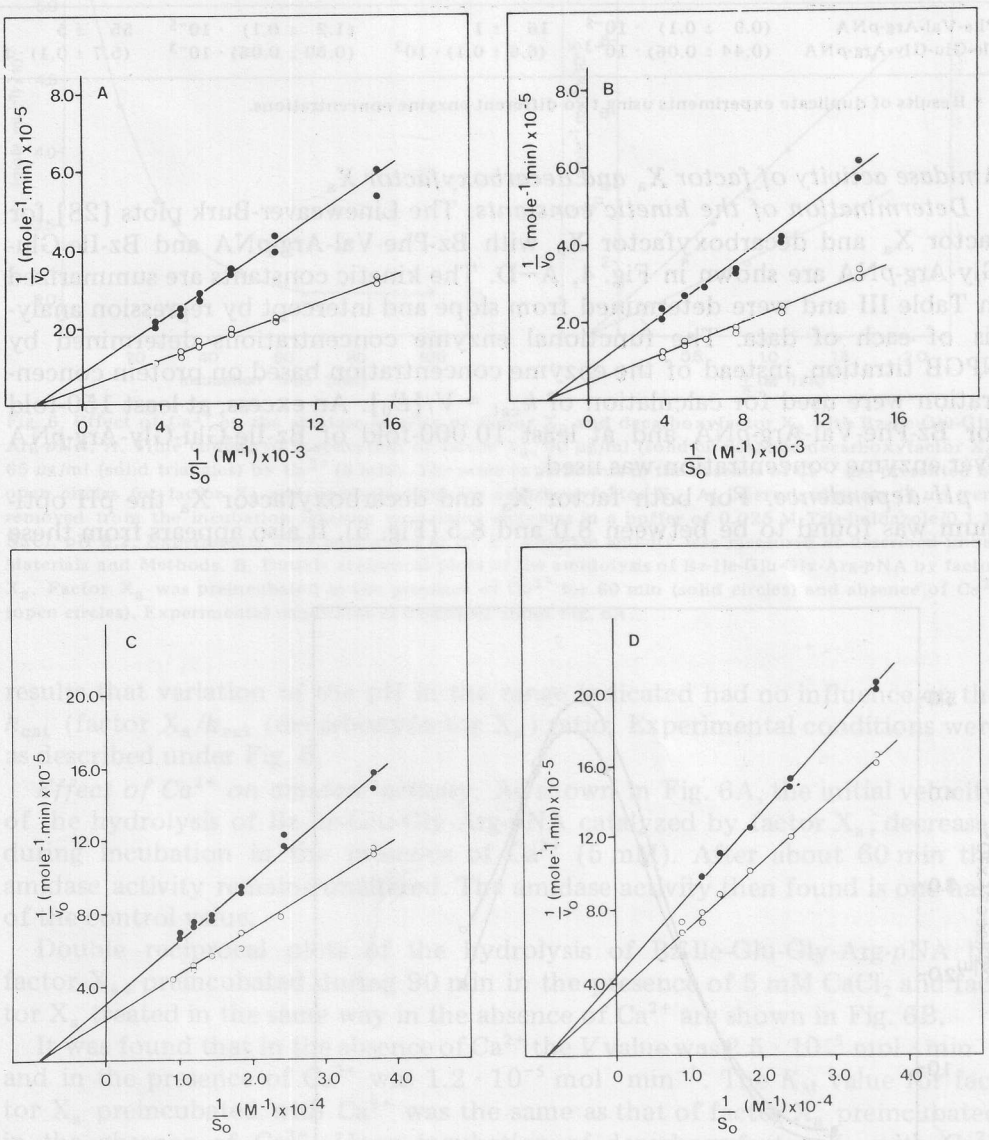

Fig. 4. Lineweaver-Burk plots for factor X_a and decarboxyfactor X_a using Bz-Phe-Val-Arg-*p*-NA and Bz-Ile-Glu-Gly-Arg-*p*NA. A. Amidolysis of Bz-Ile-Glu-Gly-Arg-*p*NA by decarboxyfactor X_a. Enzyme concentration, $5.0 \cdot 10^{-9}$ M (open circles) and $2.5 \cdot 10^{-9}$ M (solid circles). B. Amidolysis of Bz-Ile-Glu-Gly-Arg-*p*NA by factor X_a. Enzyme concentration, $4.0 \cdot 10^{-9}$ M (open circles) and $2.0 \cdot 10^{-9}$ M (solid circles). C. Amidolysis of Bz-Phe-Val-Arg-*p*NA by decarboxyfactor X_a. Enzyme concentrations, $9.0 \cdot 10^{-8}$ M (open circles) and $6.1 \cdot 10^{-8}$ M (solid circles). D. Amidolysis of Bz-Phe-Val-Arg-*p*NA by factor X_a. Enzyme concentrations, $1.9 \cdot 10^{-7}$ M (open circles) and $1.3 \cdot 10^{-7}$ M (solid circles). Experimental conditions as described under Materials and Methods.

TABLE III

KINETIC CONSTANTS FOR THE HYDROLYSIS OF SYNTHETIC AMIDE SUBSTRATES BY ACTIVATED FACTOR X AND ACTIVATED DECARBOXYFACTOR X *

Substrate	Factor X_a		Decarboxyfactor X_a	
	K_M (M)	k_{cat} (min^{-1})	K_M (M)	k_{cat} (min^{-1})
Bz-Phe-Val-Arg-*p*NA	$(0.9 \pm 0.1) \cdot 10^{-5}$	16 ± 1	$(1.2 \pm 0.1) \cdot 10^{-5}$	55 ± 5
Bz-Ile-Glu-Gly-Arg-*p*NA	$(0.44 \pm 0.06) \cdot 10^{-3}$	$(6.6 \pm 0.1) \cdot 10^{3}$	$(0.50 \pm 0.06) \cdot 10^{-3}$	$(5.7 \pm 0.1) \cdot 10^{3}$

* Results of duplicate experiments using two different enzyme concentrations.

Amidase activity of factor X_a and decarboxyfactor X_a

Determination of the kinetic constants. The Lineweaver-Burk plots [28] for factor X_a and decarboxyfactor X_a with Bz-Phe-Val-Arg-*p*NA and Bz-Ile-Glu-Gly-Arg-*p*NA are shown in Fig. 4, A—D. The kinetic constants are summarized in Table III and were determined from slope and intercept by regression analysis of each of data. The functional enzyme concentrations determined by NPGB titration, instead of the enzyme concentration based on protein concentration were used for calculation of $k_{cat} = V/[E_0]$. An excess, at least 150-fold for Bz-Phe-Val-Arg-*p*NA and at least 10 000-fold of Bz-Ile-Glu-Gly-Arg-*p*NA over enzyme concentration was used.

pH dependence. For both factor X_a and decarboxyfactor X_a the pH optimum was found to be between 8.0 and 8.5 (Fig. 5). It also appears from these

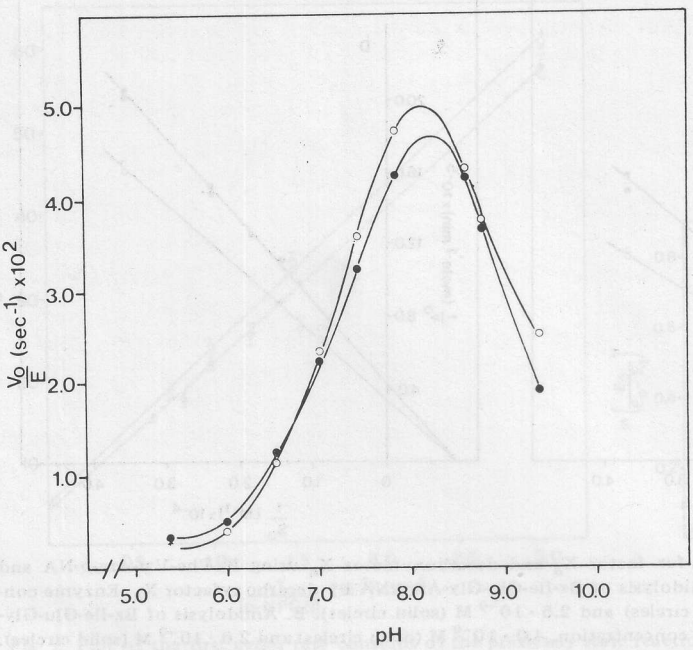

Fig. 5. pH optima of factor X_a (○) and decarboxyfactor X_a (●) with Bz-Ile-Glu-Gly-Arg-*p*NA. The pH dependence was determined in Tris-imidazole bufferes at varying pH, ionic strength of 0.15 at 37° C. Substrate concentration was $1.2 \cdot 10^{-4}$ M, enzyme concentrations were: factor X_a, $1.2 \cdot 10^{-8}$ M and decarboxyfactor X_a, $1.1 \cdot 10^{-8}$ M. Amidase activity assay as described under Materials and Methods.

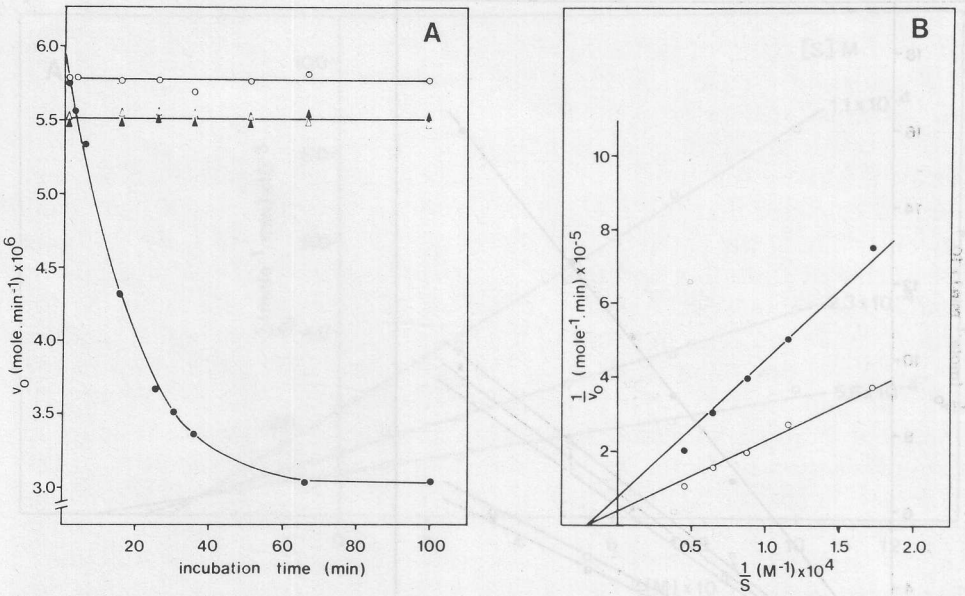

Fig. 6. Effect of Ca^{2+} on the amidase activity of factor X_a and decarboxyfactor X_a with Bz-Ile-Glu-Gly-Arg-*p*NA. A. Time courses of inactivation of factor X_a, 50 μg/ml (solid circles) and decarboxyfactor X_a, 65 μg/ml (solid triangles) by Ca^{2+} (5 mM). The same experiments in the absence of Ca^{2+} are presented by open circles for factor X_a and open triangles for decarboxyfactor X_a. At intervals aliquots (4 μl) were removed from the incubation mixture containing enzymes in a buffer of 0.025 M Tris-imidazole/0.1 M NaCl, pH 8.2. Experiments were performed at 37° C. Amidase activity was measured as described under Materials and Methods. B. Double reciprocal plots of the amidolysis of Bz-Ile-Glu-Gly-Arg-*p*NA by factor X_a. Factor X_a was preincubated in the presence of Ca^{2+} for 60 min (solid circles) and absence of Ca^{2+} (open circles). Experimental conditions as described under Fig. 6A.

results that variation of the pH in the range indicated had no influence on the k_{cat} (factor X_a/k_{cat} (decarboxyfactor X_a) ratio. Experimental conditions were as described under Fig. 5.

Effect of Ca^{2+} on amidase activity. As shown in Fig. 6A, the initial velocity of the hydrolysis of Bz-Ile-Glu-Gly-Arg-*p*NA catalyzed by factor X_a, decreases during incubation in the presence of Ca^{2+} (5 mM). After about 60 min the amidase activity remains unaltered. The amidase activity then found is one half of the control value.

Double reciprocal plots of the hydrolysis of Bz-Ile-Glu-Gly-Arg-*p*NA by factor X_a, preincubated during 90 min in the presence of 5 mM $CaCl_2$ and factor X_a treated in the same way in the absence of Ca^{2+} are shown in Fig. 6B.

It was found that in the absence of Ca^{2+} the V value was $2.5 \cdot 10^{-5}$ mol · min^{-1} and in the presence of Ca^{2+} was $1.2 \cdot 10^{-5}$ mol · min^{-1}. The K_M value for factor X_a preincubated with Ca^{2+} was the same as that of factor X_a preincubated in the absence of Ca^{2+}. Upon incubation of decarboxyfactor X_a with Ca^{2+} under identical conditions as described for factor X_a no alteration was found in the amidase activity.

Effect of factor V_a on amidase activity. Factor V_a has no significant effect upon the K_M and V values of the hydrolysis of Bz-Ile-Glu-Gly-Arg-*p*NA catalyzed by factor X_a and decarboxyfactor X_a under the conditions described in the legend to Fig. 7. However, no decrease in amidase activity of factor X_a

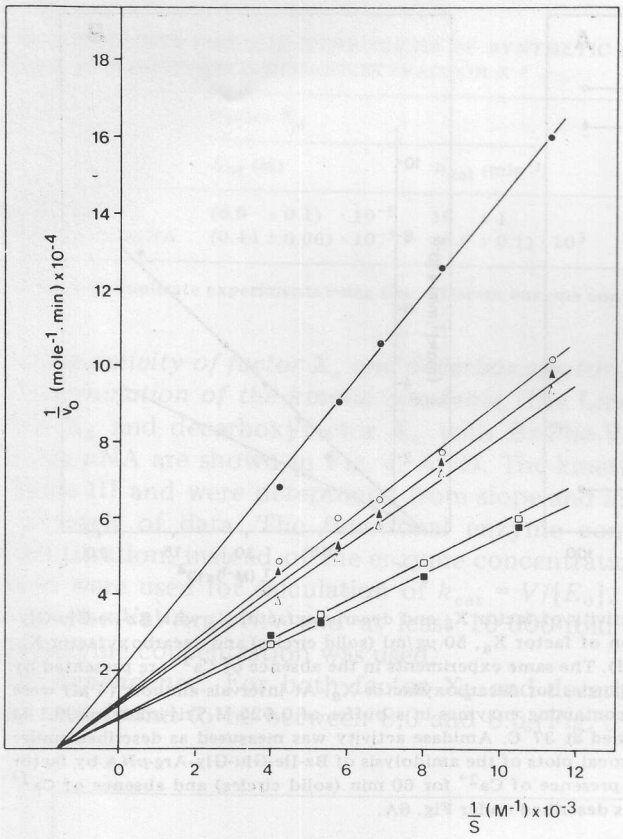

Fig. 7. Effect of factor V_a on the amidase activity of factor X_a and decarboxyfactor X_a using Bz-Ile-Glu-Gly-Arg-*p*NA as substrate. Lineweaver-Burk plots were constructed from data obtained by amidolysis of Bz-Ile-Glu-Gly-Arg-*p*NA by 1, factor X_a (○); 2, factor X_a preincubated for 60 min in the presence of Ca^{2+} (5 mM), (●); 3, factor X_a in the presence of factor V_a (10 U/ml (△); 4, factor X_a in the presence of Ca^{2+} (5 mM) and factor V_a (10 U/ml) (▲); 5, decarboxyfactor X_a, (□); 6, decarboxyfactor X_a in the presence of factor V_a (10 U/ml), (■). Enzyme concentrations were: factor X_a, $1.8 \cdot 10^{-8}$ M and decarboxyfactor X_a, $1.0 \cdot 10^{-8}$ M. Substrate concentrations ranging from $8.7 \cdot 10^{-5}$ to $2.3 \cdot 10^{-4}$ M. Experimental conditions were the same as described under Fig. 6.

upon incubation with Ca^{2+} (5 mM) was found when factor V_a (1.0 mg/ml) was present in the incubation mixture. This effect could however, be duplicated by the addition of bovine serum albumin to a concentration of 0.5 mg/ml (results not shown).

Inhibition of amidase activity by benzamidine. A plot of the reciprocal initial rates of factor X_a and decarboxyfactor X_a hydrolysis of Bz-Ile-Glu-Gly-Arg-*p*NA ($1/v_0$) versus inhibitor concentration (I) at different substrate concentrations [29] is shown in Fig. 8. For both factor X_a and decarboxyfactor X_a straight lines were obtained. The inhibition by benzamidine was observed as competitive. Competitive inhibition was also found in a double reciprocal plot of v_0 versus substrate concentration at different inhibitor concentrations. The K_i values for inhibition of the hydrolysis of Bz-Ile-Glu-Gly-Arg-*p*NA determined from Fig. 8 by regression analysis were found to be $2.4 \cdot 10^{-4}$ and $3.0 \cdot 10^{-4}$ M for factor X_a and decarboxyfactor X_a, respectively.

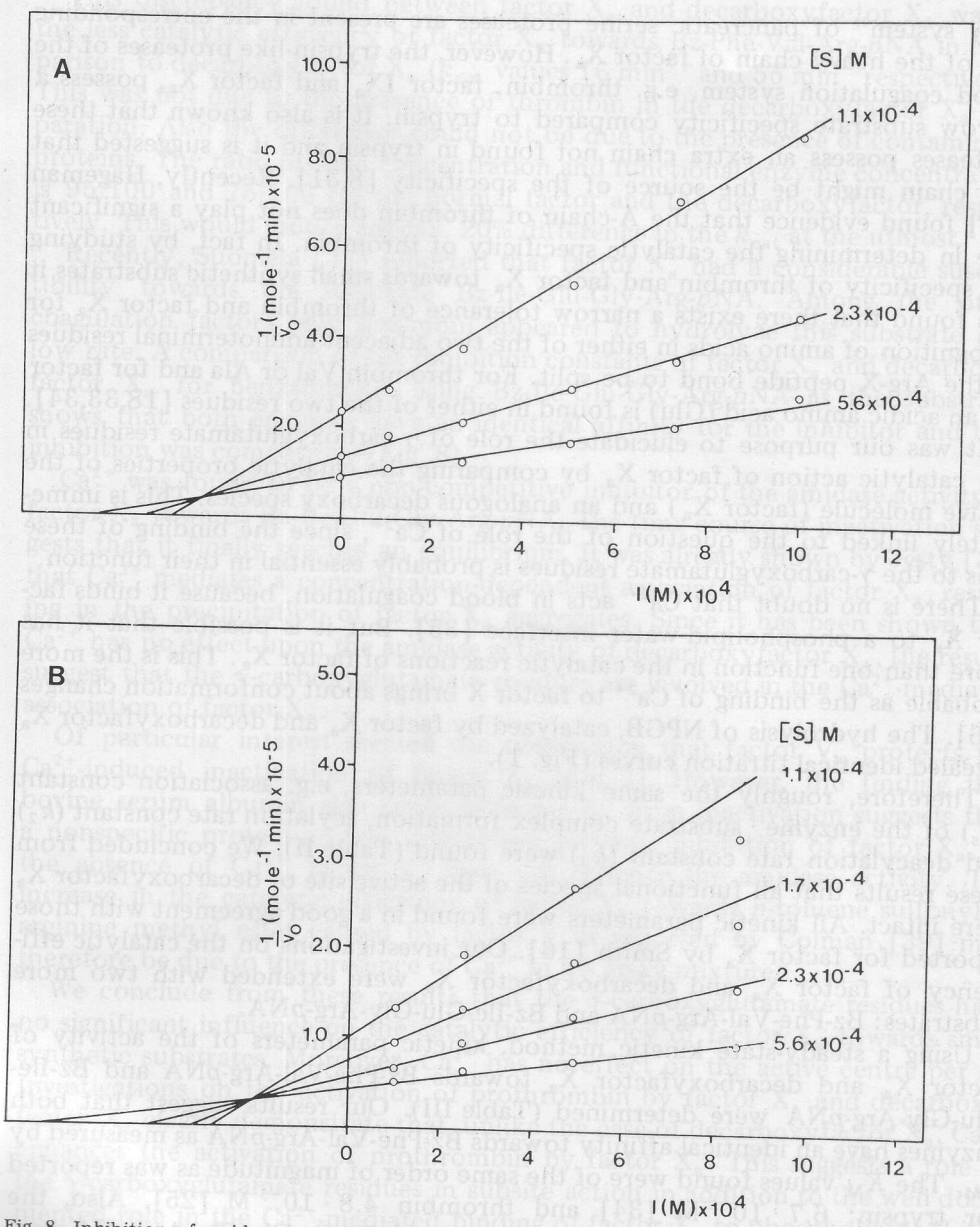

Fig. 8. Inhibition of amidase activity of factor X_a and decarboxyfactor X_a by benzamidine. Dixon plot was constructed by plotting the reciprocal initial rate of Bz-Ile-Glu-Gly-Arg-*p*NA hydrolysis by factor X_a (A) and decarboxyfactor X_a (B) versus inhibitor concentration at fixed concentrations of substrate as indicated. Enzyme concentrations were $1.0 \cdot 10^{-8}$ M for decarboxyfactor X_a and $0.4 \cdot 10^{-8}$ M for factor X_a. Amidase activity was measured as described under Materials and Methods.

Discussion

Titani [30] has shown that there exists a high degree of identity (greater than 55%) between the heavy chain of factor X_a and trypsin in a region (73 residues) which includes the active site serine. All components of the "charge-

relay system" of pancreatic serine proteases are present in the corresponding loci of the heavy chain of factor X_a. However, the trypsin-like proteases of the blood coagulation system, e.g. thrombin, factor IX_a and factor X_a, possess a narrow substrate specificity compared to trypsin. It is also known that these proteases possess an extra chain not found in trypsin and it is suggested that this chain might be the source of the specificity [8,31]. Recently, Hageman [32] found evidence that the A-chain of thrombin does not play a significant role in determining the catalytic specificity of thrombin. In fact, by studying the specificity of thrombin and factor X_a towards small synthetic substrates it was found that there exists a narrow tolerance of thrombin and factor X_a for recognition of amino acids in either of the two adjacent aminoterminal residues of the Arg-X peptide bond to be split. For thrombin Val or Ala and for factor X_a an acidic amino acid (Glu) is found in either of the two residues [18,33,34].

It was our purpose to elucidate the role of γ-carboxyglutamate residues in the catalytic action of factor X_a by comparing the catalytic properties of the native molecule (factor X_a) and an analogous decarboxy species. This is immediately linked to the question of the role of Ca^{2+}, since the binding of these ions to the γ-carboxyglutamate residues is probably essential in their function.

There is no doubt that Ca^{2+} acts in blood coagulation, because it binds factor X_a to a phospholipid-water interface [35]. But it is possible that it has more than one function in the catalytic reactions of factor X_a. This is the more probable as the binding of Ca^{2+} to factor X brings about conformation changes [36]. The hydrolysis of NPGB, catalyzed by factor X_a and decarboxyfactor X_a revealed identical titration curves (Fig. 1).

Therefore, roughly the same kinetic parameters, e.g. association constant (K_s) of the enzyme · substrate complex formation, acylation rate constant (k_2) and deacylation rate constant (k_3) were found (Table II). We concluded from these results that all functional species of the active site of decarboxyfactor X_a were intact. All kinetic parameters were found in a good agreement with those reported for factor X_a by Smith [16]. Our investigations on the catalytic efficiency of factor X_a and decarboxyfactor X_a were extended with two more substrates: Bz-Phe-Val-Arg-*p*NA and Bz-Ile-Glu-Gly-Arg-*p*NA.

Using a steady-state kinetic method, kinetic parameters of the activity of factor X_a and decarboxyfactor X_a towards Bz-Phe-Val-Arg-*p*NA and Bz-Ile-Glu-Gly-Arg-*p*NA were determined (Table III). Our results suggest that both enzymes have an identical affinity towards Bz-Phe-Val-Arg-*p*NA as measured by K_M. The K_M values found were of the same order of magnitude as was reported for trypsin: $6.7 \cdot 10^{-5}$ M [34] and thrombin $4.8 \cdot 10^{-5}$ M [25]. Also, the affinity of factor X_a and decarboxyfactor X_a for Bz-Ile-Glu-Gly-Arg-*p*NA were found to be equal, (K_M values $4.4 \cdot 10^{-4}$ and $5.0 \cdot 10^{-4}$ M respectively) and to agree very well with the data supplied by Kabi Diagnostica, Sweden ($3.0 \cdot 10^{-4}$ M).

A comparison of the K_M values determined for both substrates shows that the enzymes bind Bz-Phe-Val-Arg-*p*NA more effectively than Bz-Ile-Glu-Gly-Arg-*p*NA by one order of magnitude. However, under the conditions used in these experiments factor X_a hydrolyzes Bz-Ile-Glu-Gly-Arg-*p*NA much faster (400 times) than it does Bz-Phe-Val-Arg-*p*NA and so does decarboxyfactor X_a (100 times faster).

This discrepancy found between factor X_a and decarboxyfactor X_a was to the less catalytic efficiency of factor X_a towards Bz-Phe-Val-Arg-*p*NA in comparison to decarboxyfactor X_a (k_{cat} values 16 min^{-1} and 55 min^{-1} respectively). This was not due to the presence of thrombin in the decarboxyfactor X_a preparation. Also the difference could not be due to the presence of contaminant proteins. The ratio protein concentration and functional enzyme concentration is 95/100 and 75/100 for the normal factor and the decarboxyfactor, respectively. This would induce approx. 20% difference in the k_{cat} at the utmost.

Recently, Suomela [37] reported that factor X_a had a considerable susceptibility towards the substrate Bz-Ile-Glu-Gly-Arg-*p*NA. Among the other coagulation factors only thrombin appeared to hydrolyze this substrate at a low rate. A comparison of the inhibition constants of factor X_a and decarboxyfactor X_a for benzamidine, using Bz-Ile-Glu-Gly-Arg-*p*NA as the substrate, shows that both enzymes have an identical affinity for the inhibitor and that inhibition was competitive (Fig. 8).

Ca^{2+} was found to be a non-competitive inhibitor of the amidase activity of factor X_a towards Bz-Ile-Glu-Gly-Arg-*p*NA. The time course of inactivation suggests that it finally reaches an equilibrium. It was already shown by Jesty [38] that Ca^{2+} mediates a concentration-dependent association of factor X_a, resulting in the precipitation of factor X_a aggregates. Since it has been shown that Ca^{2+} has no effect upon the amidase activity of decarboxyfactor X_a, the results suggest that the γ-carboxyglutamate residues are involved in the Ca^{2+}-mediated association of factor X_a.

Of particular interest seemed the observation that factor V_a protects the Ca^{2+}-induced inactivation of factor X_a (Fig. 7). However, the finding that bovine serum albumin also protects factor X_a from inactivation suggests that a nonspecific protein-protein interaction prevents inhibition of factor X_a. In the absence of Ca^{2+}, factor V_a does not enhance the amidase activity. The increase in the catalytic efficiency of factor X_a towards *p*-toluene sulfonyl-L-arginine methyl ester caused by factor V as reported by Colman [39] may therefore be due to the presence of Ca^{2+} in his assay mixture.

We conclude from these results that the γ-carboxyglutamate residues have no significant influence on the catalytic efficiency of factor X_a towards small synthetic substrates. Moreover, Ca^{2+} has no effect on the active centre per se. Investigations on the activation of prothrombin by factor X_a and decarboxyfactor X_a clearly demonstrate that, unlike the case of decarboxyfactor X_a, Ca^{2+} enhances the activation of prothrombin by factor X_a. This suggests a role of the γ-carboxyglutamate residues in subsite action in addition to the well documented role in the Ca^{2+}-mediated binding of factor X_a to phospholipids (Lindhout, M.J., Kop-Klaassen, B.H.M. and Hemker, H.C. (1978), in preparation).

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