The vitamin K dependent enzymatic properties of blood coagulation factor X

Citation for published version (APA):

Lindhout, M. J. (1977). *The vitamin K dependent enzymatic properties of blood coagulation factor X*. [Doctoral Thesis, Maastricht University]. Rijksuniversiteit Limburg.
<https://doi.org/10.26481/dis.19771111ml>

Document status and date:
Published: 01/01/1977

DOI:
[10.26481/dis.19771111ml](https://doi.org/10.26481/dis.19771111ml)

Document Version:
Publisher's PDF, also known as Version of record

Please check the document version of this publication:

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- The final published version features the final layout of the paper including the volume, issue and page numbers.

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THE VITAMIN K DEPENDENT ENZYMATIC PROPERTIES OF BLOOD COAGULATION FACTOR X

PROEFSCHRIFT

ter verkrijging van de graad van doctor in de geneeskunde
aan de Rijksuniversiteit Limburg, op gezag van de Rector
Magnificus Dr. H. A. Tiddens, hoogleraar in de faculteit
der geneeskunde, volgens besluit van het college van dekanen
in het openbaar te verdedigen op vrijdag 11 november 1977
des namiddags om 4 uur precies, in de aula der universiteit.

door

Mattheus Jan Lindhout

geboren te Bergen op Zoom in 1944

~~009057~~

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Dit proefschrift werd bewerkt in het laboratorium voor Cardio-biochemie en Bloedstollingsbiochemie van het Academisch Ziekenhuis te Leiden en het laboratorium voor Biochemie van de Rijksuniversiteit Limburg, onder de leiding van Prof.Dr.H.C.Hemker.

Deze onderzoeken werden gedeeltelijk uitgevoerd onder auspiciën van de Stichting voor Medisch Wetenschappelijk Onderzoek FUNGO met financiële steun van de Nederlandse Organisatie voor Zuiver Wetenschappelijk Onderzoek

Aan mijn ouders

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Drukkerij : Krips-Repro N.V.,Meppel

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CHAPTER I

GENERAL INTRODUCTION

Thrombin generation. The enzyme thrombin plays a key role in human pathology because it stands at the centre of events that govern hemostasis and thrombosis. It causes platelets to aggregate irreversibly and it causes fibrinogen to form fibrin.

Irreversible platelet changes and fibrin formation accompany normal hemostasis as well as all forms of thrombosis. Diseases that arise from a lack of adequate thrombin formation are relatively scarce. The hemophilias and vitamin K deficiency do not account for a large percentage in the morbidity and mortality pattern of man. They are of paramount importance though, as they enable us to study the mechanism of thrombin formation and thereby the mechanism of the undue thrombin formation, that leads to thrombosis and other pathological forms of the hemostasis mechanism. The importance of the latter aberrations for human pathology can hardly be exaggerated. Thrombosis plays the central role in both the genesis and the acute complications of arterial atherosclerosis and by this fact alone accounts for about 40% of all deaths in The Netherlands. At this moment venous thrombosis, once a much feared complication of any major surgical intervention or other immobilizing disease by now can be adequately treated on basis of diminishing the thrombin forming power of the blood. Other conditions remain to be adequately treated: coagulation as mediator of immune damage and all forms of intravascular coagulation are among them.

It is clear that the study of thrombin formation is an essential part of pathology and merits to be studied in detail. Physiologically, thrombin formation can be initiated via two different pathways, e.g.:

1. the surface catalyzed transformation of the zymogen factor XII into an activated form (factor XII_a), which is the primary step

Fig. 1.

Analogies between clotting proteases (after Magnusson, ref. 2). The arrows indicate the active serine (bold: activated; small: not activated). The squiggle is the Ca^{2+} binding site.

Fig. 2.

Structures of the active form of vitamin K and coumarin based anticoagulants.

in the formation of factor XI_a . In this step also high molecular weight kininogen and prekallikrein are involved. It initiates the so-called intrinsic pathway.

2. the transformation of factor VII into factor VII_a by tissue factor (T.F.), a lipoprotein released by injury which initiates the extrinsic pathway.

After initiation, the reaction sequence leading to thrombin formation can be visualized as is shown below (1):

Two features are characteristic for this scheme:

1. The cascade sequence: the product of one reaction acts as (part of) the enzyme in the following step. Most of the reactions are characterized now as activation by limited proteolysis. Fig. 1 shows the molecular aspects of this proteolysis for several clotting factors.
2. The complex enzymes. An essential feature of the activation mechanism is the participation of phospholipid micelles by offering a phospholipid-water interface to the interacting coagulation factors.

This thesis contributes to the knowledge of prothrombinase, the

enzyme that converts prothrombin into thrombin. It is a complex enzyme and mediates the last step in the cascade.

Role of vitamin K in blood coagulation factor synthesis. The only known function of vitamin K in animals is the biosynthesis of the blood coagulation factors II, VII, IX, and X (3). Vitamin K was discovered by Dam (4) because of its relation with blood coagulation. The appearance of a haemorrhagic diathesis by chickens on a cholesterol free diet, was related to a deficiency of the fat soluble vitamin K. In 1936 it was found that vitamin K deficiency resulted in a decrease of prothrombin activity (5). In the early fifties when other proteins involved in blood coagulation were discovered, the role of vitamin K was ascertained also in the biosynthesis of the factors VII, IX, and X (6-8). The early work on the vitamin K, its chemical and physiological properties is excellently reviewed by Wiss, Dam and Grimmer (9). Anderson and Barnhart (10) demonstrated with immuno-fluorescence techniques that the four vitamin K dependent coagulation factors were synthesized in the liver parenchyma cells. The discovery of components with an anti-vitamin K activity by Campbell and Link (11) stimulated further investigations on the action of vitamin K (structures are shown in fig. 2).

Thierry et al. (12) have shown that compounds with a 4-hydroxy-coumarin structure, like dicoumarol, warfarin and phenprocoumon interfere with the vitamin K action in hepatocytes. The mode of action of these coumarins is not yet known, however. Several authors suggested a competition of these compounds with vitamin K on a receptor protein involved in the biosynthesis of coagulation factors (13,14). More recently, Bell and Matschiner (15) reported the accumulation of the 2,3-epoxide derivative of phylloquinone in rat liver after administration of warfarin. They postulated that an increase of the epoxide/vitamin K ratio in the liver causes a decrease of the activity of the four vitamin K dependent coagulation factors. Accumulation of 2,3-epoxide-phylloquinone is probably due

Fig. 3.

Involvement of vitamin K epoxidase in the formation of prothrombin.

Fig. 4.

Structure of γ -carboxyglutamic acid.

Fig. 5.

Vitamin K-dependent carboxylase. Peptide-bound glutamyl residues in the precursor are modified to γ -carboxyglutamyl residues in prothrombin.

to inhibition of phyloquinone reductase by warfarin. The hypothesis of Bell et al. (16) is summarized in fig. 3. Sadowski (17), however, reported the stimulation of prothrombin synthesis after treating vitamin K deficient rats with the epoxide. The development of an in vitro system for the synthesis of prothrombin will make it possible to study this question in more detail (see page 17 and further).

Indirect evidence for a prothrombin precursor molecule. During the last twenty years several groups of investigators worked intensively on the elucidation of the mechanism of vitamin K action and several suggestions to explain its mode of action have been put forward. The preliminary work on the role of vitamin K will not be discussed here and the reader is referred to reviews by Pennock (18) and Hemker (19). The investigations during the last ten years focussed on two alternate hypotheses, e.g.:

1. Vitamin K acts at a ribosomal site to regulate the de novo synthesis of prothrombin (20-23).
2. Vitamin K functions in the modification of the prothrombin polypeptide chain of a postribosomal stage: the precursor hypothesis (24-26).

The relation between vitamin K action and de novo synthesis has been studied with inhibitors of protein synthesis reaction at the ribosomal level, namely puromycin and cycloheximide.

It is difficult to interpret the results of these experiments and so conflicting data have been obtained on the stimulation of prothrombin synthesis by vitamin K in vitamin K deficient rats treated with cycloheximide (25, 27-29).

It is now clear that cycloheximide when administered to vitamin K deficient rats before vitamin K will only partially inhibit prothrombin synthesis, whereas general protein synthesis will be almost completely blocked (30).

These data suggest that even when the synthesis of the polypeptide

chain is inhibited, vitamin K still affects the production of prothrombin from an intracellular pool. Work from Prydz's group (31) gives also evidence for a "factor VII like" pool.

Further support for the existence of a precursor pool came from the investigations of Suttie et al. (32-34). In the microsomal fraction of liver obtained from rats treated with warfarin, they could generate thrombin with the aid of a non-physiological prothrombin activator (*Echis Carinatus* venom). The amount of thrombin was inversely proportional with the prothrombin activity in plasma. They also demonstrated a vitamin K dependent synthesis of prothrombin in microsomal preparations obtained from vitamin K deficient rats. This prothrombin synthesis could be inhibited with warfarin but not cycloheximide.

Recently, Esmon (35) was able to isolate a precursor of prothrombin from the liver of vitamin K deficient rats. Unlike the precursor of prothrombin, identified in rat livers, the precursor molecules of coagulation factors in the human and bovine plasma will appear in the circulation. The first evidence for such an inactive prothrombin precursor in human plasma was obtained by Hemker et al. (36). They reported that in plasma of patients treated with vitamin K antagonists a protein appears which inhibits the blood coagulation in specific coagulation assays, and which was called protein induced by vitamin K absence (antagonists), abbreviated to PIVKA (36). Already in 1963 they postulated that vitamin K antagonists block the prothrombin synthesis at a postribosomal step so that the polypeptide precursors reach the circulation.

Using immunochemical techniques several laboratories reported the existence of proteins analogous to each of the four vitamin K dependent coagulation factors. The precursors corresponding to the normal factors II, VII, IX, and X that appear in the plasma and are purified from there, will be referred to as PIVKA-II, PIVKA-VII, PIVKA-IX, and PIVKA-X, respectively, which is the nomenclature used

by Hemker. The name proposed for all forms of precursors of vitamin K dependent clotting factors is decarboxy protein. This refers to all forms and thus comprises several intracellular as well as plasmatic variety. PIVKA designates the plasmatic variety only.

Immunochemical evidence for precursor protein. The first immunological evidence for the existence of PIVKA-II in human plasma was reported by Ganrot (37) and Josso (38). Goodnight (39) and Denson (40) demonstrated the appearance of an inactive factor VII in the plasma of patients treated with oral anticoagulants. Levanon (41) and Brozovic (42) reported a higher level of factor VII antigen than expected from a factor VII activity assay. Patients treated with vitamin K antagonists also show higher levels of antigen related to factor IX and factor X than was to be expected on basis of bioassays (40, 43-47). Reekers (48) and Lindhout (49) demonstrated with crossed immunoelectrophoresis that treatment of cattle with phenprocoumon results in a decrease of the normal factors IX and X with a parallel increase of PIVKA-IX and PIVKA-X.

Characterization of PIVKA-II. The identification of PIVKAs stimulated further investigations on the action of vitamin K on the chemical nature of the postribosomal modification of prothrombin. It was assumed that vitamin K affects the incorporation of carbohydrates in the precursor molecule of prothrombin (50-52). Glycolipid derivatives of vitamin K, which may be the carbohydrate donors, could be formed by incubation of phylloquinone and UDP-N-acetylglucosamine (53) with smooth endoplasmic reticulum obtained from rat liver. Differences in carbohydrate content of human prothrombin and human PIVKA-II were reported by Morrison (54).

Chemical analysis of highly purified bovine prothrombin and PIVKA-II by Stenflo (55,56) and Nelsetuen (57) indicated that the carbohydrate content and the structure of both proteins are probably similar. The observation (58) that asialo- and aglycoprothrombin retain biological activity and that the carbohydrate part of thrombin

is not essential for its activity (59,60) made the hypothesis that the vitamin K dependent step in the formation of prothrombin involves glycosylation of the protein unlikely.

Although PIVKA-II has no biological activity it was possible to generate thrombin rapidly using non-physiological activators of prothrombin, like trypsin (57), Echis Carinatus venom (57), and staphylocoagulase (61,62). These observations suggest that the PIVKA-II molecule contains an intact thrombin. The differences in the rate of thrombin formation between PIVKA-II and prothrombin must account for the difference in biological activity with the physiological activator.

Prothrombin activation is enhanced by calcium and phospholipids (63,64) which is discussed in more detail in chapter V. The lack of any effect of Ca^{2+} ions on the electrophoretic mobility of PIVKA-II, PIVKA-IX and PIVKA-X (48,49,65-67) and the absence of Ca^{2+} -dependent immunochemical properties (56,68) as is found in the normal factors suggested a difference in calcium binding properties. Moreover, the observations (69) that PIVKA-II does not bind to phospholipids in the presence of Ca^{2+} ions and that addition of phospholipids has no effect on the rate of thrombin formation from PIVKA-II favor the hypothesis that a vitamin K dependent mechanism brings about a change in a precursor molecule that affects its calcium binding properties and by so doing converts it into prothrombin.

Identification of γ -carboxyglutamic acid. Nelstuen and Suttie (70) isolated an acidic peptide from a tryptic digest of bovine prothrombin (so called fragment 1 region of prothrombin) that binds calcium ions. The work of Stenflo (71) on the calcium binding region of prothrombin revealed two acidic peptides with an electrophoretic mobility much higher than would be expected from the amino acid composition. The same observation was done by Magnusson (72). An analogous peptide with an anomalous high electrophoretic mobility could not be identified in the amino terminal region of PIVKA-II.

Several other investigators have reported the existence of abnormally acidic peptides in prothrombin (67, 73). With ^1H -nuclear magnetic resonance spectroscopy and mass-spectroscopy Stenflo (74) identified two residues of γ -carboxyglutamic acid (fig. 4) in a tetrapeptide (residues 6-9) from the amino terminal region of prothrombin. At the same time Nelsetuen (75) characterized γ -carboxyglutamic acid from a dipeptide (residues 33-34).

It was shown by Magnusson (76) that all 10 of the first 33 glutamic acid residues in prothrombin are modified to a γ -carboxy derivative. It is interesting to note that several investigators (77-80) report the existence of various prothrombin-like molecules differing in calcium binding and activation properties induced in cattle by prolonged administration of warfarin. This suggests the existence of partially carboxylated forms of prothrombin.

The similarity in amino acid sequence of the aminoterminal region of the vitamin K dependent coagulation factors prothrombin, factor IX and factor X (81) suggest the presence of γ -carboxyglutamic acids in both factor IX and X. Howard et al. (82) reported that the vitamin K dependent peptide of factor X contains 14 extra carboxyl groups and it was assumed that most glutamic acid residues in this peptide were modified to γ -carboxyglutamic acids. Recently, Fryklund (83) identified γ -carboxyglutamic acids in factor IX.

Functional role of γ -carboxyglutamic acid. The similarity in structure and amino acid composition of the vitamin K dependent coagulation factors and other members of the chymotrypsin family, like trypsin, plasmin and chymotrypsin suggests that they have an ancestral gen in common. The fact that the calcium binding by the vitamin K dependent region has been conserved, suggests an important functional role of this region. The relation between structure and function might be underlined by the occurrence of the γ -carboxyglutamic acid residues in pairs. A 'cage-like' structure was suggested as a model for the calcium binding by a

pair of γ -carboxyglutamic acids (82,84). The importance of the calcium mediated binding of coagulation factors to phospholipid surfaces is well known, but an explicit mechanism to explain its function is still lacking (85).

Function of vitamin K. In the past few years it became clear that vitamin K promotes the carboxylation of a microsomal precursor to prothrombin (fig. 5). Sutties group (86) found that the post-mitochondrial supernatant of vitamin K deficient rats incorporates $\text{H}^{14}\text{CO}_3^-$ in a microsomal protein after administration of vitamin K and depending on the presence of the prothrombin precursor in the microsomal preparation. All radioactivity was found primarily in the amino terminal region of prothrombin. Moreover, they give evidence that the radioactivity is associated with the γ -carboxyglutamic acids.

The so-called vitamin K dependent carboxylase studied in microsomes required the presence of PIVKA-II, vitamin K, HCO_3^- (CO_2), oxygen, an energy source and the postmicrosomal supernatant containing NADH or NADP reductase (87-91). The stimulating factor present in the postmicrosomal supernatant could be replaced by the reduced form of vitamin K (phylloquinol) (87). Investigations (16, 92) on the metabolism of the vitamin K epoxide suggest the existence of a coupling between the vitamin K - vitamin K-epoxide conversion and vitamin K stimulated carboxylation.

INTRODUCTION TO THIS THESIS

As explained on page 7 and further, it is important to know the details of the thrombin forming reaction. In practice this reaction is damped by anticoagulation. Both oral anticoagulation by vitamin K antagonists and heparin therapy are used for this purpose. A study of the role of factor X and PIVKA-X in the function of prothrombinase thus serves a double purpose. In the first place it will help in elucidating the fine mechanism of prothrombinase action and more precisely of the role of Ca^{2+} binding γ -carboxyglutamic acid residues there. In the second place it will give an insight in the abnormalities induced by oral anticoagulation.

In chapter II the purification and characterization of PIVKA-X are described and in chapter III its Ca^{2+} binding properties. The latter by both flow dialysis and Ca^{2+} dependent spectral changes.

In chapter IV it is investigated under what conditions PIVKA-X can be converted into an active enzyme analogous to factor X_a : the role of Ca^{2+} ions and hence the role of γ -carboxyglutamic acid residues in this process is investigated.

In order to elucidate the role of γ -carboxyglutamate residues in the catalytic action of factor X_a , we compared the catalytic properties of normal factor X_a and an analogous decarboxy factor X_a , PIVKA- X_a (chapter V).

Prothrombin activation by factor X_a is enhanced several orders of magnitude by the so-called accessory components, like factor V (V_a), Ca^{2+} and phospholipids. This rate enhancement can be accounted for by several mechanisms, e.g.:

1. Interaction of factor X_a with Ca^{2+} , phospholipids and factor V_a may affect the catalytic efficiency of the proteolysis.
2. Binding of phospholipids, Ca^{2+} and factor V_a may cause changes in the susceptibility of the substrate.
3. Localization and orientation of the reaction components by protein-

protein and/or protein-phospholipid interactions.

Use of PIVKA- X_a , the factor X_a analogue which probably does not bind to phospholipids since the Ca^{2+} binding sites are lacking, in activation tests of prothrombin, may help to distinguish between these mechanisms.

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CHAPTER II

IDENTIFICATION, PURIFICATION AND PROPERTIES OF THE PHENPROCOUMON
INDUCED DECARBOXY FACTOR X (PIVKA-X) FROM BOVINE PLASMA

A comparison to normal factor X

SUMMARY

1. An abnormal blood coagulation factor (PIVKA-X) analogous to factor X was identified in plasma from phenprocoumon treated cows by immunological techniques.
2. By a procedure involving adsorption to barium sulfate, chromatography on DEAE-Sephadex and QAE-Sephadex and preparative polyacrylamide gel electrophoresis, PIVKA-X was purified from plasma of phenprocoumon treated cows. No contaminants could be detected in the final preparation by polyacrylamide gel electrophoresis and zone-electrophoresis.
3. The molecular weight of PIVKA-X as determined by SDS polyacrylamide gel electrophoresis is approximately 55,000 which is equal to that of factor X. The protein consists of two polypeptide chains with molecular weights of 44,000 and 17,000.
4. PIVKA-X has antigenic determinants in common with normal factor X.
5. The amino acid composition and amino-terminal amino acids of normal factor X and PIVKA-X are identical.
6. Less than one residue of γ -carboxyglutamate could be detected per mole of PIVKA-X
7. In the absence of Ca^{2+} ions, normal factor X has a slightly higher electrophoretic mobility than PIVKA-X. In the presence of Ca^{2+} ions the mobility of factor X decreases considerably while the mobility of PIVKA-X remains unaltered.

INTRODUCTION

In several laboratories a decarboxy prothrombin (PIVKA-II) present in plasma of dicoumarol treated cows has been extensively purified and characterized (1-4). The structural comparison of normal and dicoumarol induced prothrombin has been the subject of intensive investigation in recent years (5-9). Prothrombin contains a number of γ -carboxyglutamic acid residues in the amino-terminal region, whereas glutamic residues are found in the amino-terminal region of PIVKA-II.

Recently, it has been reported that two other vitamin K dependent coagulation factors (i.e. factor IX and factor X) also contain γ -carboxyglutamic acid residues (10-13).

Experiments on the biosynthesis of prothrombin (14-16) confirm the earlier suggestion of Hemker et al. (17) that PIVKA-II is a precursor of normal prothrombin accumulating because of a decreased turnover of the vitamin K dependent conversion of PIVKA-II into prothrombin.

Since there are four vitamin K dependent coagulation factors, one can postulate the existence of four PIVKAs. These proteins will be referred to as PIVKA-II, PIVKA-VII, PIVKA-IX, and PIVKA-X.

We reported earlier the demonstration of both PIVKA-IX and PIVKA-X (18,19). In this chapter we describe the identification, purification, and partial characterization of PIVKA-X. Because of the low concentration of factor X in plasma (10-15 mg/l) large amounts of plasma are needed as a starting material and therefore bovine plasma was used. Moreover, well established procedures are available for the purification of normal bovine factor X (20,21). Another reason to study the bovine system is that more is known about the structure of bovine factor X than that of human factor X (12,22,23).

MATERIALS

Normal bovine factors II, IX, and X used for the immunization of

rabbits were purified according to the method of Reekers (24). Common inorganic chemicals (reagent grade), barium sulfate, acrylamide, N',N'-methylene bisacrylamide and benzamidine hydrochloride were obtained from Merck, Darmstadt, GFR.

Marcoumar was a kind gift of Dr.M.Kunz (Hoffmann-La Roche, Basle, Switzerland). DEAE-Sephadex and QAE-Sephadex (Pharmacia, Uppsala, Sweden) were equilibrated according to the instructions supplied by the manufacturers. Agarose was a product of Koch-Light Laboratories Ltd. Aluminum hydroxide, moist gel was purchased from British Drug Houses Ltd. Sodium dodecyl sulfate (Koch-Light laboratories Ltd.) was recrystallized from ethanol. Bovine serum albumin, glutamate dehydrogenase, ovalbumin, carbonic anhydrase, lysozyme, myoglobin, α -chymotrypsin-A, soybean trypsin inhibitor, diisopropylfluorophosphate and dansylchloride were obtained from Sigma Chemical Co. Reference dansylamino acids were supplied by Calbiochem A.G. Polyamide layer sheets were obtained from Merck. Vitamin K and heparin were purchased from Hoffmann-La Roche.

METHODS

Preparation of rabbit antbovine factor II and IX antiserum. Antisera were prepared in rabbits by injection of purified preparations of bovine factor II and factor IX (18). Antisera were adsorbed with barium sulfate (100 mg/ml) for 30 min at room temperature and heat inactivation was performed for 30 min at 56°C in order to remove procoagulant activity. The adsorbed and inactivated antisera were centrifuged for one hour at 105,000 g at 4°C. Sodium merthiolate (0.01% final concentration) was added before storage at -20°C.

Preparation of rabbit anti-bovine factor X antiserum. The first intramuscular injection was given with 1.0 ml factor X₂ (0.2 mg/ml) mixed with 1.0 ml complete Freund adjuvant. A second and third intramuscular injection of 1.0 ml factor X₂ (0.2 mg/ml) mixed with 1.0 ml incomplete Freund adjuvant was given after intervals of 3 weeks. Plasmaphoresis was performed after the second month. Antisera were

treated as described above.

Gel electrophoresis. Analytical polyacrylamide gel electrophoresis was performed according to Ornstein (25) using a Tris-HCl-glycinate buffer system as described by Davis (26). Polyacrylamide gel electrophoresis in sodium dodecyl sulfate was carried out according to Weber and Osborn (27). Bovine serum albumin, glutamate dehydrogenase, ovalbumin, carbonic anhydrase, α -chymotrypsinogen A, myoglobin, and lysozyme were used as markers for the molecular weight estimation, using molecular weights given by Weber and Osborn (27). The gels were stained according to Fairbanks (28). Equipment for analytical polyacrylamide gel electrophoresis was obtained from Buchler Instruments (Polyanalyst).

Agarose gel electrophoresis was carried out in 1.5 mm thick 1% agarose gels in 0.05 M barbital buffer pH 8.6 containing 2.0 mM Ca-lactate or 2.0 mM EDTA according to Johansson (29). Electrophoresis was run at 15 volts per cm on a glass plate and cooled by water of 10°C. The gels were fixed in 10% trichloroacetic acid and stained with Coomassie Brilliant blue G (Sigma Chem.Co) in 10% acetic acid. A LKB Multiphor 2117 apparatus was used.

Immunochemical methods. PIVKA-X was immunochemically quantitated with the technique of Laurell (30). Electrophoresis was performed (10 volts/cm for 3 hours) into a 1% agarose gel containing 1% anti-bovine factor X antiserum, using 0.05 M barbital buffer pH 8.6 in the presence of 2.0 mM Ca-lactate. As standard was used a plasma obtained from a cow under deep anticoagulation (factor X activity less than 5%). One unit is arbitrarily defined as the concentration of factor X related antigen in 1.0 ml of standard plasma.

The crossed immunoelectrophoresis was carried out according to Laurell (31) on glass plates (10 x 20 cm). Agarose gel 1% in 0.05 M barbital buffer pH 8.6 containing 2.5 mM Ca-lactate or EDTA in the first dimension was used. After the first dimension run, the agarose gel 0.5 cm above the slit was replaced by 1% agarose containing 0.5% anti-bovine factor X antiserum and 1.0 mM

Ca-lactate. Electrophoresis in the second dimension was continued for 4 hours at 10 volts/cm. After the completion of the electrophoresis the gel was washed overnight in saline-phosphate solution, then washed in distilled water for 2 hours, dried and stained with 0.025% Coomassie Brilliant blue R in methanol-water-acetic acid (450:450:100). Analyses were performed in a LKB multiphor 2117 apparatus. Double immunodiffusion was carried out according to Ouchterlony (32).

For coagulation factor neutralization tests, plasma samples were incubated for 30 min at 37°C with antiserum. The mixture was left overnight at 4°C and centrifuged for one hour at 18,000 g. Coagulation factor activities in the supernatant were assayed in one-stage assays using human deficient plasmas as reagents. In control experiments the antiserum was replaced by normal rabbit serum treated in the same way as described for the antiserum.

Coagulation factor assay. Coagulation times specifically dependent on the concentrations of factor II, VII, and X were obtained by mixing in a test tube at 37°C: 0.1 ml bovine plasma artificially deficient in either factor II, VII, or X, 0.1 ml sample and 0.1 ml bovine brain thromboplastin, prepared according to Owren (33). The coagulation reaction was started by adding 0.1 ml CaCl_2 solution (33 mM). Coagulation factor concentrations are expressed as a percentage of the content in a normal bovine plasma pool, obtained from 20 cows. The concentrations were calculated from the coagulation times in specific tests as described by Hemker et al. (34). It is defined that 1 ml bovine plasma with a blood coagulation factor activity of 100% contains 1 unit activity of each of the factors.

Preparation of normal bovine factor X. Bovine factor X was prepared by a modified method of Jackson (20) and Fujikawa (21). Bovine blood was collected in an anticoagulant solution, containing 0.1 M sodium oxalate, 0.01 M benzamidinium hydrochloride, two units heparin per ml and 5 mg crude soybean trypsin inhibitor per litre. The plasma was

separated by centrifugation in a MSE Mistral 6 l centrifuge for 20 min at 2500 rpm and 4°C. In a representative purification 30 litres of bovine plasma were used.

Step 1: barium sulfate adsorption. Barium sulfate suspended in 0.45% (w/v) NaCl in 1 mM Tris buffer pH 7.5 was added to the plasma (10 mg/ml) and stirred for 30 min at 4°C followed by centrifugation at 1500 g for 5 min. The barium sulfate cake was dispersed with an UltraTurrax (IKA type TP 18/10) at high speed for 2 min. in 500 ml 0.45% (w/v) NaCl in 1 mM Tris buffer pH 7.5. The barium sulfate was removed by centrifugation and the dispersion-washing procedure was repeated three times. Protein was eluted from the barium sulfate with 1500 ml 0.06 M trisodium citrate buffer pH 5.8 containing 0.01 M benzamidine hydrochloride and 40 mg purified soybean trypsin inhibitor per litre, by stirring at 4°C for 30 min. This step was repeated once.

Step 2: DEAE -Sephadex adsorption and elution. The combined eluates (3000 ml) were adjusted with 1 M NaOH to pH 7.1. A slurry settled by gravity of DEAE-Sephadex (75 ml) equilibrated in 0.06 M trisodium citrate in 0.05 M Tris-HCl buffer pH 7.1 was added to the eluate and stirring was continued for about 30 min at room temperature until factor X activity was bound to the DEAE-Sephadex. The DEAE-Sephadex was removed from the solution by filtration on a Buchner funnel on a 60 mesh nylon cloth and washed with 1000 ml 0.06 M trisodium citrate in 0.05 M Tris-HCl buffer pH 6.8, followed by 1000 ml of the same buffer adjusted to pH 8.0. The DEAE-Sephadex was resuspended in last buffer and packed into a column (45 x 2.5 cm) onto a 3 cm high DEAE-Sephadex layer.

Factor X was eluted with a linear gradient of 0.1 - 0.6 M NaCl in 0.05 M Tris-HCl buffer containing 0.1 M trisodium citrate and 1 mM benzamidine hydrochloride at pH 8.0. Each gradient chamber contained 300 ml solution. A flow rate of 50 ml/hour was maintained during elution and 5 ml fractions were collected.

Step 3: QAE-Sephadex chromatography. The factor X containing fractions eluted from the DEAE-Sephadex column were pooled, dialyzed overnight against 0.05 M trisodium citrate pH 7.0 containing 1 mM benzamidine hydrochloride and applied to a column (30 x 2.5 cm) of QAE-Sephadex equilibrated with the same buffer. The column was washed with 250 ml 0.1 M trisodium citrate buffer pH 7.0. Factor X was eluted with a linear gradient of 0.10 - 0.35 M trisodium citrate buffer pH 7.0 containing 1 mM benzamidine hydrochloride with 400 ml in each gradient chamber. The flow rate was 20 ml/hour and 5 ml fractions were collected. The factor X_1 and X_2 containing fractions were pooled separately and concentrated in a Diaflow ultrafiltration cell equipped with a PM-10 membrane (Amicon Corp. model 202). The concentrate was adjusted to pH 8.1 with 1.0 M Tris-HCl buffer pH 8.6. DFP (1.0 M in 2-propanol) was added to a final concentration of 1 mM and incubated for 60 min. at 4°C. After dialysis against 0.025 M Tris-HCl buffer pH 7.4 - 0.12 M in NaCl during 48 hours at 4°C with four changes, the factor X_1 and X_2 preparations were stored at -80°C.

Induction of PIVKA-X in the cow. Four cows, weighing about 500 kg each, were orally given 600 mg Marcoumar the first day, followed by 200 mg daily for 4-6 days. Blood samples were drawn from a jugular vein each day during the treatment and collected into polyethylene tubes (50 ml) containing 0.8 ml of a 20% (w/v) trisodium citrate solution. The effect of Marcoumar was monitored by the assay of factor II, VII, and X activity and by crossed immunoelectrophoresis of plasma samples. At a factor X activity of about 10% of the normal activity, 5 litres of blood were collected from a jugular vein of each cow in 1 litre polyethylene bottles containing 100 ml 0.1 M sodium oxalate, 10 mg heparin, 10 mg benzamidine hydrochloride and 5 mg crude soybean trypsin inhibitor. Afterwards the animals were given 50 mg vitamin K intramuscularly. Plasma was obtained by centrifugation in a MSE Mistral 6 l centrifuge at 2500 rpm during 20 min. at 4°C.

Preparation of PIVKA-X. Bovine PIVKA-X was prepared by a modified method as described for bovine factor X by Jackson (20) and Fuji-kawa (21).

Step 1: Removal of normal factor X. Marcoumar plasma (21 l) was stirred with 30 g moist gel $\text{Al}(\text{OH})_3$ in 150 ml water during 10 min. at room temperature. The $\text{Al}(\text{OH})_3$ was removed by centrifugation at 2,000 g for 10 min. at 4°C.

Step 2: Adsorption of PIVKA-X on barium sulfate. The supernatant (21,150 ml) was stirred with 400 g barium sulfate suspended in a minimal amount of 0.45% (w/v) NaCl in 1 mM Tris buffer pH 7.5 for 30 min. at 4°C. BaSO_4 was removed by centrifugation at 1500 g for 5 min. The washing procedure of the BaSO_4 cake was performed as described for the factor X preparation. Protein was eluted from the BaSO_4 with 1050 ml of 0.12 M trisodium citrate in 0.05 M Tris-HCl buffer pH 5.8 containing 0.01 mM benzamidine hydrochloride and 40 mg purified soybean trypsin inhibitor per litre, by stirring at 4°C for 30 min. This step was repeated once.

Step 3: DEAE-Sephadex adsorption and elution. The combined BaSO_4 eluates (2100 ml) were diluted with an equal volume of distilled water and adjusted to pH 7.1 with 1.0 M NaOH. The batchwise adsorption and washing of the DEAE-Sephadex were carried out as described for the factor X purification. In addition the DEAE-Sephadex was washed with 1000 ml of 0.1 M NaCl in 0.02 M potassium phosphate buffer of pH 6.8. The DEAE-Sephadex was packed into a column (45 x 2.5 cm) onto a 3 cm high DEAE-Sephadex layer equilibrated in the final washing buffer. PIVKA-X was eluted using a linear gradient of 0.1 - 0.6 M NaCl in a 0.02 M potassium phosphate buffer of pH 6.8 containing 1 mM benzamidine hydrochloride. Each chamber contained 250 ml of the solution. The effluent of the DEAE-Sephadex column was monitored by rocket immunoelectrophoresis according to Laurell (30) using a monospecific anti-bovine factor X antiserum.

Step 4: QAE-Sephadex chromatography. The PIVKA-X fractions from the

DEAE-Sephadex column were pooled, dialyzed overnight against 0.05 M trisodium citrate, 1 mM benzamidine hydrochloride pH 7.0. The same QAE-Sephadex chromatography procedure as described for factor X purification was followed. PIVKA-X containing fractions were identified by rocket immunoelectrophoresis.

Step 5: Preparative polyacrylamide gel electrophoresis. The PIVKA-X containing fractions from the QAE-Sephadex column were dialyzed against 2 mM Tris-HCl-glycinate buffer pH 8.7, 10 mM in benzamidine hydrochloride and concentrated in a Diaflow ultrafiltration cell equipped with a PM-10 membrane (Amicon Corp. model 202). Generally, 40 mg of protein in 3-4 ml dialysis buffer containing 20% (v/v) sucrose was subjected to preparative polyacrylamide gel electrophoresis on a gel bed of 10 cm in the LKB 7900 Uniphor apparatus. Fractions of 3.0 ml were collected. The elution buffer was pumped at a flow rate of 30 ml/hour. Electrophoretic conditions were 700 V and 18 mA.

Composition of the gel solutions: Solution A: 30 mg acrylamide and 0.9 g bisacrylamide dissolved in 100 ml water. Solution B: 0.08 M Tris-glycine buffer pH 8.7 and 4 mM benzamidine HCl. Solution C: 140 mg ammonium persulfate dissolved in 100 ml water. Final gel solution was obtained by mixing 1 volume A, 1 volume B and 2 volumes C. According to the nomenclature of Hjerten (35) $T = 7.5\%$ and $C = 3\%$. Electrode chambers contained 20 mM Tris-glycine and 1 mM benzamidine hydrochloride at pH 8.7. The elution buffer was the same as the electrode buffer. The PIVKA-X containing fractions were pooled and concentrated in a Diaflow ultrafiltration cell equipped with a PM-10 membrane (Amicon Corp. Model 202). The concentrate was adjusted to pH 8.1 with 1.0 M Tris-HCl pH 8.6. 1.0 M DFP in 2-propanol was added to a final concentration of $10^{-3}M$. After dialysis against 0.025 M Tris-HCl pH 7.4 0.12 M NaCl during 48 hours with four changes, the PIVKA-X preparation was stored at $-80^{\circ}C$.

Determination of amino-terminal amino acid. Aminoterminal amino acids were determined qualitatively by the dansylchloride procedure of Gray (36). Dansylamino acids were identified on polyamide sheets using the procedure of Weiner et al. (37).

Amino acid analysis. The amino acid composition was determined according to Spackman (38). The proteins (0.5 - 1.5 mg) were hydrolyzed in 6 N HCl in evacuated Pyrex tubes at 110°C for 24 hours. The analyses were performed on a LKB amino acid analyzer 3201 using the one-column system of Hamilton (39).

γ -Carboxyglutamate was determined according to a modification of the method of Zytковicz (40). Prior to hydrolysis, proteins (1.0 mg) were completely reduced in ^3H -diborane after 96 hours of incubation at room temperature. The three identified ^3H -diborane reduction products are: homoserine (HSE), arising from the aspartic acid, 5-hydroxy-norvaline (HNV) arising from glutamic acid and 5,5'-dihydroxyleucine (DHL) arising from γ -carboxyglutamate. The specific activity of DHL is equal to half of the specific activity of HSE and HNV. The number of γ -carboxy glutamate residues was estimated from the calculated specific activity of DHL (49).

RESULTS

Preparation of monospecific antisera against bovine factors II, IX, and X. Undiluted rabbit anti-bovine factor X, anti-bovine factor IX, and anti-bovine factor II antiserum were allowed to diffuse against undiluted bovine plasma (fig. 1). Single precipitation lines were formed, showing a monospecificity of each of the antisera. This experiment shows that factor II, factor IX, and factor X have no antigenic determinants in common, because all reactions have the non-identity of both antigens and antibodies.

Neutralization test of coagulation factor activity by the antisera shows that the activities are specifically inhibited by their antibodies (table I).

TABLE I. Neutralization of coagulation factor activity by antisera.

	antiserum against factor	coagulation times obtained in a specifically deficient system:			
		f.II def.	f.VII def.	f.IX def. ^a	f.X.def.
bovine plasma	II	49.3	23.8	138	34.1
bovine plasma	IX	27.3	23.4	450	31.4
bovine plasma	X	28.1	25.2	142	133.7
bovine plasma	control	27.6	23.8	135	34.4
buffer time		133.0	126.3	365	135.5

a: factor IX was assayed according to the method of Veltkamp et al.(50).

Fig. 1.

Double immunodiffusion pattern of the anti-bodies used. Centre well: normal bovine plasma; wells 1 and 4: anti-factor II antiserum; wells 2 and 5: anti-factor IX antiserum; wells 3 and 6: anti-factor X antiserum.

Fig. 2.

Double immunodiffusion. Centre well: contains anti-bovine factor X antiserum. 1: normal plasma; 2: coumarin plasma; 3: $\text{Al}(\text{OH})_3$ -adsorbed coumarin plasma; 4: $\text{Al}(\text{OH})_3$ -adsorbed normal plasma; 5: normal serum; 6: coumarin serum.

Demonstration of PIVKA-X after administration of phenprocoumon.

Immunodiffusion. In a double immunodiffusion test (fig. 2) anti-bovine factor X antiserum revealed single and completely identical precipitation lines against: a. normal bovine citrated plasma; b. bovine citrated plasma obtained after oral anticoagulation of a cow (less than 5% factor X activity); c. the same plasma adsorbed with $\text{Al}(\text{OH})_3$ (2 mg/ml); d. serum of an anticoagulated cow and e. the same serum adsorbed with $\text{Al}(\text{OH})_3$ (2 mg/ml). No precipitation line is seen with normal plasma adsorbed with $\text{Al}(\text{OH})_3$ (2 mg/ml). It is shown that the factor X related antigen present in phenprocoumon plasma (PIVKA-X) has antigenic determinants in common with factor X in normal plasma.

Crossed immunoelectrophoresis. Two-dimensional immunoelectrophoresis of normal plasma (factor X activity 100%) performed in the presence of 2.5 mM Ca-lactate, showed one peak but the plasma taken 24 hours after the start of the phenprocoumon treatment (factor X activity 40%) showed a second peak with a higher anodical mobility. In the plasma of the same cow after 4 days of phenprocoumon treatment only the anodical peak remained and less than 5% factor X activity was measured (fig. 3).

The gradual increase of an abnormal factor X with antigenic determinants in common with normal factor X but with a higher electrophoretic mobility was accompanied by a decrease of factor X activity during phenprocoumon treatment.

Complete blocking of factor X synthesis, resulting in the appearance of PIVKA-X parallel with the disappearance of factor X was also confirmed by rocket immunoelectrophoresis analysis of plasmas obtained at different stages of anticoagulation. As shown in fig. 4, factor X activity decreased after starting the anticoagulant treatment, but no alterations could be demonstrated in the immunochemically determined concentration of factor X related antigen. However, after adsorption of these plasmas onto $\text{Al}(\text{OH})_3$ (2 mg/ml) an increasing amount of unadsorbable factor X related antigen (PIVKA-X) is observed during the time course of the treatment.

a

b

c

fig. 3.

Crossed immunoelectrophoresis of bovine plasma in the presence of calcium (first dimension, horizontal) and anti-factor X antiserum (second dimension, vertical). a: normal plasma, b: plasma after 24 h anticoagulation, c: plasma after 100 h anticoagulation.

Fig. 4.

Effect of phenprocoumon treatment on factor X activity.

A cow weighing 600 kg was anticoagulated with 600 mg phenprocoumon the first day and further on 200 mg daily. Factor X was determined with the one-stage assay (x). Determination of factor X related antigen in plasma by rocket immunoelectrophoresis (▲). Determination of factor X related antigen in plasma adsorbed with 0.2 (w/v) $Al(OH)_3$ by rocket immunoelectrophoresis (○). Detailed experimental conditions are described under Methods.

TABLE II. Purification of factor X from bovine oxalated plasma. Results of a representative experiment starting with 30 litres of bovine plasma.

Fraction	Volume (ml)	Concentration (units / ml)	Protein (mg/ml)	Spec.act. (units/mg)	Yield (%)	Purification (fold)
Plasma	30,000	1.0	75	0.013	100	1
BaSO ₄ eluate	3,000	4.1	1.5	2.7	40	210
DEAE-Sephadex	250	30	0.80	37	25	2,900
QAE-Sephadex X ₁	115	30	0.40	75	12	6,000
QAE-Sephadex X ₂	110	20	0.35	74	9	6,000

TABLE III. Purification of PIVKA-X from coumarin bovine oxalated plasma. Results of a representative experiment starting with 21 litres of bovine plasma.

Fraction	Volume (ml)	Concentration (units/ml)	Protein (mg/ml)	Spec.act. (Units/mg)	Yield (%)	Purification (fold)
plasma	21,000	1.0	77	0.013	100	1
BaSO ₄ eluate	2,100	2.5	5.9	0.4	25	33
DEAE-Seph.	237	13.3	1.2	11.0	15	850
QAE-Seph.	220	10.3	0.4	25.0	10	1,900
Prep.p.a.a.e. ^a	56	4.3	0.1	43.0	1	3,000

a: One half of the PIVKA-X preparation after QAE-Sephadex was submitted to preparative polyacrylamide gel electrophoresis.

Fig. 5.

Step 2 in factor X preparation. DEAE-Sephadex chromatography of BaSO_4 -eluate in 0.06 M trisodium citrate, 0.05 M Tris-HCl buffer, pH 8.0. After washing the column with 175 ml of a solution of 0.1 M NaCl, 0.1 M trisodium citrate, 0.05 M Tris-HCl buffer pH 8.0, a gradient of 2 x 300 ml was started, ranging from 0.1 M to 0.6 M NaCl in 0.1 M trisodium citrate, 0.05 M Tris-HCl buffer, pH 8.0. Fraction volume 5 ml. Bed dimension of anion exchanger 40 x 2.5 cm. Flow rate of 50 ml/hour. Absorbance at 280 nm (●); factor X activity (○); gradient (—).

Fig. 6.

Step 3 in factor X preparation. QAE-Sephadex chromatography of factor X containing fractions from DEAE-Sephadex. 250 ml of protein solution (0.6 mg/ml) in 0.05 M trisodium citrate buffer pH 7.0 was applied to QAE-Sephadex equilibrated in the same buffer. After washing the column with 250 ml of 0.1 M trisodium citrate buffer pH 7.0, a gradient was applied ranging from 0.1 - 0.35 M trisodium citrate buffer pH 7.0. Each chamber contains 400 ml solution. Bed dimensions of anion-exchanger 30 x 2.5 cm. Flow rate of 20 ml/hour and fraction volume 5 ml. Absorbance at 280 nm (●), factor X activity (○), gradient (—).

Preparation of factor X. The preparation of normal factor X is summarized in table II. The purification is based on the method of Jackson (20) and Fujikawa (21). The amount of BaSO_4 (10 mg/ml) used, adsorbed nearly quantitatively factor X activity, leaving 40% factor II activity in the supernatant. In our hands elution of the BaSO_4 cake with 0.06 M trisodium citrate buffer pH 5.8 results in a considerable loss in yield. Higher citrate concentrations and higher pH of the elution buffer does not give better results. Inactivation by BaSO_4 may be a possibility (41).

Batchwise adsorption of factor X activity from the BaSO_4 eluate onto DEAE-Sephadex was chosen to make possible handling of larger volumes of solution. Moreover, under the conditions used, factor II activity does not adsorb and factor VII activity adsorbs approximately for 60% to DEAE-Sephadex. In fig. 5 the elution profile of the DEAE-Sephadex column is shown. Factor X activity was eluted as a double peak at 0.18 M NaCl and 0.22 M NaCl.

Chromatography of the combined factor X eluted from the DEAE-Sephadex on QAE-Sephadex equilibrated in 0.05 M trisodium citrate buffer pH 7.0 revealed two factor X preparations eluted at 0.20 and 0.25 M trisodium citrate respectively (fig. 6). According to Jackson (42) and Fujikawa (21) they were identified as factor X_1 and X_2 . Both factor X preparations have about the same specific activity. The work of Neal et al. (43) suggested a difference in γ -carboxyglutamate content of the factor X preparations.

The bovine factor X_1 and X_2 preparations were free of the vitamin K dependent coagulation factors II, VII, and IX. Jackson reported a high susceptibility of factor X to proteolytic enzymes (44). In our hands a proper yield of factor X could only be obtained by collection of the blood in the presence of heparin, benzamidine and soybean trypsin inhibitor and by addition of benzamidine to all buffers used.

Preparation of PIVKA-X. The purification of PIVKA-X was based on the method described for factor X preparations. A representative purification

Fig. 7.

Adsorption studies of factor II, IX, and X and their analog PIVKAs onto Al(OH)_3 from oxalated plasma.

- factor II activity (●), factor VII activity (▲), factor IX activity (○), and factor X activity (x). Activity in the supernatant was determined by one-stage assays.
- PIVKA-II (●), PIVKA-IX (○), and PIVKA-X (x), concentrations in the supernatant. The adsorption experiments were carried out with coumarin plasma with less than 5% of the normal factors II, IX, and X. The concentration of the PIVKAs was determined by rocket immunoelectrophoresis (30).

Fig. 8.

BaSO_4 adsorption of PIVKA-II (○), PIVKA-IX (▲), and PIVKA-X (●) from oxalated coumarin plasma and factor X (★) from oxalated normal plasma. Concentrations in the supernatant as determined by rocket immunoelectrophoresis (30). Detailed experimental conditions are described under Methods.

of PIVKA-X is summarized in table III.

The fact that PIVKAs not adsorb to insoluble barium salts has been reported by several investigators (4, 45-48). Fig. 2 shows that PIVKA-X differs from factor X since it does not adsorb to $\text{Al}(\text{OH})_3$ up to 2 mg/ml. Adsorption of the factors II, IX, and X and their analog PIVKAs onto $\text{Al}(\text{OH})_3$ from oxalated plasma was studied further as is shown in fig. 7. It was found that an $\text{Al}(\text{OH})_3$ concentration of 2.5 mg/ml quantitatively adsorbs factor X, while about 20% factor IX activity and 40% factor II activity remains in the supernatant. In comparison with the normal factors, the PIVKAs have a much lower affinity for $\text{Al}(\text{OH})_3$ (fig. 7B). At an $\text{Al}(\text{OH})_3$ concentration of 10 mg/ml, PIVKA-X is quantitatively adsorbed, while about 90% of the factor II and factor IX related antigen remains in the supernatant.

From these results it is obvious that normal factor X activity may be removed from oxalated plasma obtained by phenprocoumon treated cows by adsorption with 1.5 mg/ml $\text{Al}(\text{OH})_3$, without a loss of PIVKA-X.

Unlike reported by Gaudernack (47,48), fig. 8 shows the possibility to remove the factor X related antigen from the plasma by adsorption onto BaSO_4 . Addition of 30 mg BaSO_4 /ml plasma results in a quantitative adsorption of PIVKA-X. It is further shown in fig. 8 that PIVKA-II and PIVKA-IX at this BaSO_4 concentration are only adsorbed for about 10%. Batchwise adsorption of the plasma, treated with BaSO_4 , with QAE-Sephadex, equilibrated in 0.02 M Tris-HCl buffer pH 7.4 containing 0.1 M NaCl and subsequently elution by a gradient of 0.1 M to 0.4 M NaCl only revealed PIVKA-II and PIVKA-IX (eluted both at 0.2 M NaCl). No PIVKA-X could be detected in the eluant of the QAE-Sephadex column even after washing the column with 1.0 M NaCl solution of pH 7.4. After dilution of the BaSO_4 eluant 1:1 with distilled water and adjusted to pH 7.1 with 1 N NaOH, PIVKA-X was quantitatively adsorbed on DEAE-Sephadex equilibrated in 0.02 M potassium phosphate buffer pH 6.8 containing 0.1 M NaCl. Fig. 9 shows the elution pattern of PIVKA-X from DEAE-Sephadex. PIVKA-X

Fig. 9.

Step 3 in PIVKA-X preparation. DEAE-Sephadex chromatography of BaSO_4 eluate in potassium phosphate 0.02 M + NaCl 0.1 M pH 6.8. After washing the column with 400 ml of buffer, a gradient was applied ranging from 0.1 - 0.6 M NaCl in 0.02 M K-phosphate buffer pH 6.8. Each gradient chamber contains 250 ml of the gradient solutions. Bed dimensions of the anion-exchanger 45 x 2.5 cm. Flow rate of 40 ml/hour and fraction volume 5 ml. Absorbance at 280 nm (●), PIVKA-X concentration (○), gradient (—).

Fig. 10.

Step 4 in PIVKA-X preparation. QAE-Sephadex chromatography of PIVKA-X containing fractions obtained after DEAE-Sephadex chromatography. 240 ml of protein solution (1.0 mg/ml) in 0.05 M sodium citrate buffer pH 7.0 was applied to QAE-Sephadex equilibrated in the same buffer. After washing the column with 600 ml of 0.1 M trisodium citrate buffer pH 7.0, a gradient (2 x 300 ml) was applied ranging from 0.1 M - 0.35 M trisodium citrate buffer pH 7.0. Bed dimensions of anion-exchanger 30 x 2.5 cm. Flow rate of 10 ml/hour and fraction volume 5 ml. Absorbance at 280 nm (●), PIVKA-X concentration (○), gradient (—).

Fig. 11.

Analytical polyacrylamide gel electrophoresis of normal bovine plasma and several fractions, obtained during the purification procedures.

1. normal bovine plasma, 70 µg; 2. BaSO₄-eluate from PIVKA-X purification, 100 µg; 3. PIVKA-X after DEAE-Sephadex, 45 µg; 4. PIVKA-X after QAE-Sephadex, 50 µg; 5. PIVKA-X after preparative polyacrylamide gel electrophoresis, 50 µg; 6. factor X after QAE-Sephadex, 30 µg.

Fig. 12.

Purification of PIVKA-X by preparative polyacrylamide gel electrophoresis. 3 ml of PIVKA-X containing fractions after QAE-Sephadex (10 mg/ml) were applied on a gel ($T = 7.5$ and $C = 3 : (35)$) of 10 cm length. Fraction volume 3.0 ml. Absorbance at 280 nm (●), PIVKA-X concentration (○), t.d. = tracking dye. Detailed experimental conditions are described under Methods.

was eluted as a single peak at 0.3 M NaCl. Fig. 10 shows the elution pattern of PIVKA-X from QAE-Sephadex. Like factor X, PIVKA-X is eluted in a double peak at 0.18 M and 0.22 M trisodium citrate. Only in fraction 180 a trace amount of factor X activity ($\approx 5\%$) could be detected.

Polyacrylamide gel electrophoresis shows that the PIVKA-X preparation contains several protein bands after the QAE-Sephadex step, in contrast to the factor X preparation after QAE-Sephadex, which is almost pure (fig. 11). A further purification of PIVKA-X was obtained by preparative polyacrylamide gel electrophoresis. The elution profile is shown in fig. 12. PIVKA-X is eluted in the first major protein peak. Less than 5% factor X activity could be detected in the pooled PIVKA-X fractions. Analytical polyacrylamide gel electrophoresis shows that this final PIVKA-X preparation is homogeneous and has the same electrophoretic mobility as factor X (gels 5 and 6 of fig. 11, respectively).

Like factor X, PIVKA-X is highly susceptible to proteolytic enzymes. A proper yield of PIVKA-X could only be obtained by adding benzamidine hydrochloride to all buffers. Omission of benzamidine in the last purification step results in the appearance of three different degradation products of PIVKA-X.

Relative electrophoretic mobility of PIVKA-X and factor X. As shown by Stenflo (4) the electrophoretic mobility of normal factor II in agarose gel electrophoresis is lower in a Ca^{2+} containing buffer than in an EDTA containing buffer, whereas the mobility of PIVKA-II is unaffected by Ca^{2+} ions. Agarose gel electrophoresis of factor X and PIVKA-X in the presence of Ca^{2+} ions (fig. 13B) or EDTA (fig. 13A) also demonstrates a difference in affinity to Ca^{2+} ions for PIVKA-X and factor X. It is also shown that PIVKA-X has a somewhat lower electrophoretic mobility than factor X in the presence of EDTA, which indicates a lower net negative charge of PIVKA-X.

Fig. 13.

agarose gel electrophoresis pattern in 0.05 M barbital buffer, pH 8.6 of factor X and PIVKA-X.

A. in the presence of EDTA (2 mM); 1. factor X, 100 μg; 3. PIVKA-X, 50 μg.

B. in the presence of Ca^{2+} (2 mM); 1. factor X, 100 μg; 3. PIVKA-X, 50 μg.

The electrophoretic pattern of bovine plasma (2) is included as a reference.

Fig. 14.

Sodium dodecyl sulfate gel electrophoresis of factor X and PIVKA-X. Gel electrophoretic system of Weber and Osborn (27).

1. factor X, disulfide reduced; 2. factor X; 3.

PIVKA-X, disulfide reduced; 4. PIVKA-X. Approximate loads for each gel: 15 - 20 μg of protein.

Fig. 15.

Double immunodiffusion. Centre well: anti-factor X antiserum. Well 1: coumarin bovine plasma; 2: PIVKA-X; 3: factor X; 4: normal bovine plasma.

A		B	
	+		+
		1	-
		-	+
1	-		+
	+		
		2	-

Fig. 16.

Crossed immunoelectrophoresis of purified factor X and purified PIVKA-X.

A. First dimension in the presence of Ca^{2+} (2 mM). 1. factor X; 2. PIVKA-X.

B. First dimension in the presence of EDTA (2 mM). 1. factor X; 2. PIVKA-X.

Molecular size of factor X and PIVKA-X. Electrophoresis on polyacrylamide gel in the presence of sodium dodecyl sulfate according to Weber (27) of both factor X and PIVKA-X revealed a major single protein band with a molecular weight around 55,000 (fig. 14). After reduction of the S-S bonds with β -mercaptoethanol both proteins show two protein bands with molecular weights of approximately 44,000 and 17,000. The molecular weights of PIVKA-X and factor X estimated by this method, are in good agreement with the molecular weight reported for factor X (20,21).

Immunochemical properties. A double immunodiffusion test of purified factor X, PIVKA-X, normal bovine plasma and phenprocoumon plasma with less than 5% factor X activity against anti-bovine factor X antiserum shows one precipitation line. A reaction of complete identity was seen between all samples and the antiserum (fig. 15).

When purified factor X and purified PIVKA-X were analyzed by crossed immunoelectrophoresis in calcium containing buffer, single symmetrical arcs were obtained with both proteins (fig. 16A), showing a higher electrophoretic mobility for PIVKA-X. By this technique factor X could not be detected in the PIVKA-X preparation. In the presence of EDTA the precipitation arc of PIVKA-X is at the same position as that found for factor X (fig. 16B). The immunoelectrophoretic patterns obtained with the purified proteins were not different from those obtained with unfractionated plasma.

Amino acid analysis. The amino acid composition of PIVKA-X and factor $X_{1,2}$ appeared to be identical (table IV), and is in good agreement with published data (21,42). The composition given is the average of two different preparations of each of the proteins. The number of γ -carboxyglutamate residues was determined in PIVKA-X, factor X_1 and factor X_2 by a modification of the method of Zytkevich (40) as described in Methods. Less than one γ -carboxyglutamate residue was detectable in PIVKA-X. No differences were found in the γ -carboxyglutamate content of factor X_1 and X_2 (table V). These results are in close agreement with the number of γ -carboxyglutamate residues

TABLE IV. Amino acid composition of factor X and PIVKA-X.

Amino acid	factor X mole/50,000 ^a	PIVKA-X mole/50,000 ^a	factor X (ref. 42) ^a mole/50,000
lysine	20	21	21
histidine	11	11	10
arginine	25	25	25
aspartic acid	40	42	40
threonine	28 ^b	28	30
serine	31 ^b	30	29
glutamic acid	61	61	61
proline	20	18	20
glycine	42	37	41
alanine	31	29	31
half-cystine	19	20	24
valine	20	22	24
methionine	6	6	6
isoleucine	11	13	11
leucine	33	29	32
tyrosine	11	11	9
phenylalanine	22	22	21

a: Molecular weights do not include the carbohydrate content (42).

b: Values determined by extrapolation to zero-time hydrolysis.

TABLE V. γ -Carboxyglutamate (Gla) content in factor X_1 , factor X_2 , and PIVKA-X

	Factor X_1 mole/50,000	Factor X_2 mole/50,000	PIVKA-X mole/50,000
Gla	12 8 ^a	13 13 ^a	<1

a: Values taken from Neal et al. (43).

Gla content was determined by a modified method according to Zytkovicz (40) as described under Methods.

suggested by several authors from analysis of the N-terminal portion of the light chain, prepared from a mixture of factor X_1 and factor X_2 (10,12).

Aminoterminal amino acid analysis. Qualitative amino terminal amino acid determination of PIVKA-X and factor X by a method according to Gray (36) showed a single intensive spot and several very weak spots by chromatography on polyamide sheets as described by Weiner et al. (37). In both factor X and PIVKA-X the main spot was identified as DNS-alanine. Fujikawa (21) reported the sequence of the NH_2 -terminal region of the heavy chain to contain Trp-Ala-Ile-His. By the method of Gray (36) tryptophane degrades and therefore cannot be detected.

DISCUSSION

A protein induced by vitamin K antagonists, analogous to prothrombin (PIVKA-II, as defined in the introduction) has been demonstrated and isolated by many investigators (1-4). The data presented here, clearly demonstrate the existence of an abnormal factor X in plasma of phenprocoumon treated cows (defined as PIVKA-X) which is ineffective in blood coagulation. The following arguments can be brought forward in support of the existence of PIVKA-X besides PIVKA-II.

- a. The antigen precipitable with monospecific antibodies against factor X, found in normal plasma, coumarin plasma, coumarin serum, $Al(OH)_3$ -adsorbed coumarin plasma and $Al(OH)_3$ -adsorbed coumarin serum shows immunochemically a reaction of complete identity (fig. 2). No precipitable material is found when normal plasma is adsorbed with $Al(OH)_3$ under conditions known to remove all factor X activity.
- b. Quantitation of antigen reacting with anti-bovine factor X antibodies in the rocket immunoelectrophoresis according to Laurell (30) shows that more material is present in coumarin and in $Al(OH)_3$ -adsorbed coumarin plasma than can be accounted for by the factor X activity (fig. 4). Moreover, it is shown, that during the phenprocoumon treatment, the concentration of the non-adsorbable factor X related antigen increases while factor X activity decreases.

c. By a different technique, the crossed immunoelectrophoresis in the presence of Ca^{2+} (31), the appearance of PIVKA-X and parallel disappearance of factor X during the phenprocoumon treatment was also established (fig. 3).

Recent advances in chemical analysis of the differences between prothrombin and PIVKA-II suggest that the vitamin K dependent mechanism serves to modify glutamate residues into γ -carboxyglutamate residues (6,8). This fact may cause the differences between prothrombin and PIVKA-II in biological activity, calcium binding and adsorption to insoluble inorganic salts. In addition, our results on PIVKA-IX and PIVKA-X may represent a situation parallel to that found for PIVKA-II. However, our adsorption studies of PIVKA-X on $\text{Al}(\text{OH})_3$ and BaSO_4 (fig. 7 and fig. 8) appear to be in contradiction with the statement of Nelsestuen et al. (3) that " Ca^{2+} binding sites are responsible for the quantitative and very selective adsorption of prothrombin onto insoluble barium salts". Similar comparison of the electrophoretic mobility of prothrombin and factor X may reveal a larger net negative charge on factor X (51). The more anodical position of PIVKA-X to that of PIVKA-II also indicates the same difference in net negative charge (18). These facts may explain the higher affinity of PIVKA-X towards insoluble barium salts as compared with PIVKA-II.

The isolation procedure for PIVKA-X described in this chapter yields a preparation which is highly pure as judged by several criteria, e.g. gel electrophoresis and amino terminal amino acid analysis. Essentially the same purification procedure as described for factor X could be used for the preparation of PIVKA-X. We performed one minor modification namely the use of a potassium phosphate buffer instead of the citrate-Tris buffer in the DEAE-Sephadex step. It was found that no resolution was obtained between PIVKA-X and the bulk protein using the former buffer in the gradient. The heterogeneity of PIVKA-X as demonstrated in the QAE-Sephadex elution profile is of interest. It is unlikely that this phenomenon is due to the presence of factor X, because the factor X activity is far less than the amount of

precipitable antigen.

It was reported by Neal et al. (43) that factor X_1 and X_2 differ in their γ -carboxyglutamate content (respectively 8 and 13 residues) and that this difference may be accounted for by the different behaviour on anion-exchange chromatography. However, in our hands an equal amount of γ -carboxyglutamate residues was found in both factor X_1 and factor X_2 . This result was confirmed by Magnusson (personal communication). Moreover, PIVKA-X which contains less than one residue of γ -carboxyglutamate also shows heterogeneity on anion-exchange chromatography under certain conditions. Unlike the purification of factor X, after the QAE-Sephadex step an additional step was required for PIVKA-X, e.g. polyacrylamide gel electrophoresis. The final purification of factor X is about twice the purification of PIVKA-X. Probably this discrepancy is due to the different methods used in determination of concentration (clotting activity versus an immunochemical assay). As judged by gel electrophoresis techniques both factor X and PIVKA-X preparations are homogeneous.

PIVKA-X is very similar to factor X on the basis of molecular weight, polypeptide chains composition, amino acid composition, amino terminal amino acid, immunochemical reaction and its behaviour on polyacrylamide gel electrophoresis. Furthermore, it is shown that the purified PIVKA-X and the native protein in unfractionated plasma are similar as they have the same immunochemical and electrophoretic properties.

Based on amino acid analysis the differences between factor X and PIVKA-X in biological activity and Ca^{2+} binding may be explained by the difference found in γ -carboxyglutamate content (table V).

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CHAPTER III

THE ROLE OF γ -CARBOXYGLUTAMYL RESIDUES IN THE POSITIVE COOPERATIVE BINDING OF Ca^{2+} TO BLOOD COAGULATION FACTOR X

SUMMARY

1. The Ca^{2+} binding properties of factor X and its analogous decarboxy factor X (PIVKA-X) have been compared with the aid of flow rate dialysis and ultraviolet difference spectroscopy.
2. Factor X binds about 20 moles of Ca^{2+} per mole of protein. The first four sites exhibit positive cooperativity.
3. Changes in the ultraviolet difference spectrum when Ca^{2+} ions are bound, suggest a conformational change.
4. In PIVKA-X a very low affinity of Ca^{2+} and no ligand induced conformational change was observed. Both phenomenons lack a cooperative character.
5. It is concluded that the presence of γ -carboxyglutaryl residues in factor X is a prerequisite for positive cooperative Ca^{2+} binding.

INTRODUCTION

The binding of Ca^{2+} to prothrombin requires the presence of γ -carboxyglutamyl residues (1-3). Carboxylation of 10 glutamyl residues in the amino-terminal part of prothrombin has been shown to occur by action of vitamin K at a post-ribosomal stage (4-6).

The Ca^{2+} mediated binding of prothrombin to phospholipid surfaces, a prerequisite of normal thrombin generation is thought to occur via these residues (7,8). It was reported that an abnormal prothrombin isolated from the plasma of cattle treated with vitamin K antagonists and lacking γ -carboxyglutamyl residues, does not bind to phospholipid vesicles (9).

Calcium binding to prothrombin (3, 10-14) and prothrombin fragment 1 (3,13) has been investigated in a number of laboratories. It is shown that prothrombin can bind 10-14 moles of Ca^{2+} , of which about four sites with a high affinity.

Factor X, another vitamin K dependent clotting factor, shows positive cooperativity until 3-5 of the 20 sites are occupied (3).

However, for both prothrombin and factor X, a considerable divergence in form of the binding isotherms was obtained. Positive cooperativity (2,12,15) as well as the presence of two distinct classes of binding sites (11,13,16) were suggested.

In this chapter, we compare Ca^{2+} binding to factor X and to de-carboxy factor X (PIVKA-X) that is obtained from plasma of cattle under phenprocoumon treatment. In this way it will be possible to demonstrate that γ -carboxyglutamate residues are necessary for cooperative Ca^{2+} binding. The increased affinity for binding of successive Ca^{2+} ions implies that a protein conformational change must be associated with Ca^{2+} binding. This was investigated with ultraviolet difference spectroscopy.

MATERIALS AND METHODS

Factor X and PIVKA-X were purified according to the methods described in chapter II. All chemicals were analytical grade and obtained from Merck. $^{45}\text{Ca}^{2+}$ was obtained from the Radiochemical Centre, Amersham. Extinction coefficient ($E_{1\text{cm}}^{1\%}$ at 280 nm) and molecular weights used for calculation of the protein concentrations were respectively 12.4 and 55,000 for both factor X and PIVKA-X (17). Standard CaCl_2 solutions in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl and 0.02% Na-azide were prepared from reagent grade $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. The Ca^{2+} concentration in these solutions was determined by titration (18).

Flow rate dialysis. The Ca^{2+} binding properties of factor X and PIVKA-X were studied by the flow rate dialysis technique according to Colowick (19). The method is based on measuring the rate of dialysis of $^{45}\text{Ca}^{2+}$ from a protein- Ca^{2+} equilibrium mixture across a membrane.

The upper chamber of the dialysis cell containing the protein solution and $^{45}\text{Ca}^{2+}$ was separated by a Visking dialysis membrane from the lower chamber. Both chambers have a volume of 2.5 ml. A buffer solution, identical to the solvent for the protein was pumped through the lower chamber. Under the conditions used, the withdrawal of ligand from the upper chamber is a negligible fraction of the total ligand. When a steady state is reached, i.e. the rates of ligand entering and leaving the lower chamber are equal, the concentration in the effluent becomes a measure of the concentration of free ligand in the upper chamber.

With this method a series of equilibrium measurement can be performed at increasing ligand concentrations in the same protein solution. The volume of buffer that has to be passed to reach a steady state is given by:

$$\frac{dN}{dt} = S_1 D - N \cdot \frac{f}{V} \quad (1)$$

where: N = number of ligand molecules in the lower chamber

S_1 = initial concentration of ligand ($^{45}\text{Ca}^{2+}$) in the upper chamber

D = diffusion constant (dependent on properties of the dialysis apparatus and the nature of the diffusion ligand)

V = volume of lower chamber

f = volume of buffer passing through the lower chamber per unit of time.

In the steady state:

$$\frac{N}{V} = \frac{S_1 D}{f} \quad (2)$$

The time required to reach a steady state concentration of Ca^{2+} in the lower chamber can be calculated from the integrated form of equation (1):

$$\frac{N_t}{V} = \frac{S_1 D}{f} (1 - e^{-(ft/V)}) + \frac{N_{t=0}}{V} e^{-(ft/v)} \quad (3)$$

When the volume flowing through the lower chamber, ft , is more than four times the volume of the lower chamber, V , the exponential term is negligible and therefore the concentration of ligand in the lower chamber, N_t/V , becomes constant ($> 98\%$ of the steady state volume). Subsequent addition of ligand to the upper chamber alters the concentration of free ligand from S_1 to S_2 . Then, equation (3) becomes:

$$\frac{N_t}{V} = \frac{S_2 D}{f} (1 - e^{-(ft/V)}) + \frac{S_1 D}{f} e^{-(ft/V)}$$

Again the additional flow required to reach the new steady state concentration $S_2 D$ is the same as that to reach the first steady state.

In a representative experiment the upper chamber was filled with 2 ml of a solution containing about 1.5 mg protein in 0.1 M NaCl, 0.05 M Tris-HCl, pH 7.5. 10 μ l of $^{45}\text{Ca}^{2+}$ solution (specific activity 8 $\mu\text{Ci}/\mu\text{g}$) was added to the upper chamber (final concentration 12 μM). The steady state was established after 12 ml of 0.05 M Tris-HCl buffer, pH 7.5, containing 0.1 M NaCl and 0.02% Na-azide passed through the lower chamber at a flow rate of 4 ml/min. After the steady state is reached, small volumes (5 - 15 μ l) of a non-radioactive Ca^{2+} were added to the upper chamber to increase the total Ca^{2+} concentration stepwise.

Fractions of 3 ml were collected and a 0.5 ml aliquot was mixed with 5 ml of a gelling scintillation solution (Aquasol, New England Nuclear) and counted in a Packard Tricarb liquid scintillation spectrometer. All experiments were performed at room temperature.

In an identical experiment, the steady state concentration of $^{45}\text{Ca}^{2+}$ was determined in the absence of protein. This control value will be a measure of the concentration of 100% free Ca^{2+} in the upper chamber (designated the 100% F value).

The steady state concentration of $^{45}\text{Ca}^{2+}$ in the effluent is always proportional to the free $^{45}\text{Ca}^{2+}$ concentration in the upper chamber. Therefore, the steady state concentration of $^{45}\text{Ca}^{2+}$ in the presence of protein divided by the 100% F value represents the fraction of the total Ca^{2+} concentration that is in the free state. The total Ca^{2+} concentration in the upper chamber is known, so that the concentration of the free and bound Ca^{2+} can be calculated.

However, the 100% F value will decrease during the time course of the experiment as a result of dilution after each addition of Ca^{2+} and loss of radioactivity in the effluent. The effect of dilution can be determined in a control experiment. The effect of cpm loss on the 100% F value must be calculated. For each step the 100% F value was corrected in the following way:

$$\text{corrected control value} = \frac{\text{actual cpm in upper chamber}}{\text{initial cpm in upper chamber}} \times 100\% - F$$

The actual counts in upper chamber are calculated by subtracting the total amount of counts of the effluent collected at any moment from the total counts in the upper chamber present at the start of the experiment.

The Ca^{2+} binding properties of factor X and PIVKA-X were evaluated by plotting $\bar{v}$ (mole Ca^{2+} bound / mole protein) versus $\bar{v}$ / free Ca^{2+} -concentration (F)) according to the method of Scatchard (20).

The ligand binding properties were also expressed by the equation given originally by Hill (21):

$$\frac{\bar{v}}{N} = K[F]^{n_H} / (1 + K[F]^{n_H}) \quad (1)$$

in which $\bar{v}/N$ is the fraction of the total number of binding sites occupied by ligand, $[F]$ is the free ligand concentration and K and n_H are constants. Equation (1) can be written as:

$$\log \frac{\bar{v}}{N - \bar{v}} = \log K + n_H \log [F]$$

giving a straight line when $\log \bar{v}/(N - \bar{v})$ is plotted versus $\log [F]$, which is known as the Hillplot.

Although the Hill equation is known to be an oversimplification(22), the slope (n_H , Hill coefficient) indicates the presence of any site-site interaction. That is, binding is cooperative ($n_H \neq 1$) or non-cooperative ($n_H = 1$).

An alternative method with which the contributions of site-site interactions in multiple ligand binding can be assessed, is given by De Meyts and Roth (23). They attempted to overcome the problems caused by the unknown stoichiometry of the binding process, i.e. unknown number of sites of different affinities or sites of

changing affinity, by taking an average affinity ($\bar{K}$) at each level of occupancy ($\bar{Y}$). The assumption is that when binding sites are not occupied, they are all in the same conformation and an identical affinity for the ligand is expected. Due to site-site interaction, the affinity will vary as a function of occupancy, as can be shown by plotting $\bar{K}$ versus $\bar{Y}$. The average affinity ($\bar{K}$) is given by the negative slope of a line joining $\bar{v}/F$ at a particular $\bar{v}$ to the number of total sites occupied (N). The fractional occupancy, $\bar{Y}$, is given by $\bar{v}/N$.

Ultraviolet difference spectroscopy. Ultraviolet difference spectra were measured on an Aminco DW-2 double beam spectrophotometer equipped with a thermostated cell holder maintained at 25°C. A set of matched, rectangular quartz cells (Hellma 104 QS) with light path of 1.0 cm were used. To study the protein- Ca^{2+} interaction, a Ca^{2+} solution was added in the sample cell and an equal volume of buffer in the reference cell, using Hamilton microsyringes. Both cells contain about 1.5 mg factor X or PIVKA-X, in 0.1 M NaCl, 0.01 M Tris-HCl at pH 7.5. Difference spectra were recorded from 240 nm to 340 nm on a full scale of 0.05 absorbance unit.

RESULTS AND DISCUSSION

Flow rate dialysis

Evaluation of the flow rate dialysis technique. In order to obtain a correct interpretation of the data obtained, some control experiments had to be carried out in order to evaluate the influence of several factors on the experimental system. The following requirements have to be met:

1. The steady state has to be reached after an amount of buffer corresponding to 4 times the volume of the lower chamber was pumped through.
2. The steady state concentration of $^{45}\text{Ca}^{2+}$ in the effluent has to be directly proportional to the concentration of free $^{45}\text{Ca}^{2+}$ in the upper chamber.

Fig. 1.

Flow dialysis profile of $^{45}\text{Ca}^{2+}$. At the first two arrows $^{45}\text{Ca}^{2+}$ was introduced into the upper chamber containing 2.0 ml of 0.05 M Tris-HCl, 0.1 M NaCl pH 7.5. At the third arrow non-radioactive Ca^{2+} was added at a concentration of 20 mM. For further experimental details see materials and methods.

Fig. 2.

Steady state concentration of $^{45}\text{Ca}^{2+}$ in the effluent as a function of the $^{45}\text{Ca}^{2+}$ concentration in the upper chamber. Six subsequent additions of $^{45}\text{Ca}^{2+}$ were made to the upper chamber which contained 2.0 ml of 0.05 M Tris-HCl, 0.1 M NaCl pH 7.5. Experimental conditions as described under Materials and Methods.

3. The time required for the binding reaction to equilibrate must be much faster than the time required to reach a steady state concentration of $^{45}\text{Ca}^{2+}$ in the effluent.

4. Non-specific Ca^{2+} binding must be absent, e.g.: Ca^{2+} binding to the dialysis membrane and other parts of the apparatus.

As demonstrated in fig. 1 the steady state at any step was reached after collection of about 12 ml of effluent, which is 5 times the volume of the lower chamber.

Aliquots of $^{45}\text{Ca}^{2+}$ solution (5.7×10^{-4} M) were added in succession to the upper chamber containing 2 ml of 0.05 M Tris-HCl, 0.1 M NaCl buffer pH 7.5.

Fig. 2 shows a linear relationship between the steady state concentration of $^{45}\text{Ca}^{2+}$ in the effluent and the concentration of free $^{45}\text{Ca}^{2+}$ in the upper chamber. It is concluded that these results fit the theoretical model described under Materials and Methods.

Indication that no errors are introduced by a possible slow approach to equilibrium of the binding reaction was obtained from the following experiment. An aliquot (2.0 ml) of factor X preincubated for 30 min. with 0.5 mM Ca^{2+} was added to the upper chamber, immediately followed by the addition of 10 μl $^{45}\text{Ca}^{2+}$ which makes the final Ca^{2+} concentration 0.512 mM. The time required to reach the steady state was the same as in an experiment where non-radioactive Ca^{2+} and $^{45}\text{Ca}^{2+}$ were added simultaneously to the upper chamber containing the factor X solution.

Binding of Ca^{2+} to the membrane may cause a considerable error in the estimation of the amount Ca^{2+} bound to the protein (24). However, the flow dialysis profile of $^{45}\text{Ca}^{2+}$ in the absence of protein (fig. 1) shows that after addition of high excess of Ca^{2+} to the upper chamber to a final concentration of 20 mM, the steady state concentration in the effluent shows only a small spike. This shows that the amount of $^{45}\text{Ca}^{2+}$ bound to

Fig. 3.

A. Flow dialysis profiles of $^{45}\text{Ca}^{2+}$ in the presence (open circles) and absence (solid circles) of factor X.

B. Flow dialysis profiles of $^{45}\text{Ca}^{2+}$ in the presence (open circles) and absence (solid circles) of PIVKA-X. For further experimental details see Materials and Methods.

the membrane can be neglected.

The final conclusion of these control experiments may be that several factors which can induce errors in the binding experiments can be neglected.

Ca²⁺ binding by factor X and PIVKA-X. The flow dialysis profiles of ⁴⁵Ca²⁺ in the presence and absence of factor X are shown in fig. 3A. The initial addition of ⁴⁵Ca²⁺ to the factor X solution (0.036 mM) in the upper chamber resulted in a final Ca²⁺ concentration of 0.012 mM. Subsequent additions of non-radioactive Ca²⁺ are indicated by the arrows. The concentration of Ca²⁺ shown represents the final Ca²⁺ concentration in the upper chamber. Up to sample no. 10 the control curve (solid circles) represents experimental values. The dotted part of the curve is the calculated control curve compensated for dilution and cpm loss as described under Materials and Methods. Addition of excess Ca²⁺ (17 mM) resulted in a rise of the steady state, concentration of ⁴⁵Ca²⁺ to the level of the corrected control value. The radioactivity in the effluent, therefore is indeed a measure for the free Ca²⁺ concentration. It also indicates that the steady state concentration in the effluent does not depend on the total Ca²⁺ concentration in the upper chamber.

The binding curve for factor X (solid circles) obtained by plotting $\bar{v}/\text{Ca}^{2+}$ versus $\bar{v}$ according to Scatchard (20) is shown in fig. 4. The Scatchard plot shows a positive slope between 0 and 3-4 mole Ca²⁺ bound per mole factor X, which indicates positive cooperative Ca²⁺ binding. Extrapolation of the curve to the $\bar{v}$ axis shows the presence of 20 Ca²⁺ binding sites per mole of factor X.

The flow dialysis profiles of ⁴⁵Ca²⁺ in the presence and absence of PIVKA-X are shown in fig. 3B. The first additions of ⁴⁵Ca²⁺ resulted in final concentrations of 0.012 and 0.024 M respectively. Subsequently, non-radioactive Ca²⁺ was added to final concentrations as indicated in the figure. At a final concentration of 30 mM Ca²⁺ in the upper chamber all ⁴⁵Ca²⁺ bound to PIVKA-X is exchanged

Fig. 4.

Scatchard plot of Ca^{2+} binding by factor X (solid circles) and PIVKA-X (open circles). Proteins are dissolved in 0.05 M Tris-HCl, 0.1 M CaCl_2 buffer pH 7.5.

Fig. 5.

The "average affinity" profile of the factor X Ca^{2+} binding. Data are those calculated from fig. 4 as described under Materials and Methods.

since the steady state concentration in the binding experiment reaches the corrected control value. The results of this experiment were used to construct a Scatchard plot (open circles, fig. 4).

PIVKA-X as compared to factor X gives a different Scatchard plot. Only low affinity binding sites were detected. Because of the very low affinity of PIVKA-X for Ca^{2+} , the total number of binding sites and binding constant cannot be determined.

The complexity of the factor X binding curve due to site-site interaction does not allow to calculate the binding constants from the slopes of the Scatchard plot.

In order to describe the site-site interaction in a different way, we plotted the average affinity of a Ca^{2+} binding site, $\bar{K}$, versus the fractional occupancy, $\bar{Y}$, according to the method of De Meyts et al. (23). Such a plot for the Ca^{2+} binding sites in factor X is shown in fig. 5. It clearly demonstrates that when the sites are empty a very low affinity is observed. When occupancy increases, the $\bar{K}$ values also increase until about 20% of the sites are occupied, i.e. when 4.0 Ca^{2+} per mole factor X are bound. The plot also suggests the presence of negative cooperativity, i.e. decrease of $\bar{K}$ with increase of $\bar{Y}$. However, the scattering of the data points in that region and the uncertainty in determining the number of total binding sites (N), prevent definite conclusions.

Evidence for cooperative Ca^{2+} binding to factor X was also obtained from the Hill plot shown in fig. 6. Assuming 20 Ca^{2+} binding sites, the binding data of fig. 4 gave two n_H values: 1.5 for the binding of the first four moles of Ca^{2+} and 0.90 for the subsequent ones. From these results we conclude that there are at least two different classes of binding sites, e.g.: positive cooperativity in the binding of the first 3-4 Ca^{2+} ions and about 16 sites showing non site-site interaction.

Fig. 6.

Hill plot of the factor X Ca^{2+} binding data. Data are those of fig. 4 which were obtained with factor X.

Ultraviolet difference spectroscopy. Interaction of Ca^{2+} with factor X changes the ultraviolet difference spectra as is shown in fig. 7. The two absorption peaks at 286 nm and 293 nm indicate a significant environmental change for tryptophanyl and possibly tyrosyl chromophores during Ca^{2+} binding. The changes are not observed in a similar experiment with PIVKA-X. This fact confirms that interaction of Ca^{2+} with γ -carboxyglutamate residues perturbs the tryptophane surrounding. The ultraviolet difference spectrum obtained upon binding of Ca^{2+} to the γ -carboxyglutamate residues indicates a polar perturbation of the tryptophane residues, because such changes are reported to induce a red shift of the $^1\text{L}_a$ band, resulting in a difference spectrum extending into the 300 nm region and a broad negative band around 260 nm (30).

As can be seen in fig. 8, the height of the absorption peak at 293 nm increases with Ca^{2+} concentration. Of interest is the curvature of the plot at low Ca^{2+} concentration. It implies the positive cooperative character of the Ca^{2+} binding. We conclude that the results obtained by the ultraviolet difference spectroscopy are qualitatively comparable with that obtained by the flow rate dialysis technique. The maximal height of the absorption peak at 293 nm was obtained at a Ca^{2+} concentration above 2 mM. It was calculated from the data presented in fig. 4, that at this concentration the fractional occupancy is about 0.5. This result suggests that there is no relationship between the number of sites showing cooperativity and the number of binding sites involved in the ultraviolet difference spectrum change.

CONCLUDING REMARKS

The results of our Ca^{2+} binding studies indicate that Ca^{2+} binds positively cooperatively to factor X. They are in good agreement with those reported by Henriksen and Jackson (3). PIVKA-X, which is identical to factor X but for the γ -carboxyglutamate residues, does not show positive cooperativity. We therefore conclude that

Fig. 7.

Ultraviolet difference spectrum produced by the interaction of factor X with Ca^{2+} .
 Experimental conditions: 1.5 mg factor X dissolved in 0.01 M Tris-HCl, 0.1 M NaCl buffer pH 7.5. Final Ca^{2+} concentration in the sample cuvette: 5 mM.

Fig. 8.

Increase in absorption maximum at 293 nm produced upon interaction of factor X (1.5 mg/ml) with Ca^{2+} . Experimental conditions: 0.01 M Tris-HCl, 0.1 M NaCl buffer pH 7.5.

γ -carboxyglutamate residues are involved in this binding. This is strongly supported by the results obtained with the ultra-violet difference spectroscopy experiments with factor X and PIVKA-X. Indeed, only in case of factor X evidence for a conformational change on binding of Ca^{2+} is obtained. It is further shown that this Ca^{2+} induced conformational change exhibits cooperativity.

Although several models for the geometry of the Ca^{2+} binding to γ -carboxyglutamic acid are proposed (25-27), no conclusion can be put forward about the relationship between the different sets of sites (positive cooperative and non-cooperative) and the number of γ -carboxyglutamate residues involved in Ca^{2+} binding.

From recent studies (28,29) on metal ion induced transitions of prothrombin fragment 1, it appears that prothrombin fragment 1 has two classes of metal binding sites. One set of sites (about 4) apparently non-selective with respect to the metal ion bound, probably triggers a conformational transition, which leads to unmasking of a subsequent set of sites. This second set of sites must be filled in order to permit binding of fragment 1 to phospholipids and to obtain Ca^{2+} mediated self-association of prothrombin fragment 1.

The question arises, whether the phenomenon of self-association causes the changes in the ultraviolet difference spectrum. If there is any effect, a reasonable explanation can be given for the observed difference between the number of sites showing positive cooperativity (3-4 Ca^{2+} bound per mole factor X) and the number of sites which have to be filled in order to obtain a maximal change in the ultraviolet spectrum (about 10). Perturbation of the tryptophanyl residues caused by self-association may be superimposed on the perturbation due to site-site interaction of the initial binding sites.

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CHAPTER IV

ACTIVATION OF DECARBOXY FACTOR X (PIVKA-X) BY A PROTEIN FROM
RUSSELL'S VIPER VENOM (RVV-X)

Purification and partially characterization of activated PIVKA-X

SUMMARY

1. Incubation of PIVKA-X with the factor X activating enzyme from Russell's Viper venom revealed the generation of amidase activity towards Bz-Ile-Glu-Gly-Arg-pNA but not of activity in blood coagulation.
2. The rate of activation of both factor X and PIVKA-X depends on the ability of the zymogens to bind Ca^{2+} . The relationship between Ca^{2+} concentration and velocity of the activation reaction is sigmoid in case of factor X but hyperbolic with PIVKA-X.
3. Activated PIVKA-X was purified by powder column electrophoresis.
4. Identical changes of primary structure accompanied the activation of factor X and PIVKA-X. Identical molecular weight and common antigenic determinants were found in factor X_a and PIVKA- X_a . The amino acid composition was identical except for 10 glutamic acid residues in PIVKA- X_a that are γ -carboxyglutamic acid residues in factor X_a .
5. Unlike factor X, activated factor X remains in the same position in the presence of calcium at pH 8.6. This is probably due to self-association of factor X_a under the influence of Ca^{2+} . The electrophoretic mobility of activated PIVKA-X is only slightly decreased compared to PIVKA-X in the presence of Ca^{2+} .

Fig. 1.

The mechanism of activation for bovine factor X (14). The solid arrow refers to the major pathway. The active centre serine is circled.

INTRODUCTION

Two alternative modes of activation of factor X exist in the physiological blood coagulation process: the extrinsic and the intrinsic system. The principal intrinsic pathway requires tissue thromboplastin and factor VII (1-17). Via the intrinsic pathway factor X is activated by a product of activated factor IX, factor VIII, phospholipids and Ca^{2+} ions (18-30).

Several non-physiological modes of activation of factor X have also been described, utilizing Russell's Viper venom (RVV) (15,28,31-41), trypsin (13, 42,43) and various other reagents (44). It is generally agreed that the activation products obtained with each of these systems exhibit functional similarity in that they rapidly convert prothrombin to thrombin in the presence of factor V, phospholipids and Ca^{2+} ions. Conflicting results are reported concerning physical and chemical properties of the activation products. Seegers and coworkers give evidence which favors different molecular forms of factor X_a as obtained by different conditions of activation (36, 45, 46). Bajaj and Mann (47) showed that trypsin activation of factor X resulted in an eight fold higher yield of factor X_a than obtained with the venom. Whether this is due to the formation of a different species of factor X_a is unknown.

Radcliff and coworkers concluded that the different ways of activation yield essentially the same product (13). Recent studies (14,34,35) showed that the conversion of factor X (M.W. 55,000) to factor X_a by factor IX_a and factor VIII, factor VII and tissue factor, a protease from Russell's Viper venom or pancreatic trypsin is due to cleavage of an activation peptide from the NH_2 -terminal region of the heavy chain. This cleavage occurs between Arg-51 and Ile-52, giving rise to factor $X_{a\alpha}$ (M.W. 45,000) and an activation peptide (M.W. 9,500). Factor $X_{a\alpha}$ is converted into factor $X_{a\beta}$ (M.W. 42,600) by hydrolysis of a second peptide bond between Arg-290 and Gly-291 at the C-terminal region

of the heavy chain. Jesty et al. noted that the conversion of factor $X_{a\alpha}$ to factor $X_{a\beta}$ could be catalyzed by factor $X_{a\alpha}$ in the presence of Ca^{2+} and phospholipids (16,48).

A minor pathway involving the cleavage of more than one peptide bond has also been reported. In a slow reaction, factor X is converted into factor X_{β} (M.W. 51,000) by intrinsic activators, factor VII and tissue thromboplastin or trypsin by cleavage of the peptide bond between Arg-290 and Gly-291. Factor X_{β} has no enzymatic activity. It can be further hydrolyzed into factor $X_{a\beta}$. The mechanism of activation for factor X is summarized in figure 1 (from Fujikawa et al. ref. 14).

Factor X activated by one of the agents described above is the protein that contributes the active site to prothrombinase, the physiological activator of prothrombin consisting of factor V, phospholipids and Ca^{2+} (49). Factor X_a alone has a small prothrombinase activity. Unlike factor X, PIVKA-X lacks the ability to bind Ca^{2+} , and does not function normally in blood coagulation. Whether this is because PIVKA-X cannot be split by factor X activation agents or because "activated" PIVKA-X is still inactive in prothrombinase was as yet unknown.

This chapter describes the activation mechanism of PIVKA-X by Russell's Viper venom. The role of Ca^{2+} in the activation process is studied, and the purification and partially characterization of activated factor X and PIVKA-X are described.

MATERIALS AND METHODS

All chemicals were analytical grade and obtained from Merck. Factor X and PIVKA-X were obtained according to the purification procedures described in chapter II. Russell's Viper venom was obtained from Sigma Chemical Co., St. Louis, USA and the factor X activator (RVV-X) was purified with the method according to Schiffman (50). The chromogenic substrate Bz-Ile-Glu-Gly-Arg-pNA (S-2222) was a kind gift of Dr.G.Claeson, A.B. Bofors Nobel Division, Peptide Research, Mölndal, Sweden. p-Nitrophenyl-

guanidino-benzoate (NPGB) was purchased by Biochemical Nutrition Comp.

Amino acid and NH_2 -terminal amino acid analysis. The amino acid composition was determined by means of the method according to Spackman (51). Amino terminal amino acid analysis was performed according to Gray (52), γ -carboxyglutamic acid was determined with the modified method of Zytkevich (53). More details about the analysis are described in chapter II.

Protein concentration determination. Concentration of total protein in solutions was determined with absorbance at 280 nm, assuming $E_{1\text{cm}}^{1\%}$ for factor X = 12.4 (54). The same value could be used for determination of PIVKA-X concentration because the molecular weight and amino acid composition for factor X and PIVKA-X are equivalent. An $E_{280\text{ nm}}^{1\%}$ of 10.0 was employed for the venom coagulation protein (39) and $E_{280\text{ nm}}^{1\%}$ of 12.2 for factor $X_a(\text{RVV})$ and PIVKA- $X_a(\text{RVV})$ (15).

Gel electrophoresis. Analytical polyacrylamide gel electrophoresis, disc-gel electrophoresis in the presence of sodium dodecyl sulphate and agarose gel electrophoresis were performed as described in chapter II.

Immunochemical methods. The one-dimensional crossed immuno-electrophoresis, employed for PIVKA-X determination using mono-specific anti-bovine factor X antiserum, was performed with the method of Laurell (55) as described in more detail in chapter II. Double immunodiffusion was performed according to Ouchterlony (56).

Factor X_n assay. Factor X was activated in suitable mixtures as described under Methods. At intervals, 10 μl aliquots were removed from the activation mixtures and diluted with 1.0 ml Michaelis buffer containing 10 mM EDTA. The samples were further diluted with Michaelis buffer depending on the degree of activation.

Subsequently, an aliquot (0.1 ml) of the final dilution was incubated at 37°C for 30 sec with 0.1 ml cephalin prepared with the method of Bell (57) from bovine brain and 0.1 ml factor X deficient bovine plasma. Clotting was initiated by adding 0.1 ml of 33 mM CaCl_2 solution to the incubation mixture. The clotting times were converted into units obtained by a reference curve of the logarithm of clotting time against the logarithm of dilutions of activated factor X. One unit factor X_a activity per ml was defined as the activity of 10 μg factor X_a per ml, i.e. about the amount that can be generated in 1 ml normal plasma.

Active site titration. Titration of factor X_a and PIVKA- X_a with p-NPGB was performed according to Smith (58). In a typical titration the sample cuvette contains 0.3 mg per ml of factor X_a or PIVKA- X_a in 0.1 M sodium barbital pH 8.3 with a final volume of 300 μl . The reference cuvette contains the same volume of buffer solution alone. To each cuvette 10 μl of a 0.01 M solution of p-NPGB in dimethyl-formamide-acetonitrile (1:3 v/v) was 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 absorbance. In the Aminco DW-2 spectrophotometer ϵ_{410} for p-nitrophenol at pH 8.3 was calculated as 17,500.

Amidase activity assay. The amidase activity of activated factor X and activated PIVKA-X was measured according to Svendsen et al. (59). At intervals, 10 μl aliquots were removed from the incubation mixture and diluted with 90 μl of 0.05 M Tris-HCl 0.05 M imidazole buffer (pH 8.3) containing 0.1 M NaCl and 10 mM EDTA. To obtain a final volume of 350 μl , 320 μl 0.05 M Tris-HCl, 0.05 M imidazole buffer (pH 8.3) containing 0.1 M NaCl, 25 μl solution of Bz-Ile-Glu-Gly-Arg-pNA (S-2222) in water (2 mM) and 5 μl of the diluted sample were mixed.

The initial rate of hydrolysis was measured in an Aminco DW-2 spectrophotometer, operating in the dual wavelength mode. Analysis was conducted at 37°C and amidase activity is expressed as

$\Delta A_{391-344}/\text{min}$.

Rate of conversion of factor X and PIVKA-X. The following incubation procedure was used when factor X and PIVKA-X were activated by RVV-X. Buffer solution of 0.05 M Tris-HCl 0.05 M imidazole (pH 8.3), containing 0.1 M NaCl, RVV-X, S-2222 and factor X or PIVKA-X was added to a microcuvet (Helma, type 105 OS with 10 mm lightpath). The final concentrations of the reaction components are given in the legend to figure 4. The endogenous rate of hydrolysis of S-2222 was measured before the reaction was initiated by addition of Ca^{2+} .

All assays were performed in an Aminco DW-2 spectrophotometer operating in the dual wavelength mode. The hydrolysis of S-2222 was measured at 391 nm with 344 nm as the reference wavelength. The determination was carried out at 37°C and amidase activity was expressed as $\Delta A_{391-344}/\text{min}$.

Preparation of factor X_a . A solution containing 48 mg of factor X in 120 ml of 0.025 M Tris-HCl buffer (pH 8.3), 0.2 M in NaCl was made 5 mM with respect to CaCl_2 and incubated at 37°C for 30 min. with 0.8 mg of the factor X converting protein from Russell's Viper venom. Complete activation of factor X was achieved as judged by factor X_a activity assay of the incubation mixture. The reaction mixture was then diluted two times with 0.05 M Tris-HCl (pH 7.1) containing 0.06 M trisodium citrate and dialyzed overnight against the same buffer. Then the incubation mixture was applied to a column (30 x 1.5 cm) of DEAE-Sephadex equilibrated in the dialysis buffer. Factor X_a was eluted by a linear NaCl gradient (0 -0.6 M) in 0.05 M Tris-HCl buffer pH 8.0 containing 0.1 M trisodium citrate.

Fig. 2.

Activation of factor X and PIVKA-X by RVV-X.

A. Factor X (0.8 mg/ml) was incubated with RVV-X (10 μg/ml) and Ca²⁺, 10 mM final concentration.

B. PIVKA-X (1.0 mg/ml) was incubated with RVV-X (25 μg/ml) and Ca²⁺, 10 mM final concentration.

-○- factor X_a activity as determined by bioassay.

-●- amidase activity towards Bz-Ile-Glu-Gly-Arg-pNA.

-▲- amidase activity. Activation carried out in the presence of 10 mM EDTA.

Fig. 3.

Time courses of activation of factor X and PIVKA-X by RVV.

A. Factor X, 0.7 mg/ml in 0.025 M Tris-HCl buffer pH 7.4 - 0.2 M NaCl, was incubated with RVV-X, 7 μg/ml, in the presence of 10 mM Ca²⁺. At several time intervals, samples, 50 μl, were removed and added to 50 μl of 5% sodium dodecyl sulfate, followed by heating to 100°C for 2 min. before electrophoresis.

B. PIVKA-X, 1.0 mg/ml in 0.025 M Tris-HCl buffer pH 7.4 - 0.2 M NaCl, was incubated with RVV-X, 10 μg/ml, in the presence of 10 mM Ca²⁺. Samples, 50 μl, were removed at the times shown and treated as described for factor X.

Preparation of activated PIVKA-X. Semi-purified PIVKA-X obtained after QAE-Sephadex chromatography (fig. 10, chapter II) was dialyzed against 0.025 M Tris-HCl buffer pH 7.4 containing 0.1 M NaCl.

PIVKA-X, 40 mg in 25 ml of dialysis buffer was incubated with 1 mg RVV-X in the presence of 10 mM Ca^{2+} at 37°C for 100 min. The reaction was stopped by the addition of EDTA to a final concentration of 10 mM. Then the reaction mixture was dialyzed against 2 mM Tris-glycine buffer pH 8.3 containing 1 mM EDTA. Activated PIVKA-X was isolated by powder column electrophoresis according to Rosenbaum (60). All buffers contained 5 mM calcium lactate, in order to obtain a separation between PIVKA-X_a and residual amounts of factor X_a. The column of the LKB 7900 Uniphor apparatus was packed with a slurry of Biogel P-300 (100-200 mesh) in 0.025 M Tris-glycine buffer pH 8.3. A volume of 25 ml of 0.025 M Tris-HCl buffer, pH 8.2 was allowed to flow into the column and then the sample, 30 mg protein dissolved in 25 ml Tris-HCl buffer was allowed to flow into the column. After the sample had entered the column, Tris-glycine buffer pH 8.3 was placed on the top of the column. A potential difference of 600 V was applied and the power was maintained at 16 W throughout the course of the separation. The sample was eluted from the bottom of the column with Tris-glycine buffer pH 8.3 at a flow rate of 30 ml per hour. Fractions of 3 ml were collected. PIVKA-X_a was determined by amidase assay as described elsewhere.

RESULTS

Activation of bovine factor X and PIVKA-X by RVV-X. Activation of factor X and PIVKA-X by the purified protease from Russell's Viper venom (RVV-X) was followed in time. Fig. 2A shows the curves for factor X_a activity (open circles) and amidase activity (solid circles) upon action of RVV-X. The weight ratio of RVV-X to factor X in this experiment was 80:1. The results show a good

agreement between formation of factor X_a activity and amidase activity. The reaction is essentially complete within 3 min. It is also demonstrated that EDTA completely inhibits the activation reaction (solid triangles).

Fig. 2B shows the time course for PIVKA-X activation by RVV-X as measured for factor X_a activity (open circles) and amidase activity (solid circles). For the activation of PIVKA-X the weight ratio of PIVKA-X to RVV-X was 50:1. In contrast with an almost negligible formation of factor X_a activity within 2 min., a progressively increase in amidase activity is demonstrated. The small factor X_a activity generated by RVV-X from PIVKA-X is most likely to be due to a contamination of factor X (< 5%). Under the conditions used, PIVKA-X is almost completely activated within 150 min. As in the activation of factor X, EDTA inhibits the reaction completely. The final yield of amidase activity per mole of zymogen with PIVKA-X is about 90% of that obtained with factor X.

Molecular weight changes in factor X and PIVKA-X upon activation were also monitored. At intervals aliquots were removed from the activation mixture and analyzed by SDS gel electrophoresis (fig. 3). Fig. 3A gives the result of factor X activation with RVV-X. The large protein band at zero time decreases in intensity during the first minutes of activation. Parallel with the disappearance of this band a new faster moving protein band appears. Appearance of this band coincides with the generation of factor X_a and amidase activity (fig. 2), and can be assigned to as factor $X_{a\alpha}$ (14). At longer incubation time a slightly faster moving protein band was also observed, this will be referred to as factor $X_{a\beta}$ (14). Because the X_a activity and amidase activity remain unchanged, factor $X_{a\alpha}$ and $X_{a\beta}$ have the same specific activity.

Qualitative the same molecular changes, as described for factor X were observed in PIVKA-X upon incubation with RVV-X (fig. 3B).

Fig. 4.

The effect of Ca^{2+} on factor X and PIVKA-X activation by RVV-X.

A. The time courses of activation of factor X. Factor X, 13 $\mu\text{g/ml}$, was incubated with RVV-X, 0.12 $\mu\text{g/ml}$ in the presence of Bz-Ile-Glu-Gly-Arg-pNA ($1.1 \times 10^{-4}\text{M}$). The final concentration of Ca^{2+} in the activation mixture was:

*, none; ○, 0.6 mM; ●, 0.7 mM; ▲, 0.8 mM; △, 1.0 mM; ■, 1.5 mM; □, 2.8 mM; x, 5.6 mM.

B. The time courses of activation of PIVKA-X. PIVKA-X, 13 $\mu\text{g/ml}$, was incubated with RVV-X, 0.12 $\mu\text{g/ml}$, in the presence of Bz-Ile-Glu-Gly-Arg-pNA ($1.1 \times 10^{-4}\text{M}$). The final concentration of Ca^{2+} in the activation mixture was:

*, none; ○, 0.3 mM; ●, 0.6 mM; ▲, 1.0 mM; △, 1.5 mM; ■, 3.0 mM; x, 6.0 mM.

Experimental conditions as described under Methods.

The appearance of a new faster moving protein band coincided with the generation of amidase activity. Both occurred at a slower rate with PIVKA-X than with factor X.

Influence of Ca^{2+} on rate of activation of factor X and PIVKA-X by RVV-X. The rate of factor X and PIVKA-X activation by RVV-X was measured by a continuous spectrophotometric assay as described under Methods. In case of a linear rate of factor X_a or PIVKA- X_a formation, the increase of amidase activity towards Bz-Ile-Glu-Gly-Arg-pNA will be parabolic in time. A plot of absorbance versus t^2 yields a straight line the slope of which indicates the initial velocity of the activation reaction (28). The linear relationship between absorbance and t^2 obtained from activation experiments at variable Ca^{2+} concentrations is shown in fig. 4. The effect of Ca^{2+} on the initial rate of the activation of factor X and PIVKA-X by RVV-X is shown in fig. 5. It is shown that under optimal conditions the initial rate of factor X_a formation is about 60 times higher than the initial rate of PIVKA- X_a formation. Of particular interest in these studies were the differences in nature of the Ca^{2+} dependence: sigmoid and hyperbolic in case of factor X and PIVKA-X activation, respectively (fig. 5). The Hill plot (fig. 6) of the data presented in fig. 5A and 5B suggests cooperativity and non-cooperativity in the Ca^{2+} dependent activation of respectively factor X and PIVKA-X.

Preparation of activated factor X. Factor X_a was separated from the activation mixture described under Methods by a single step of DEAE-Sephadex chromatography. To prevent a further conversion of factor X_{a_i} into factor $\text{X}_{a_{is}}$ (14) the activation was stopped by addition of trisodium citrate instead of EDTA. The latter was found to precipitate factor X_a with considerable loss of activity. Precipitation of protein material during the incubation was observed when factor X concentrations greater than 500 μg per ml were used in the activation mixture. This is probably due to the Ca^{2+} mediated aggregation of factor X_a .

Fig. 5.

Rate of generation of amidase activity from factor X (panel A) and PIVKA-X (panel B) by RVV-X at various Ca^{2+} concentrations, as determined from fig. 4.

Fig. 6.

Hill plot of the data presented in fig. 5A and B., where v = initial velocity at any Ca^{2+} concentration, $V_{\max}$ = initial velocity at optimal Ca^{2+} concentration, $\circ$ -, factor X; $\bullet$ -, PIVKA-X.

Fig. 7.

Purification of factor X_a .

Factor X, 48 mg in 120 ml of a reaction mixture containing 0.025 M Tris-HCl buffer, pH 8.0 - 0.2 M NaCl and 5 mM Ca^{2+} was activated by RVV-X, 0.8 mg, for 30 min. at 37°C. After dialysis against 0.05 M Tris-HCl buffer, 0.06 M trisodium citrate, pH 7.1, the solution was applied to a DEAE-Sephadex column (30 x 1.5 cm), equilibrated with the dialysis buffer. The column was eluted with a linear gradient, 250 ml per gradient chamber, from 0 - 0.6 M NaCl in 0.05 M Tris-HCl buffer, 0.1 M trisodium citrate pH 8.0, at 30 ml per hour. Fractions of 4.5 ml were collected. —●—, protein ($E_{280 \text{ nm}}$); —○—, factor X_a activity; —, NaCl gradient.

Factor X_a was eluted from the DEAE-Sephadex column at a NaCl concentration of 0.24 M in a single peak (fig. 7). The factor X_a prepared by this method is homogeneous as is shown by disc gel electrophoresis. It is further shown that factor X_a has a higher electrophoretic mobility than factor X (fig. 8). The specific activity of the preparation as determined by bioassay was found to be 3.0×10^5 U/mg protein. The functional enzyme concentration as determined by NPGb titration was about 90% of the actual protein concentration (5.5×10^{-6} M).

Preparation of activated PIVKA-X. The activation procedure of PIVKA-X was performed as described under Methods. After maximal amidase activity was formed, activated PIVKA-X was purified from the activation mixture by powder column electrophoresis with continuous elution. Ca^{2+} was present in the electrophoresis buffer in order to decrease the electrophoretic mobility of factor X_a which may be present as a contamination in the activation mixture.

The relevant part of the elution profile of the electrophoresis is shown in fig. 9. The first large protein peak contained amidase activity and was found to crossreact with monospecific anti-bovine factor X antiserum. The PIVKA- X_a containing fractions were pooled and concentrated to about 5 ml (Amicon PM-10 membrane).

The final yield is about 30%, as calculated from protein recovery. The specific activity as determined by bioassay for factor X_a activity was approximately 6×10^2 U/mg protein. This is less than 1% of the specific activity general determined for purified factor X_a . The functional PIVKA- X_a concentration as assayed by NPGb titration was about 75% of the calculated protein concentration.

The PIVKA- X_a prepared with this method was homogeneous in polyacrylamide gel electrophoresis and has in absence of Ca^{2+} an electrophoretic mobility equal to factor X_a (fig. 8).

Fig. 8.

Polyacrylamide disc gel electrophoresis of purified factor $X_{(a)}$ and PIVKA- $X_{(a)}$.

Gel 1: factor X, 50 μ g; gel 2: factor X_a , 50 μ g; gel 3: PIVKA-X, 50 μ g; gel 4: PIVKA- X_a , 30 μ g.

Fig. 9.

Purification of activated PIVKA-X by preparative powder column electrophoresis. Experimental conditions as described under Methods. $\bullet$ —, protein ($E_{280 \text{ nm}}$); $\circ$ —, amidase activity towards Bz-Ile-Glu-Gly-Arg-pNA, t.d., tracking dye (bromophenol blue).

Zone electrophoresis of factor X_a and PIVKA- X_a . In order to study the electrophoretic mobility of factor X_a and PIVKA- X_a in the presence of EDTA or Ca^{2+} we performed an electrophoresis in agarose gel at pH 8.6 as described in chapter II. Fig. 10 (left) shows the electrophoretic mobility in the presence of EDTA. It is demonstrated that factor X_a and PIVKA- X_a have identical electrophoretic mobilities, that are somewhat lower than that of factor X. In the presence of Ca^{2+} factor X_a has lost its electrophoretic mobility and even tends to migrate to the cathode. It is also shown that the electrophoretic mobility of PIVKA- X_a is retarded in the presence of Ca^{2+} (fig. 10, right panel).

Immunodiffusion. In double immunodiffusion experiments according to Ouchterlony (56) single precipitation lines were observed for anti-factor X against activated factor X and activated PIVKA-X. Reactions of partial identity were observed. The results indicate that the two proteins have antigenic determinants in common (fig. 11).

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate. Electrophoresis of purified factor X_a and PIVKA- X_a on polyacrylamide in the presence of sodium dodecyl sulphate according to Weber and Osborn (61) shows that both enzymes have an identical molecular weight of about 48,000 (fig. 12, gels 1 and 3). Electrophoresis after disulfide reduction demonstrates that the proteins consist of two polypeptide chains with molecular weights around 33,000 and 17,000 (fig. 12 gels 2 and 4). A comparison with reduced factor X or reduced PIVKA-X indicates that the molecular change by activation with RVV-X occurs by splitting off a peptide from the heavy chain. The molecular weight of the light chain remains unchanged in the activation process.

Amino-terminal amino acid analysis. Amino-terminal amino acid analysis was performed with the method according to Gray (52). For both factor X_a and PIVKA- X_a alanine and isoleucine were found as the amino-terminal amino acids. In chapter II alanine

Fig. 10.

Left: agarose gel electrophoresis pattern in barbital buffer containing EDTA of factor X_a (A), PIVKA- X_a (B), normal factor X (C). The electrophoretic pattern of bovine plasma is included as a reference (D).

Right: agarose gel electrophoresis pattern in barbital buffer containing Ca^{2+} of factor X_a (A), PIVKA- X_a (B). The electrophoretic pattern of bovine plasma is included as a reference.

Fig. 11.

Double immunodiffusion.
Centre well: anti-bovine factor X-antiserum. Well 1, normal bovine plasma; well 2, factor X; well 3, factor X_a ; well 4, coumarin bovine plasma; well 5, PIVKA-X; well 6, PIVKA- X_a .

Fig. 12.

Sodium dodecyl sulfate polyacrylamide gel electrophoresis.

Gel 1, factor X_a , 15 µg; gel 2, factor X_a , 30 µg, disulfide reduced prior to electrophoresis; gel 3, PIVKA- X_a , 15 µg; gel 4, PIVKA- X_a , 30 µg, disulfide reduced prior to electrophoresis.

was reported as the amino-terminal amino acid of the light chain while tryptophane was probably found as the amino-terminal amino acid of the heavy chain of PIVKA-X. It is obvious that after activation of PIVKA-X isoleucine becomes the new amino-terminal amino acid of the heavy chain, as in the case with factor X (34).

Amino acid analysis. The amino acid compositions for factor X_a and PIVKA- X_a are summarized in table I. No significant differences in amino acid composition were observed. These amino acid analyses are in good agreement with the data published by Fujikawa (34).

γ -Carboxyglutamate residues were determined by a modified method according to Zytkevich (53) as described in chapter II. The number of γ -carboxyglutamate residues detected in factor X_a was about the same as in factor X, indicating that no γ -carboxyglutamate residues were lost during the activation and subsequent purification of factor X_a . Less than one residue of γ -carboxyglutamate was found in PIVKA- X_a . The presence of γ -carboxyglutamate in the PIVKA- X_a preparation might be due to a contamination with factor X_a or to a partial carboxylation of glutamic acid residues in PIVKA- X_a .

DISCUSSION

In this chapter experiments are described by which we demonstrate the conversion of PIVKA-X into an active enzyme analogous to factor X_a . Preliminary communications of this phenomenon were made by us earlier (62,63).

The aim of the experiments was to find out whether the inability of PIVKA-X to participate in blood coagulation is due to an impaired activation mechanism and/or impaired enzymatic function in prothrombinase.

The synthetic chromogenic substrate, Bz-Ile-Glu-Gly-Arg-pNA has been shown to be a useful tool in studying the reactions involved in activation of factor X (64,66). It also appeared

TABLE I. Amino acid composition of factor X_a and PIVKA- X_a

Amino acid	factor X_a mole/48,000	PIVKA- X_a mole/48,000	factor X_a (ref.14) mole/45,000
lysine	21	16	20
histidine	8	8	8
arginine	22	23	23
aspartic acid	38	41	33
threonine ^a	25	23	26
serine ^a	35	27	24
glutamic acid	63	58	58
proline	12	11	15
glycine	44	38	38
alanine	28	25	26
half-cystine	17	15	24
valine	18	17	26
methionine	6	6	5
isoleucine	9	11	11
leucine	26	26	23
tyrosine	8	8	9
phenylalanine	21	21	21

a: Values determined by extrapolation to zero time hydrolysis.

Acid hydrolysate after reduction with [^3H] -diborane

Amino acid	factor X_a	PIVKA- X_a
γ -carboxyglutamic acid	10	< 1

suitable for studying the activation of PIVKA-X.

The factor X activating protein from Russell's Viper venom (RVV-X) was suitable as an activator of PIVKA-X, because of its well documented reaction mechanism (14,16) and the inability of RVV-X to bind Ca^{2+} (41). The latter is important since any Ca^{2+} effect on activation will primarily be due to interaction of Ca^{2+} with factor X or PIVKA-X.

In contrast to activation by the physiological activators, factor VII_a and factor IX_a , the activation of factor X with RVV-X does not require phospholipids (14). However, the mechanism of activation of factor X by physiological and non-physiological activators has been shown to be identical (14).

Under the conditions used, factor X was completely activated within 3 minutes and clotting and amidase activity increased in parallel (fig. 2A). Under the same conditions the complete activation of PIVKA-X as measured by amidase activity requires about 180 min (fig. 2B).

After that time about the same amidase activity as in the factor X activation was reached. The possibility to convert PIVKA-X in an enzyme analogous to factor X_a was also confirmed by the comparable time course of generation of amidase activity and limited proteolysis, as visualized by SDS-gel electrophoresis. The time course of generation of clotting activity is qualitatively and quantitatively quite different. Therefore, it is concluded that no clotting activity can be generated from PIVKA-X. The factor X_a activity, which is generated, must be due to a contamination with factor X.

To our surprise the activation of PIVKA-X depends on the presence of Ca^{2+} . This Ca^{2+} requirement is unexpected because it is known that RVV-X binds no Ca^{2+} (41) and PIVKA-X has a very low affinity for Ca^{2+} , due to the absence of γ -carboxyglutamate residues (this thesis). It appeared that the maximal rate of factor X activation is about 60 times higher than that of PIVKA-X,

whereas the velocity of activation of factor X is dependent upon Ca^{2+} concentration in a sigmoidal fashion, that of PIVKA-X is hyperbolic.

A Hill plot of the data obtained, indicates that only in case of factor X the increase in Ca^{2+} concentration has an allosteric effect on the reaction velocity ($n_H=4.3$).

We conclude that the substrate for RVV-X is the protein complexed with Ca^{2+} and Ca^{2+} binding to γ -carboxyglutamate residues is essential for a rapid conversion to factor X_a .

In view of the Hill coefficient obtained it is tempting to speculate that a Ca^{2+} induced conformational change in factor X is important for the activation reaction. Evidence for a Ca^{2+} dependent conformation in factor X was obtained by ultraviolet difference spectroscopy analysis (chapter III). The fact that addition of Ca^{2+} to PIVKA-X has no effect on the spectra of the aromatic amino acids and the low rate of activation of PIVKA-X clearly demonstrates the importance of a Ca^{2+} dependent conformational change in the substrate for a rapid activation.

From the Ca^{2+} binding experiments it is assumed that cooperativity in the Ca^{2+} dependent activation of factor X is caused by a positive cooperative binding of the first four Ca^{2+} ions to factor X (chapter III). However, the concentration of Ca^{2+} , 2 mM, required for a maximal rate of activation of factor X, suggests that in addition to the occupancy of the Ca^{2+} binding sites mentioned above, a second set of sites must be filled. This interpretation is consistent with the observation of Prendergast and Mann (65), who showed that the maximal rate of activation of prothrombin is found at a Ca^{2+} concentration that greatly exceeds that required to fill the Ca^{2+} binding sites which exhibit positive cooperativity.

Recently, Kosow (66) reported studies on the role of Ca^{2+} in the activation of human factor X by RVV-X. Based on a sigmoidal re-

relationship between Ca^{2+} concentration and initial reaction velocity, he proposed that there are at least three cooperative binding sites for Ca^{2+} .

Separation of activated factor X from the activation mixture could be achieved by DEAE-Sephadex chromatography as described by Jesty (48). Using this procedure we did not succeed to separate activated PIVKA-X from its activation mixture. Using powder column electrophoresis, a homogeneous preparation as judged by polyacrylamide electrophoresis was obtained. Addition of Ca^{2+} to the electrophoresis buffer was carried out in order to obtain a separation between PIVKA- X_a and contaminating factor X_a . In spite of this, a residual factor X_a activity (about 6×10^2 U/mg protein) can be detected in the purified PIVKA- X_a preparation. The presence of factor X_a activity in the purified product may be due to a persistent contamination or to an intrinsic activity of PIVKA- X_a , like in the case of PIVKA-II (67). Differentiation will only be possible when high yields of PIVKA- X_a enable us to carry out rigorous tests for purity of this product.

As measured by sodium dodecyl sulfate gel electrophoresis, factor X_a and PIVKA- X_a have equal molecular weights. In fig. 12 it is shown that two forms of the enzymes may exist, with molecular weights of approximately 48,000 and 44,000 daltons. Upon reduction, prior to electrophoresis it was shown that the heterogeneity is due to a difference in molecular weight of the heavy chain. Because the only amino-terminal amino acids found were isoleucine and alanine, the difference must be caused by splitting off a peptide at the carboxy-terminal end of the heavy chain. These results are consistent with the two forms of factor X_a reported by Fujikawa (35) and Jesty (68).

Further proof for the conversion of PIVKA-X into an active enzyme analogous to factor X_a was obtained with an immunological technique and amino acid analysis. It has been shown that PIVKA- X_a and factor X_a have common antigenic determinants (fig. 11). Differences in

amino acid composition were only found with respect to γ -carboxyglutamate content (table I). Moreover, the γ -carboxyglutamate content of factor X_a is unaltered as compared with factor X. This result indicates that all γ -carboxyglutamate residues are conserved after activation with RVV-X. A striking difference of electrophoretic mobility of factor X_a and factor X was found in the presence of Ca^{2+} . The most likely explanation for this phenomenon might be the tendency of factor X_a to aggregate in the presence of Ca^{2+} (48). Also the electrophoretic mobility of PIVKA- X_a in the presence of Ca^{2+} is somewhat decreased. Whether this can be due to aggregation is not yet clear.

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CHAPTER V

THE EFFECT OF γ -CARBOXYGLUTAMYL RESIDUES ON THE ENZYMATIC PROPERTIES OF THE ACTIVATED BLOOD CLOTTING FACTOR X

A comparison of the normal and the decarboxy enzyme

SUMMARY

The esterolytic and amidolytic properties of activated blood coagulation factor X (factor X_a) and the analogous decarboxy enzyme were compared in order to find out if the γ -carboxyglutamyl residues influence the function of the active centre. It was found that the two proteins

1. Showed similar kinetic parameters when titrated with p-NPGB.
2. Had a similar K_M and k_{cat} for various synthetic chromogenic substrates.
3. Were inhibited in the same way by benzamidine.

Further it was observed that:

4. Ca^{2+} inactivates factor X_a , presumably by inducing the formation of a dimer, but has no influence on PIVKA- 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 PIVKA- X_a .
6. The prothrombin activation in the absence of Ca^{2+} by factor X_a and PIVKA- X_a is similar. Identical distribution of activation products is observed. Evidence is obtained that the conversion of prothrombin by both factor X_a and PIVKA- X_a is enhanced by Ca^{2+} , but that this effect is markedly greater with factor X_a .

We conclude that the interaction of the γ -carboxyglutamic residues with Ca^{2+} in factor X_a has no measurable influence on the properties of the active centre.

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 structures 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, activated 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-toluene sulfonyl-L-arginine methylester (11,15), p-nitrophenyl-p'-guanidinobenzoate (16) and benzoxy-isoleucyl-glutamyl-glycyl-arginyl-p-nitroanilide (17). Contrary to other serine proteases (with the exception of clotting factor IX $_a$) factor X_a has an 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 hydrolyzed 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 Arg²⁷⁴-Thr²⁷⁵ and Arg³²³-Ile³²⁴ bonds (18-21), whereas there are 75 bonds that 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 made 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" interactions are largely responsible for highly specific interactions like these of factor X_a and prothrombin (22-25).

Studies employing small molecule substrates and peptide substrates in determining kinetic behaviour indicate that the specificity of thrombin, chymotrypsin and trypsin towards these substrates is reflected in the rate determining acylation step rather than in the enzyme-substrate complex formation (26-33).

As was mentioned above, in its physiological function factor X_a is a highly specific protease which activates prothrombin to thrombin (19, 34-40). The activation of prothrombin by factor X_a alone or the complexed enzyme containing factor X_a , V_a , phospholipid and Ca^{2+} ions in vitro occurs in several steps. Data from numerous studies focussed upon the activation mechanism in vitro suggest an activation pathway as shown in fig. 1 (19,21,34,43-50).

Factor X_a cleaves peptide bonds 2 and 3. In addition to the two factor X_a catalyzed cleavages, thrombin rapidly cleaves prothrombin and fragment 1.2. The data now available give evidence that the main, if not only, pathway for prothrombin activation by the complex enzyme can be summarized by the following equations (42, 51-53):

Fig. 1.

Schematic structure for prothrombin. The three peptide bonds that are cleaved in bovine prothrombin during or as a result of prothrombin activation are designated by ①, ②, and ③. Another bond, ①', can be cleaved in both bovine and human prothrombins; however, neither the precise location nor the agent has been determined. The nomenclature system for prothrombin proteolysis products, proposed by a Task Force of the International Committee on Thrombosis and Haemostasis is designated as follows: if bond ① is cleaved in prothrombin, two products are formed: prothrombin fragment 1 and prethrombin 1. Bovine prothrombin 1 consists of the region from the amino terminus to bond ① (Arg 156 - Ser 157); prethrombin 1 is the polypeptide from bond ① to the carboxyl terminus of prothrombin. By convention, prethrombin implies that the amino acid sequence ultimately giving rise to thrombin is contained in the polypeptide; fragment implies that the amino acid sequence of the polypeptide is not finally associated with thrombin. Cleavage of bond ② (Arg 274 - Thr 275) gives rise to prothrombin fragment 1.2 and prethrombin 2. Cleavage of bond ① in prothrombin fragment 1.2 yields prothrombin fragment 1 and prothrombin fragment 2. Cleavage of bond ③ (Arg 323 - Ile 324) in prethrombin 2 yields thrombin, a disulfide-linked, two-chain enzyme in which the polypeptide chain between bonds ② and ③ is designated the chain A and the polypeptide chain between bond ③ and the carboxyl terminus is designated the B chain. In human prothrombin an additional bond ②' is cleaved which results in the formation of an A chain of bovine thrombin that is 13 residues shorter than the A chain of bovine thrombin.

fragment 1.2 $\xrightarrow{\text{thrombin}}$ fragment 1 + fragment 2

The direct formation of prethrombin 2 from prothrombin was not recognized in the earlier investigations due to the complicating feature of thrombin catalyzed proteolysis of prothrombin as well as fragment 1.2 (19,34,43,49,50).

During slow prothrombin activation thrombin catalyzed proteolysis exceeds the factor X_a catalyzed prothrombin activation, resulting in the appearance of large quantities of prethrombin 1:

The rate of thrombin generation by factor X_a alone is less than 0.1 % of that obtained with the complex prothrombinase (41,42). Factor V_a alone does not catalyze peptide bond cleavage or activation in either prothrombin or the prethrombins (19,54). A particularly interesting feature of the prothrombin activation is the release of a large activation fragment (i.e. fragment 1.2) (21,43). Esmon et al. (52) have clearly shown that in the pure aqueous phase reaction (factor X_a alone) neither fragment 1 or fragment 2 alone or together effects the reaction rate.

Addition of factor V_a and phospholipids to factor X_a and Ca^{2+} results in an increased rate of prothrombin activation. This is almost completely dependent on the presence of fragment 1 and fragment 2 as sites for respectively the Ca^{2+} mediated binding of prothrombin to phospholipids and non-covalent protein interaction with factor V_a (51,53).

It is the purpose of the experiments described in this chapter to elucidate a possible role of γ -carboxyglutamate residues in the catalytic activity of factor X_a by comparing it with the activity of decarboxy factor X_a (PIVKA- X_a). Experiments are described concerning the role of Ca^{2+} in the activation of prothrombin by both enzymes.

MATERIALS AND METHODS

Materials and methods, except for those described below, are reported in the preceding chapters.

Chemicals. p-Nitrophenyl-p'-guanidinobenzoate hydrochloride (NPGb) was purchased from Nutritional Biochemicals Corp. Benzoyl-phenylalanyl-valyl-arginyl-p-nitroanilide (Bz-Phe-Val-Arg-pNA) and benzoyl-isoleucyl-glutamyl-glycyl-arginyl-p-nitroanilide (Bz-Ile-Glu-Gly-Arg-pNA) were products of A.B. Bofors, Nobel Pharma, Sweden. Sulfopropyl (SP)-Sephadex was obtained from Pharmacia Fine Chemicals.

Proteins. Prothrombin was prepared according to the method of Owen et al. (19). Small amounts of thrombin possibly formed during purification procedures and other proteolytic enzymes were removed by passing the prothrombin preparation through a column (0.9 x 20 cm) of SP-Sephadex and soybean trypsin inhibitor-Sepharose (55). Soybean trypsin inhibitor was coupled to Sepharose 4B with the method of Cuatrecasas (56). Factor V_a prepared with the method of Smith et al. (57) was a kind gift of Dr. J. Rosing. Activated factor X and activated PIVKA-X were prepared with the method described in chapter IV.

ACTIVE SITE TITRATION

Theory of titration. The general reaction scheme for the interactions of serine proteases with substrates is:

where in the case of NPGB as the substrate (S), E is the enzyme, ES is the enzyme-substrate complex, ES' is the guanidinobenzoyl enzyme, P₁ is the product of the acylation reaction (p-nitro-phenol) and P₂ is the product of the deacylation reaction (p-guanidinobenzoate).

As shown by Bender et al. (58) the expression for the formation of p-nitrophenol is:

$$P_1 = \frac{k_{cat}[E_0][S_0]t}{[S_0] + K_M} + [E_0] \left[\frac{k_{cat}/k_3}{1 + K_M/[S_0]} \right]^2 (1 - e^{-bt}) \quad (2)$$

$$\text{Here, } k_{cat} = \frac{k_2 \cdot k_3}{k_2 + k_3} \quad ; \quad K_M = \frac{K_S \cdot k_3}{k_2 + k_3} \quad ; \quad b = k_3 + \frac{k_2}{1 + K_S/[S_0]}$$

This equation holds under the assumption $[S_0] \gg [E_0]$

We put in equation (1):

$$A = \frac{k_{cat}[E_0][S_0]}{[S_0] + K_M} \quad (3)$$

$$\pi = E_0 \left[\frac{k_{cat}/k_3}{1 + K_M/[S_0]} \right]^2 \quad (4)$$

$$\text{then } P_1 = At + \pi(1 - e^{-bt}) \quad (5)$$

When the transient phase is over, e^{-bt} approaches zero, so that in the steady state P₁ is given by

$$P_1^* = At + \pi \quad (6)$$

From this equation it can be seen that the line of steady state

production of P_1 will intercept the ordinate in π .

When $k_2 \gg k_3$ and $S_0 \gg K_M$, $\frac{k_{cat}/k_3}{1+K_M/[S_0]}$ will approach unity and $\pi \approx E_0$.

If only the first assumption holds π must be determined at several substrate concentrations. A plot of $1/\sqrt{\pi}$ versus $1/[S_0]$ will intercept the ordinate at $1/\sqrt{[S_0]}$ as can be seen from a rearrangement of equation (4).

Determination of K_S and k_2 . Rearrangement of equation (5) and substitution of (6) gives

$$P_1 - P_1^* = -\pi e^{-bt} + \pi$$

A plot of $\ln(P_1^* - P_1)$ versus t thus provides b from its slope. As

$$b = k_3 + \frac{k_2}{1+K_S/S_0}$$

under the assumption that $k_2 \gg k_3$ may be arranged to

$$1/b = 1/k_2 + K_S/k_2 \cdot S_0$$

a plot of $1/b$ versus $1/S_0$ will provide K_S and k_2 .

Determination of k_3 and K_M . The rate constant of deacylation can be determined by isolation of the acylenzyme by gel filtration, incubation under the conditions of titration and rate assay of the recovered enzymatic activity.

A plot of $\ln \frac{[ES']}{[E_0]}$ versus t provides k_3 from the slope.

K_M arises from the substitution of known values of k_2 , k_3 , and K_S in the expression

$$K_M = \frac{K_S \cdot k_3}{k_2 + k_3}$$

Determination of K_{pb} . A second factor involved in the postburst p-nitrophenol production may be the nonspecific nitrophenol production, presumably by the imidazole and amino groups of the protein (59).

A plot of NPGB concentration versus the rate constant of the postburst p-nitrophenol production, K_{pb} , provides the first order rate constant for deacylation (k_3) from the intercept and the second order rate constant of the nonspecific nitrophenol production from the slope.

Titration of factor X_a and PIVKA- X_a . Titration of activated factor X and activated PIVKA-X was performed according to Smith (16).

In a typical titration the sample cuvette contains 150 μ g of factor X_a or 180 μ g of PIVKA- X_a in 300 μ l 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 μ l of a 0.01 M solution of p-NPGB in dimethylformamide-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 of factor X_a and PIVKA- X_a with p-NPGB occurs slowly enough to follow the reaction by continuous recording, therefore ($P_1^* - P_1$) values at various intervals of the presteady state reaction can easily be determined.

Enzyme concentrations were 3.2×10^{-6} M factor X_a and 2.2×10^{-6} M PIVKA- X_a . The NPGB concentrations ranged from 3.3×10^{-5} M to 13.3×10^{-5} M. The operational first order rate constant for the presteady state reaction, b , was determined as described above, at each concentration of p-NPGB by linear regression analysis. $1/k_2$ and K_S/k_2 were determined from the intercept and slope of a plot of $1/b$ versus $1/[p\text{-NPGB}]$.

Determination of k_3 and K_{pb} . Acylation of factor X_a and PIVKA- X_a was performed by addition of 10 μ l of 0.01 M p-NPGB to 1.2 ml of a solution of factor X_a or PIVKA- X_a in 0.1 M sodium barbital buffer pH 8.3. The final concentration of p-NPGB was 8.3×10^{-5} M. The final enzyme concentrations were 3.2×10^{-6} M factor X_a and 2.2×10^{-6} M PIVKA- X_a .

The acylenzyme solutions with excess p-NPGB were applied to a column (0.9 x 30 cm) of Sephadex G-25 equilibrated with 0.1 M 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 S-2222. The time 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

$\ln \frac{[E_0]}{[E_0] - [E_t]}$ versus time, where $[E_0]$ is the concentration

acylenzyme at zero time and $[E_t]$ the concentration deacylated enzyme at time t . Amidase activity is determined as described under Methods.

The rate constant for the postburst p-nitrophenol production is determined at 1.6×10^{-4} M p-NPGB from the slope of the steady state line and enzyme concentration.

Amidase activity of factor X_a and PIVKA- X_a

Introduction. The many properties that factor X_a shares with

α -chymotrypsin permit us to assume that qualitatively the mechanism of amidolysis will be identical in both enzymes. As shown by Gutfreund and Sturtevant (60,61), the chymotrypsin catalyzed amidolysis proceeds according to the following reaction pathway.

When the substrate S is Bz-Phe-Val-Arg-pNA, the first product P_1 is p-nitro-aniline (pNA) and the second product P_2 is Bz-Phe-Val-Arg-COOH. ES is the reversible enzyme-substrate complex and K_s the dissociation constant of this complex. EP_2 is the acyl-enzyme, k_2 and k_3 are the rate constants pertaining to the liberation of P_1 and P_2 , respectively. When $[S_0] \gg [E_0]$, the Michaelis-Menten rate equation holds:

$$v = \frac{V_{\max} [S]}{K_M + [S]}, \text{ where } K_M = K_s \frac{k_3}{k_2 + k_3} \quad \text{and} \quad \frac{V_{\max}}{[E_0]} = \frac{k_2 k_3}{k_2 + k_3}$$

A plot of v (the velocity of the catalyzed hydrolysis of the substrate) versus $[S]$ provides the determination of two steady state parameters: $V_{\max}$ (the velocity of the reaction at complete saturation of enzyme with substrate) and the Michaelis-Menten constant K_M .

In the chymotrypsin catalyzed hydrolysis of amides the acylation rate constant (k_2) $\ll$ deacylation rate constant (k_3) (62,63). When the formation of the acylenzyme is rate limiting, the steady state kinetic parameters become $k_{\text{cat}} = k_2$ and $K_M = K_s$.

Amidase activity assay. 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 PIVKA- X_a were measured in

TABLE I. Titration of factor X_a and PIVKA- 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 (μ M)	
	factor X_a	PIVKA- X_a	factor X_a	PIVKA- X_a
3.3×10^{-5}	5.30×10^{-2}	3.64×10^{-2}	3.03	2.08
6.6×10^{-5}	5.68×10^{-2}	4.06×10^{-2}	3.25	2.32
9.9×10^{-5}	5.53×10^{-2}	3.85×10^{-2}	3.16	2.20
13.2×10^{-5}	5.28×10^{-2}	3.94×10^{-2}	3.02	2.25

TABLE II. Kinetic constants of reaction of factor X_a and PIVKA- X_a with p-NPGB.

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

Tris-imidazole buffer of 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 $\lambda_r = 344$ nm and $\lambda_s = 391$ nm at 37°C. The $\epsilon_{391-344\text{nm}}$ of p-nitroaniline in the buffer solution of pH 8.2 was determined as 11,400 OD/mol.

Activation of prothrombin and prethrombin 1. The conversion of prothrombin and prethrombin 1 by factor X_a and PIVKA- X_a were studied in the liquid phase activation process. The activations were performed in 0.02 M Tris-HCl 0.1 M NaCl buffer, pH 7.5 at 37°C. The final concentrations for the activation components in the activation mixture are given in the figure legends. The functional enzyme concentrations of factor X_a and PIVKA- X_a were determined by active site titration as described under Methods.

At several time intervals aliquots were removed from the activation mixture and added to a microcuvette containing 5.7×10^{-5} M of Bz-Phe-Val-Arg-pNA in a final volume of 350 μ l of 0.025 M Tris-imidazole, 0.1 M NaCl buffer, pH 8.2. The rate of p-nitroaniline formation, proportional to the amount of thrombin activity was measured as described above. Aliquots of the reaction mixture were heated at 100°C for 2 min in 5 mM sodium phosphate buffer pH 7.0 containing 1.5% sodium dodecyl sulfate and 50% glycerol, followed by an incubation during 60 min at 37°C in the presence of β -mercaptoethanol. Sodium dodecylsulfate gel electrophoresis of these prepared aliquots was performed by the method of Weber and Osborn (64). The gels were stained with the method of Fairbanks (65).

RESULTS

Titration of factor X_a and PIVKA- X_a

Functional enzyme concentration. Factor X_a and PIVKA- X_a were

Fig. 2.

Time courses of the reaction between p'-nitrophenyl-p-guanidino-benzoate and factor X_a (open circles) and PIVKA- X_a (solid circles). Procedure is as described under Methods. The actual protein concentration is 3.6×10^{-6} M and 3.1×10^{-6} M for respectively factor X_a and PIVKA- X_a . The p-NPGB concentration is 9.9×10^{-5} M.

Fig. 3.

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

Fig. 4.

Deacylation of guanidinobenzoyl-factor X_a (open circles) and guanidinobenzoyl-PIVKA- X_a (solid circles). Experimental conditions are described under Methods.

titrated with p-NPGB as described under Methods. As shown in fig. 2, the presteady state p-nitrophenol production was complete after about 2 minutes for both factor X_a and PIVKA- X_a and is followed by a very low linear postburst production of p-nitrophenol. From this type of curves π was determined as described under Methods. As can be seen from table I, π does not vary significantly with the NPGB concentration. This fact, and the low postburst production of p-nitrophenol indicate that $S_0 \gg K_M$ and $k_2 \gg k_3$. Therefore $[E_0] \approx \pi$ and the molarity of the enzyme solutions can be calculated as $(3.1 \pm 0.1) \cdot 10^{-5}$ M and $(2.1 \pm 0.1) \cdot 10^{-5}$ M for factor X_a and PIVKA- X_a , respectively.

Determination of kinetic constants. The kinetic constants of the hydrolysis of p-NPGB catalyzed by factor X_a and PIVKA- X_a are summarized in table II. The linear relationship between $1/b$ versus $1/p\text{-NPGB}$ (fig. 3) and $\ln \left[\frac{E_0}{ES} \right]$ versus time (fig. 4) shows that the kinetics of both factor X_a and PIVKA- X_a reacting with p-NPGB are adequately described by the theory as summarized under Methods. The rate constant for the postburst p-nitrophenol production, K_{pb} , determined at a NPGB concentration of 1.6×10^{-4} M was about two times the deacylation constant k_3 for both factor X_a and PIVKA- X_a . However, the ratio k_2/k_3 is very large: therefore the postburst p-nitrophenol production has no serious effect on the accuracy of the factor X_a and PIVKA- X_a titration under the conditions used.

Amidase activity of factor X_a and PIVKA- X_a

Determination of the kinetic constants. The Lineweaver-Burk plots (66) for factor X_a and PIVKA- X_a with Bz-Phe-Val-Arg-pNA and Bz-Ile-Glu-Gly-Arg-pNA are shown in fig. 5A-5D. The kinetic constants are summarized in table III and were determined from slope and intercept by regression analysis of each set of data. The functional enzyme concentrations determined by NPGB titration, instead of the enzyme concentration based on protein concentration, were used for calculation of

Fig. 5.

Lineweaver-Burk plots for factor X_a and PIVKA- X_a using Bz-Phe-Val-Arg-pNA and Bz-Ile-Glu-Gly-Arg-pNA.

- Amidolysis of Bz-Ile-Glu-Gly-Arg-pNA by PIVKA- X_a . Enzyme concentrations: 5.0×10^{-9} M (open circles) and 2.5×10^{-9} M (solid circles).
- Amidolysis of Bz-Ile-Glu-Gly-Arg-pNA by factor X_a . Enzyme concentrations: 4.0×10^{-9} M (open circles) and 2.0×10^{-9} M (solid circles).
- Amidolysis of Bz-Phe-Val-Arg-pNA by PIVKA- X_a . Enzyme concentrations: 9.0×10^{-8} M (open circles) and 6.1×10^{-8} M (solid circles).
- Amidolysis of Bz-Phe-Val-Arg-pNA by factor X_a . Enzyme concentrations: 1.9×10^{-7} M (open circles) and 1.3×10^{-7} M (solid circles).

Experimental conditions as described under Methods.

Fig. 6.

pH optima of factor X_a (—○—) and PIVKA- X_a (—●—) with Bz-Ile-Glu-Gly-Arg-pNA. The pH dependence is determined in Tris-imidazole buffers at varying pH, ionic strength of 0.15 at 37°C. Substrate concentration is 1.2×10^{-4} M, enzyme concentrations are: factor X_a , 1.2×10^{-8} M and PIVKA- X_a , 1.1×10^{-8} M. Amidase activity assay as described under Methods.

Fig. 7.

Effect of Ca^{2+} on the amidase activity of factor X_a and PIVKA- X_a with Bz-Ile-Glu-Gly-Arg-pNA.

- A. Time courses of inactivation of factor X_a , 50 $\mu\text{g/ml}$ (solid circles) and PIVKA- X_a , 65 $\mu\text{g/ml}$ (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 PIVKA- X_a . At intervals aliquots (4 μl) were subsampled from the incubation mixture into a buffer of 0.025 M Tris-imidazole 0.1 M NaCl, pH 8.2 and amidase activity was measured as described under Methods.
- B. Double reciprocal plots of the amidolysis of Bz-Ile-Glu-Gly-Arg-pNA by factor X_a . Factor X_a was preincubated in the presence of Ca^{2+} for 60 minutes (solid circles) and absence of Ca^{2+} (open circles). Experimental conditions as described in the legend to fig. 7A.

Fig. 3.

Effect of factor V_a on the amidase activity of factor X_a and PIVKA- X_a using Bz-Ile-Glu-Gly-Arg-pNA as substrate. Lineweaver-Burk plots were constructed from data obtained by amidolysis of Bz-Ile-Glu-Gly-Arg-pNA by: 1. factor X_a , -○-; 2. idem preincubated for 60 min. in the presence of Ca^{2+} (5 mM), -●-; 3. idem in the presence of factor V_a (10 U/ml), -▲-; 4. idem in the presence of Ca^{2+} (5 mM) and factor V_a (10 U/ml), -▲·-; 5. PIVKA- X_a , -□-; 6. idem in the presence of factor V_a , -■-. Enzyme concentrations were factor X_a : 1.3×10^{-8} M and PIVKA- X_a : 1.0×10^{-8} M. Substrate concentrations ranging from 8.7×10^{-5} M to 2.3×10^{-4} M.

Experimental conditions were the same as described in the legend to fig. 7A.

$$k_{\text{cat}} = V_{\text{max}} / [E_0]$$

An excess, at least 150 fold for Bz-Phe-Val-Arg-pNA and at least 10,000 fold of Bz-Ile-Glu-Gly-Arg-pNA over enzyme concentration was used.

pH dependence. For both factor X_a and PIVKA- X_a the pH optimum was found to be between 8.0 and 8.5 (fig. 6). It also appears from those results that variation of the pH in the range indicated, did not have any influence on the k_{cat} (factor X_a)/ k_{cat} (PIVKA- X_a) ratio. Experimental conditions were as described under fig. 6.

Effect of factor V_a on amidase activity. As shown in fig. 7A, the initial velocity of the hydrolysis of Bz-Ile-Glu-Gly-Arg-pNA catalyzed by factor X_a , decreases during incubation in the presence of Ca^{2+} (5 mM). After about 60 minutes the amidase activity remains unaltered. The amidase activity then found is about one half of the control value. Double reciprocal plots of the hydrolysis of Bz-Ile-Glu-Gly-Arg-pNA by factor X_a , pre-incubated during 90 minutes 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. 7B.

It was found that in the absence of Ca^{2+} the V_{max} value was $2.5 \times 10^{-5} \text{ mole.min}^{-1}$ and in the presence of Ca^{2+} the V_{max} value was $1.2 \times 10^{-5} \text{ mole.min}^{-1}$. The K_M value for factor X_a preincubated with Ca^{2+} was the same as in case of factor X_a preincubated in the absence of Ca^{2+} . Upon incubation of PIVKA- 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_{max} values of the hydrolysis of Bz-Ile-Glu-Gly-Arg-pNA catalyzed by factor X_a and PIVKA- X_a under the conditions as described in the legend to fig. 8. However, no decrease in amidase activity of factor X_a upon incubation with Ca^{2+} (5 mM) was found when factor V_a (1.0 mg/ml) was

Fig. 9.

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

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 PIVKA- X_a hydrolysis of Bz-Ile-Glu-Gly-Arg-pNA ($1/v_0$) versus inhibitor concentration (I) at different substrate concentrations is shown in fig. 9. For both factor X_a and PIVKA- X_a straight lines were obtained. The inhibition by benzamidine was 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-pNA determined from fig. 9 by regression analysis were found to be 2.4×10^{-4} M and 3.0×10^{-4} M for respectively factor X_a and PIVKA- X_a .

The proteolytic action of factor X_a and PIVKA- X_a on prothrombin and prethrombin 1

Time course of activation of prethrombin 1. The time courses of activation of prethrombin 1 by factor X_a and PIVKA- X_a are shown in fig. 10. Similar and linear rates of thrombin formation were obtained. It can be concluded that in the presence of Ca^{2+} , factor X_a and PIVKA- X_a have the same catalytic activity towards prethrombin 1.

Time course of activation of prothrombin. The time courses of activation of prothrombin in the absence of Ca^{2+} by factor X_a and PIVKA- X_a are shown in fig. 11A. It can be seen that identical activation curves were obtained. An interesting feature of these time courses is that two phases can be distinguished, e.g.: an apparent slow rate of thrombin formation, followed by a faster linear reaction rate. The final rate of thrombin formation per mole enzyme, was found to be the same as was obtained with prethrombin 1 in the presence of Ca^{2+} (table IV). The time courses of prothrombin activation under the same conditions, but visual-

TABLE III. Kinetic constants ^[1] for the hydrolysis of synthetic amide substrates by activated factor X_a and activated PIVKA-X.

Substrate	factor X _a		PIVKA-X _a	
	K _M (M)	k _{cat} (min ⁻¹)	K _M (M)	k _{cat} (min ⁻¹)
Bz-Phe-Val-Arg-pNA	(0.9±0.1)10 ⁻⁵	16±1	(1.20±0.1)10 ⁻⁵	55±5
Bz-Ile-Glu-Gly-Arg-pNA	(0.44±0.06)10 ⁻³	(6.6±0.1)10 ³	(0.5±0.06)10 ⁻³	(5.7±0.1)10 ³

[1] Results of duplicate experiments using two different enzyme concentrations.

TABLE IV. Rate of thrombin formation ^[1] on prothrombin and prethrombin 1 activation by factor X_a and PIVKA-X_a. Experimental conditions as described in the legend to figures 10 and 11.

Activator	Substrate	Activation rate/mole enzyme ^[2]
factor X _a	prothrombin	0.85 ± 0.05
PIVKA-X _a	prothrombin	0.84 ± 0.01
factor X _a +Ca ²⁺	prothrombin	1.18 ± 0.03
PIVKA-X _a +Ca ²⁺	prothrombin	1.03 ± 0.04
factor X _a +Ca ²⁺	prethrombin 1	0.93
PIVKA-X _a +Ca ²⁺	prethrombin 1	0.85

[1] Determined at two enzyme concentrations, except in case of prethrombin 1.

[2] Unit thrombin/min/mole, where 1 unit is defined as the amount of thrombin which hydrolysis 1 mole substrate/min under the conditions used.

Fig. 10.

Time courses of the activation of prothrombin 1 by factor X_a and PIVKA- X_a in the presence of Ca^{2+} . Prothrombin 1 (0.5 mg/ml) was incubated with factor X_a , 1.3×10^{-7} M (open circles) and PIVKA- X_a , 1.0×10^{-7} M (solid circles). The incubation mixtures contain 5 mM $CaCl_2$. Thrombin assay and experimental conditions as described under Methods.

Fig. 11.

Time courses of activation of prothrombin by factor X_a and PIVKA- X_a .

A. Comparison of prothrombin activation by factor X_a and PIVKA- X_a . Prothrombin (1.4 mg/ml) was incubated with factor X_a , 7.2×10^{-8} M, $\circ$ - $\circ$ -; and 3.1×10^{-8} M, Δ - Δ -; PIVKA- X_a , 7.2×10^{-8} M, $\bullet$ - $\bullet$ -; and 2.9×10^{-8} M, $\blacktriangle$ - $\blacktriangle$ -.

B. Comparison of activation of prothrombin by factor X_a and PIVKA- X_a in the presence of 5 mM Ca^{2+} . Prothrombin (1.4 mg/ml) was incubated with factor X_a , 7.2×10^{-8} M, $\circ$ - $\circ$ -; and 3.1×10^{-8} M, Δ - Δ -; PIVKA- X_a , 7.2×10^{-8} M, $\bullet$ - $\bullet$ -; and 2.9×10^{-8} M, $\blacktriangle$ - $\blacktriangle$ -.

Thrombin assay and experimental conditions as described under Methods.

ized by sodium dodecyl sulfate gel electrophoresis, as shown in fig. 12A, revealed that prethrombin 1 becomes the substrate after about 30 minutes.

No differences in the evolution of activation products were found between factor X_a and PIVKA- X_a . Moreover, the distribution of the activation products agrees very well with the data reported by Owen et al. (19). That is, prothrombin the largest molecular weight component (mol.wt. around 74,000) at the initial stage of the reaction is rapidly converted into prethrombin 1 (mol.wt. around 48,000) and fragment 1 (mol. wt. around 24,000). Pre-thrombin 1 is converted into prethrombin 2 (mol. wt. around 38,000) by splitting off fragment 2 (mol. wt. around 13,000), which is, however, not adequately stained and barely visible. Finally pre-thrombin 2 is converted into thrombin (mol.wt. around 38,000), visible in the electrophoresis as the B-chain (mol.wt. around 35,000). The time courses of activation of prothrombin in the presence of Ca^{2+} is shown in fig. 11B. A comparison with the time courses in the absence of Ca^{2+} revealed that the lag-time in thrombin formation is obviously shortened by Ca^{2+} in case of factor X_a and to a much lesser extent for PIVKA- X_a . A slight increase of the final linear rate of thrombin formation is observed, 1.5 and 1.2 times respectively for factor X_a and PIVKA- X_a (table IV).

The time courses of prothrombin activation in the presence of Ca^{2+} were also monitored by sodium dodecyl sulfate gel electrophoresis (fig. 12B). The activation product fragment 1.2 (mol.wt. around 36,000), not detectable in the time course in the absence of Ca^{2+} , appears just above prethrombin 2. As yet known, in all probability only the prothrombin activation pathway by factor X_a , defined by the cleavage order 2-3 (see fig. 1) occurs. However, when thrombin proteolysis was allowed to occur (fig. 12 A-B) the cleavage order seems to be 1-2-3, in addition to the pathway 2-3.

In order to eliminate the conversion of prothrombin and fragment 1.2 by the proteolytic action of thrombin, activation of prothrombin was performed in the presence of diisopropylphosphofluoridate (DFP). The time course of activation, as visualized by sodium dodecyl sulfate gel electrophoresis is shown in fig. 12C. DFP inhibits factor X_a much less effectively than it inhibits thrombin. It was found that under the conditions used (1 mM DFP) no thrombin activity developed. The amidase activity of factor X_a and PIVKA- X_a dropped to 85% in the course of one hour. Examination of the gels (fig. 12C) revealed that when thrombin activity is inhibited, prethrombin 2 is directly formed from prothrombin upon activation with factor X_a and PIVKA- X_a . The absence of any stained material in the fragment 1 region obviously confirmed this. Unlike than the time courses shown in fig. 11B, no visible differences can be observed in the time courses as monitored by sodium dodecyl sulfate gel electrophoresis (fig. 12C).

A comparison of the effectiveness of phospholipids on the prothrombin activation by factor X_a or decarboxy factor X_a in the presence of Ca^{2+} is shown in fig. 13. It is clearly demonstrated that only in case of factor X_a , phospholipids markedly increase the rate of activation of prothrombin.

In view of the observations that Ca^{2+} mediates the binding of factor X to phospholipids (40) and the role of γ -carboxyglutamic acid in the Ca^{2+} binding properties of the protein, it is reasonable to conclude that the phospholipids are ineffective in the activation of prothrombin by decarboxy factor X_a because of the absence of γ -carboxyglutamic acids in the latter.

1 2 3 4 5 6 7 8 9 1 2 3 4 5 6 7 8 9

1 2 3 4 5 6 7 8 9 1 2 3 4 5 6 7 8 9

Abbreviations:

P	prothrombin
P-1	prethrombin 1
P-2	prethrombin 2
T	thrombin
F-1	fragment 1
F-2	fragment 2
F 1.2	fragment 1.2

B

Fig. 12.

Time course of prothrombin activation by factor X_a and PIVKA- X_a . At several time intervals aliquots were removed from the activation mixture and analyzed by sodium dodecyl sulfate gel electrophoresis after disulfide reduction. Detailed conditions are given under Methods.

- A. Left: prothrombin (1.3 mg/ml) was incubated with 6.0×10^{-8} M factor X_a , 1: 0 min., 2: 30 min., 3: 60 min., 4: 90 min., 5: 120 min., 6: 150 min., 7: 195 min., 8: 255 min., 9: 305 min.
 Right: prothrombin (1.3 mg/ml) was incubated with 5.9×10^{-8} M PIVKA- X_a , samples were removed from the activation mixture at the times given for factor X_a .
- B. Left: prothrombin (1.3 mg/ml) was incubated with 7.0×10^{-6} M factor X_a in the presence of 10 mM Ca^{2+} ; 1: 0 min., 2: 30 sec., 3: 2 min., 4: 5 min., 5: 10 min., 6: 20 min., 7: 30 min., 8: 40 min., 9: 60 min., 10: 90 min., 11: 120 min., 12: 240 min.
 Right: prothrombin (1.3 mg/ml) was incubated with 7.0×10^{-6} M PIVKA- X_a in the presence of 10 mM Ca^{2+} . The samples were removed from activation mixture at times given for factor X_a .
- C. Left: prothrombin (1.3 mg/ml) was incubated with 6.2×10^{-8} M factor X_a in the presence of 10 mM Ca^{2+} and 1 mM DFP. 1: 0 min., 2: 5 min., 3: 12 min., 4: 30 min., 5: 60 min., 6: 105 min., 7: 168 min., 8: 175 min., 9: 255 min.
 Right: prothrombin (1.3 mg/ml) was incubated with 6.0×10^{-8} M PIVKA- X_a in the presence of 10 mM Ca^{2+} and 1 mM DFP, samples were removed from activation mixture at times as given for factor X_a .

Fig. 13.

A comparison of the effectiveness of phospholipids in the prothrombin activation.

Prothrombin (0.4 mg/ml) was activated in the presence of 10 mM Ca^{2+} with:

- PIVIA-X_a, 3.6 g/ml
- ▲-PIVIA-X_a, 3.6 g/ml; phospholipids, 15 g/ml
- factor X_a, 3.6 g/ml
- △-factor X_a, 3.6 g/ml; phospholipids, 15 g/ml

All other conditions are as described in the experiments of Fig. 11. Thrombin was assayed by its ability to clot fibrinogen. Clotting times were converted to thrombin activity by comparison with a reference curve, prepared from a standard thrombin solution. Phospholipids were prepared according to Bell from bovine brain (ref. 57, chapter IV).

DISCUSSION

Titani (68) 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, 69). Recently, Hageman (70) found evidence that the A-chain of thrombin does not play a significant role in determining the catalytic specificity of thrombin. 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 amino-terminal 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 (18,72,72).

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 (PIVKA- X_a). This is immediately linked to the question of the role of Ca^{2+} , since the binding of these ions to the γ -carboxyglutamate residues appears to be 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 (40). Ca^{2+} does also play a role in other liquid-phase proteolytic reactions though. It is thus conceivable that it has more than one function in the catalytic reactions of factor X_a . This is more feasible as the binding of Ca^{2+} to factor X brings about allosteric conformational changes (chapter III).

The hydrolysis of p-NPGB, catalyzed by factor X_a or PIVKA- X_a , showed roughly the same kinetic parameters for both enzymes (table II). We concluded from these results that all functional species of the active site of PIVKA- X_a are comparable with that of factor X_a . The kinetic parameters found were in a good agreement with these reported for factor X_a by Smith (16).

Our investigations on the catalytic efficiency of factor X_a and PIVKA- X_a were extended with two more substrates: Bz-Phe-Val-Arg-pNA and Bz-Ile-Glu-Gly-Arg-pNA. The K_M and k_{cat} values of the activity of factor X_a and PIVKA- X_a towards Bz-Phe-Val-Arg-pNA and Bz-Ile-Glu-Gly-Arg-pNA were determined (table III). Our results suggest that both enzymes have an identical affinity towards Bz-Phe-Val-Arg-pNA as measured by K_M . The K_M values found

were of the same order of magnitude as was reported for trypsin, 6.7×10^{-5} M (72) and thrombin 4.8×10^{-5} M (25). Also the affinity of factor X_a and PIVKA- X_a for Bz-Ile-Glu-Gly-Arg-pNA were found to be equal. K_M values respectively 4.4×10^{-4} M and 5.0×10^{-4} M and agrees very well with the data supplied by Kabi Diagnostica, Sweden (3.10^{-4} M).

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

This discrepancy found between factor X_a and PIVKA- X_a is due to the less catalytic efficiency of factor X_a towards Bz-Phe-Val-Arg-pNA in comparison to PIVKA- X_a (k_{cat} values respectively 16 min^{-1} and 55 min^{-1}). This was not due to the presence of thrombin in the PIVKA- X_a preparations.

A comparison of the inhibition constants of factor X_a and PIVKA- X_a for benzamidine, using Bz-Ile-Glu-Gly-Arg-pNA as the substrate, shows that both enzymes have an identical affinity for the inhibitor and that inhibition was competitive (fig. 9).

Recently, Suomela (73) reported that factor X_a had a considerable susceptibility towards the substrate Bz-Ile-Glu-Gly-Arg-pNA. Among the other coagulation factors only thrombin appeared to hydrolyse this substrate at a low rate. Therefore, it may be concluded that both enzymes have the same catalytic activity towards small synthetic substrates and the same affinity to amidine and guanidine inhibitors. Without Ca^{2+} γ -carboxyglutamate residues do not seem to influence the catalytic activity with those substrates. Ca^{2+} was found to be a non-competitive inhibitor of the amidase activity of factor X_a towards Bz-Ile-Glu-Gly-Arg-pNA. The time

course of inactivation suggests that it finally reaches an equilibrium. It was already shown by Jesty (74) that Ca^{2+} mediates a concentration dependent association of factor X_a , resulting in the precipitation of factor X_a aggregates. Prendergast (75) reported a concentration dependent association of prothrombin fragment 1 by Ca^{2+} . Since it has been shown that Ca^{2+} has no effect upon the amidase activity of PIVKA- X_a , the results suggest that the γ -carboxy-glutamate residues are involved in the Ca^{2+} mediated association of factor X_a .

Of particular interest was the observation that factor V_a protects the association of factor X_a (fig. 8). The finding that also bovine serum albumin protects factor X_a for inactivation, may suggest that a non-specific protein-protein interaction prevents factor X_a for inhibition. 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 (TAME) caused by factor V as reported by Coleman (76) may be due to the presence of Ca^{2+} . No evidence from prothrombin activation experiments has been obtained that peptide bonds in prothrombin are more susceptible to factor X_a when bound to factor V_a .

To investigate the activity of factor X_a and PIVKA- X_a towards prothrombin is more complicated than in case of small synthetic substrates for two reasons:

1. the activation of prothrombin occurs via a multiple step mechanism;
2. the high susceptibility of prothrombin for proteolysis by its own activation product, thrombin.

In the absence of Ca^{2+} the activation of prothrombin by factor X_a and PIVKA- X_a is similar (fig. 11A). It can be assumed that both enzymes have an identical activity towards the substrate. The finding also indicates that Ca^{2+} is not absolutely necessary in this reaction.

Of particular interest is the demonstration of a lag-phase in the activation, that hitherto, as far as we know, has passed unnoticed.

This phenomenon merits further investigations, because this lag-phase is shortened by Ca^{2+} and more so when factor X_a is the enzyme than when PIVKA- X_a is the enzyme (fig. 11B).

From our experimental results, we are unable to give a straight forward explanation for the origin of the lag-phase. Nevertheless, they provide a few indications. It is known that prothrombin 2 is converted by factor X_a in thrombin approximately four times faster than prothrombin (51). However, it is observed that prethrombin 2 accumulates during the time course of activation (fig. 12C). This fact can be accounted for the competition of prothrombin and prethrombin 2 for factor X_a . At the initial stage of the reaction, prothrombin is far in excess over prethrombin 2. It is likely that the lag-phase can be explained in terms of a competitive effect between prothrombin and prethrombin 2. This is consistent with the following findings:

1. activation of prethrombin 1 by factor X_a or PIVKA- X_a does not show any lag-phase (fig. 10);
2. the rate of thrombin formation becomes linear after all prothrombin is converted into prethrombin 1 (fig. 11A and 12 A).

From these experiments, an other important conclusion can be drawn. As shown in table IV, the final rate of thrombin formation from prothrombin by factor X_a or PIVKA- X_a in the absence of Ca^{2+} (actually the prethrombin 1 activation) is similar to the rate of thrombin formation from prethrombin 1 in the presence of Ca^{2+} . This indicates that the interaction between γ -carboxyglutamate residues of factor X_a with Ca^{2+} does not influence the factor X_a activity. The question arises then, by what mechanism Ca^{2+} effects the lag-phase. It is tempting to speculate about an interaction via Ca^{2+} between factor X_a and the Ca^{2+} binding γ -carboxyglutamate residues in prothrombin. It could explain at least the differences between prethrombin 1 and prothrombin activation.

Prethrombin 2 is assumed to be non-covalently associated with Ca^{2+} binding fragment 1.2 (53). Therefore, Ca^{2+} may also effect the conversion of prethrombin 2 by factor X_a .

Jackson et al. (53) have shown that only the fragment 2 part of fragment 1.2 is able to associate with prethrombin 2. Therefore, when thrombin proteolysis exceeds the factor X_a proteolysis, the Ca^{2+} binding properties of the prethrombin 2-fragment 1.2 complex have been lost. The identical rates of thrombin formation determined after the lag-phase in all time courses may therefore be explained by the proteolytic action of thrombin. The appearance of this proteolytic action of thrombin is clearly demonstrated in fig. 11B. During the time course the protein bands in the prethrombin 1 fragment 1 and fragment 2 regions increase in density. It is also shown that fragment 1.2 is a transient component. Unfortunately, the results obtained in the time course, as was determined by the thrombin activity assay (fig. 11B) cannot be related to the results shown in fig. 12B, because of the crudeness of the technique used in the latter experiment. However, two important features can be mentioned:

1. at the early stage of the reaction, when factor X_a activity was expected to exceed the thrombin activity, Ca^{2+} enhances the activation of prothrombin;
2. at the end of the time course, in the reverse situation, the rate of thrombin formation is nearly the same in the presence as in the absence of Ca^{2+} (table IV).

In conclusion can be said that

1. Interaction between Ca^{2+} and γ -carboxyglutamate residues has no influence in the active centre of factor X_a per se;
2. In the interaction of prothrombin and/or prethrombin 2-fragment 1.2 complex with factor X_a , γ -carboxyglutamate residues likely play a role in a Ca^{2+} mediated complex formation.

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Summary

A vitamin K deficiency results in a decrease in the concentration of the four blood coagulation factors II, VII, IX, and X. The possibility to affect the concentration of blood coagulation factors is used in oral anticoagulation therapy in order to prevent and combat arterial and venous thrombosis. Parallel with the disappearance of blood coagulation activity, precursors of these factors appear in the blood circulation. These precursors differ from the normal factors by lacking γ -carboxyglutamic acids, essential for their proper function. These precursors are called decarboxy coagulation factors or Proteins Induced by Vitamin K Absence (PIVKAs).

This thesis compared the enzymatic properties of factor X and decarboxy factor X, isolated from bovine plasma. This offers the opportunity to study the role of γ -carboxyglutamic acids in the mechanism of blood coagulation.

Chapter I is a review of the investigations that have lead to the elucidation of the function of vitamin K in the biosynthesis of the blood coagulation factors.

In chapter II we describe the induction, purification and characterization of decarboxy factor X. A homogeneous preparation, as judged by different gel electrophoretic techniques, was obtained by a purification procedure slightly different from that used for the purification of factor X.

According to our investigations no other differences are found between factor X and decarboxy factor X than the presence of about 12 γ -carboxyglutamyl residues in the former, where unmodified glutamyl residues are found in decarboxy factor X. In contrast to factor X, decarboxy factor X has no blood coagulation activity and a very low affinity for Ca^{2+} .

Studies on the interaction between Ca^{2+} and factor X and decarboxy factor X by a flow rate dialysis technique and ultraviolet difference spectroscopy are described in chapter III. It is shown that 3-4 moles Ca^{2+} bind per mole factor X in a positive cooperative manner. A total of 20 Ca^{2+} binding sites was determined. The decarboxy factor X has a very low affinity for Ca^{2+} without any sign of cooperativity.

Changes in the ultraviolet spectrum caused by Ca^{2+} were demonstrated in case of factor X but not with decarboxy factor X. These findings suggest a conformational change upon interaction of Ca^{2+} with γ -carboxyglutamic acids in factor X.

The role of γ -carboxyglutamic acid in the activation of factor X by a protease from Russell's Vipervenom was investigated as described in chapter IV. Similar experiments with decarboxy factor X makes it clear that the specific interaction between Ca^{2+} and γ -carboxyglutamic acid is not an absolute requirement for the conversion of the zymogen. However, the large enhancement of the rate of activation of factor X by Ca^{2+} compared with decarboxy

factor X points to the importance of the Ca^{2+} binding sites in factor X, i.e. the γ -carboxy glutamic acids. Also the sigmoidal relationship between Ca^{2+} concentration and rate of activation agrees with the Ca^{2+} binding properties of factor X.

The only difference between activated decarboxy factor X and normal activated factor X is the presence of γ -carboxy glutamic acids in the latter which very likely causes the inability of decarboxy factor X_a to participate in blood coagulation. As a consequence, decarboxy factor X_a has no blood coagulation activity. Furthermore, it is shown that the activity of decarboxy factor X_a towards small synthetic substrates is similar to that of factor X_a .

The enzymatic properties of factor X_a and decarboxy factor X_a are reported in chapter V. Kinetic experiments on both enzymes with different synthetic substrates revealed similar kinetic parameters. In addition both enzymes also have the same catalytic efficiency towards prothrombin in the absence of Ca^{2+} . These findings indicate that there are no differences in the active center of normal factor X_a and decarboxy factor X_a .

In contrast to factor X_a , activation of prothrombin by decarboxy factor X_a is not stimulated by phospholipids and Ca^{2+} . This finding confirms the proposed role of the γ -carboxyglutamic acids in the Ca^{2+} mediated binding of prothrombin to phospholipids.

However, we also observed that Ca^{2+} has a marked influence on the reaction rate of the activation of factor X by RVV-X and the activation of prothrombin by factor X_a , but not in the analogous situation with decarboxy factor X_a . This strongly suggests that Ca^{2+} , apart from its well documented role in the binding of vitamin K dependent clotting factors to phospholipids, also functions by inducing conformational changes via its binding to γ -carboxyglutamic acids.

Samenvatting

Bij een vitamine K deficiëntie is de lever niet in staat de vier stollingsfactoren II, VII, IX, en X voldoende aan te maken. Hiervan wordt gebruik gemaakt bij de zgn. orale antistollingstherapie ter bestrijding en preventie van arteriële en veneuze thrombose. Niet alleen neemt de concentratie van de stollingsfactoren af, ook komen voorstadia van deze factoren in het bloed. Deze onderscheiden zich van de normale factoren doordat zij niet de γ -carboxyglutaminezuren bevatten die voor een normale functie noodzakelijk zijn. Zij worden decarboxy stolfactoren genoemd of ook Proteins Induced by Vitamin K Absence or Antagonists (PIVKAs).

Dit proefschrift vergelijkt de enzymatische eigenschappen van factor X en van decarboxy factor X, geïsoleerd uit runderplasma. Dit maakt het mogelijk de rol van de γ -carboxyglutaminezuren in het stollingsproces nader te omschrijven en een nader inzicht te krijgen in het bloedstollingsdefekt dat wordt geïnduceerd door orale antistollingstherapie.

Hoofdstuk I geeft een literatuuroverzicht van het onderzoek dat uiteindelijk geleid heeft tot de opheldering van de functie van vitamine K in de biosynthese van stollingsfactoren.

In het tweede hoofdstuk wordt het induceren, isoleren en karakteriseren van het decarboxy factor X beschreven. Een homogeen preparaat, zoals aangetoond met verschillende gel elektroforetische technieken, werd verkregen d.m.v. een zuiveringsmethode, welke op enkele punten afwijkt van die welke gebruikt wordt voor de zuivering van factor X.

Voor zover wij hebben kunnen nagaan bestaan er geen structurele verschillen tussen de normale factor X en het decarboxy factor X, anders dan een twaalfal γ -carboxyglutaminezuren in de eerst genoemde, waar in decarboxy factor X glutaminezuren worden gevonden. In tegenstelling tot factor X heeft het decarboxy factor X geen bloedstollings-activiteit en geen affiniteit voor Ca^{2+} ionen. Ook remt het de bloedstolling niet, zoals dat voor menselijk decarboxy factor X wel wordt beschreven.

Onderzoek naar de interactie tussen Ca^{2+} en factor X en decarboxy factor X d.m.v. de "flow rate dialysis" techniek en ultraviolet spectroscopie wordt beschreven in hoofdstuk III. De Ca^{2+} bindings experimenten met factor X laten zien dat 3-4 Ca^{2+} ionen per mole factor X positief cooperatief binden. Een totaal aantal van 20 Ca^{2+} bindingsplaatsen werd gevonden. Het decarboxy factor X daarentegen heeft een lage affiniteit voor Ca^{2+} zonder enig teken van cooperativiteit.

Een verandering in het ultraviolet spectrum onder invloed van Ca^{2+} wordt gevonden met factor X en niet met decarboxy factor X. Dit doet vermoeden dat de interactie tussen γ -carboxyglutaminezuren in factor X met Ca^{2+} een conformatieverandering teweeg brengt.

De rol van γ -carboxyglutaminezuren in de aktivatie van faktor X door een protease uit het gif van de *Vipera Russellii* werd onderzocht zoals beschreven in hoofdstuk IV. Analoge experimenten met decarboxy faktor X maken duidelijk dat de specifieke interactie tussen Ca^{2+} en γ -carboxyglutaminezuren geen strikte voorwaarde is voor de omzetting van het zymogen in enzym. De snelheid van de aktivatie is echter sterk afhankelijk van de mogelijkheid van het zymogen om Ca^{2+} ionen te binden. Anders gezegd, de interactie tussen Ca^{2+} en γ -carboxyglutaminezuren in faktor X verhoogt de reaktiesnelheid van de enzymvorming. Het sigmoïdale karakter van de relatie tussen Ca^{2+} konzentratie en reaktiesnelheid is in overeenstemming met de gevonden Ca^{2+} bindings eigenschappen van faktor X.

Het geaktiveerde decarboxy faktor X (decarboxy faktor X_a) is struktureel identiek met het geaktiveerde faktor X (faktor X_a), met dit verschil dat in de laatst genoemde een tiental γ -carboxyglutaminezuren gevonden worden waar in decarboxy faktor X_a glutaminezuren voorkomen. Het gevolg hiervan is dat het decarboxy faktor X_a geen bloedstollingsaktiviteit heeft. Aanwijzingen werden gevonden dat de aktiviteit van decarboxy faktor X_a waar het kleine synthetische substraten betreft, identiek is aan die van faktor X_a .

In hoofdstuk V wordt hier nader op ingegaan. Kinetische experimenten van faktor X_a en decarboxy faktor X_a met verschillende synthetische substraten resulteerden in gelijke kinetische parameters. Ook werd een identieke katalytische efficiëntie van beide enzymen t.a.v. het natuurlijke substraat van faktor X_a , i.c. prothrombine, waargenomen. Dit alles wijst er op dat er geen funktionele verschillen bestaan tussen het aktieve centrum van normaal faktor X_a en de decarboxy vorm. Ca^{2+} ionen hebben geen invloed op het aktivatiemechanisme van prothrombine door faktor X_a in oplossing, noch op het funktioneren van het aktieve centrum *per se*.

Er werden evenwel aanwijzingen verkregen dat Ca^{2+} ionen de omzetting van prothrombine door faktor X_a versnellen. Het is waarschijnlijk dat Ca^{2+} een rol speelt bij de vorming van het enzym-substraat kompleks. De specifieke Ca^{2+} bindingseigenschappen van de γ -carboxyglutaminezuren in zowel het substraat als het enzym hebben hierin een zekere funktie.

In tegenstelling tot faktor X_a wordt de aktivatie van prothrombine door decarboxy faktor X_a niet gestimuleerd door fosfolipiden en Ca^{2+} . Hiermee bevestigen we de rol van γ -carboxyglutaminezuren in de Ca^{2+} -afhankelijke binding van vitamine K afhankelijke stolfactoren aan fosfolipiden.

We namen bovendien waar dat Ca^{2+} een sterke invloed heeft op de snelheid van aktivatie van faktor X door RVV-X en de aktivatie van prothrombine door faktor X_a . Dit geeft aan dat naast de bekende rol van Ca^{2+} in de fosfolipide binding, Ca^{2+} ook een funktie heeft in het induceren van een conformatie verandering via hun binding met γ -carboxyglutaminezuren.

Dankwoord

Bij de voltooiing van dit proefschrift wil ik graag mijn dank betuigen aan allen die aan de totstandkoming een daadwerkelijke, danwel morele steun hebben gegeven. In het bijzonder wil ik Bernadette Kop danken voor haar waardevolle bijdrage aan dit onderzoek.

Prof.Dr.H.C.Hemker ben ik zeer erkentelijk voor zijn stimulerende invloed en de geboden faciliteiten, welke van wezenlijk belang zijn geweest.

Dr.P.P.M.Reekers, beste Paul, de wijze waarop jij mij ingewijd hebt in de bloedstolling heb ik erg gewaardeerd.

Dr.M.P.Esnouf en Prof.Dr.G.H. de Haas wil ik danken voor hun bereidheid om als co-referenten op te treden.

Jan Rosing en George Willems dank ik voor de stimulerende diskussies. De prettige samenwerking met de leden van de capaciteitgroep Biochemie waardeer ik erg.

Verder gaat mijn dank uit naar:

Jos Kop voor de aminozuur analyses;

Helene Vermeer voor het typen van het manuscript;

Piet Laterveer, Ruud Krüger, Kees Verlaan en K.Limpens voor hun onmisbare bijdrage aan de dierexperimenten.

Ook de niet genoemde medewerkers van het Biomedisch Centrum wil ik graag bedanken.

Curriculum vitae

Zoals gebruikelijk in de faculteit der geneeskunde volgt hier een kort overzicht van mijn academische studie.

Na het behalen van het eind-examen H.B.S.-B aan de Rijks-h.b.s. te Bergen op Zoom in 1963, begon ik in 1965 met de scheikunde-studie aan de Rijksuniversiteit in Utrecht. Het kandidaats-examen, letter S_2 , werd afgelegd in januari 1969. Het doctoraal-examen scheikunde, met als hoofdvak biochemie (Prof.Dr.L.L.M. van Deenen) en als bijvak klinische chemie (Dr.S.K.Wadman) werd behaald in juni 1971.

Van 1972 tot juli 1975 was ik als wetenschappelijk medewerker verbonden aan het laboratorium voor Cardiobiochemie en Bloedstollingsbiochemie van het Academisch Ziekenhuis in Leiden, onder leiding van Prof.Dr.H.C.Hemker.

Sinds juli 1975 ben ik als wetenschappelijk medewerker verbonden aan de Rijksuniversiteit Limburg.