Biochem. J. (1974) **143**, 29–37
Printed in Great Britain

The Localization of a Vitamin K-Induced Modification in an *N*-Terminal Fragment of Human Prothrombin

By TORE SKOTLAND, TURID HOLM, BJARNE ØSTERUD, RAGNAR FLENGSRUD and HANS PRYDZ
Institute of Medical Biology, University of Tromsø, Tromsø, Norway

(*Received* 21 *February* 1974)

1. The *N*-terminal fragment (PF-I) split off from prothrombin during coagulation was purified to homogeneity from human serum. 2. The apparent molecular weight is 27000±2000 in sodium dodecyl sulphate–polyacrylamide-gel electrophoresis, whereas a value of about 19600 is obtained by calculation based on amino acid and carbohydrate analyses. The *N*-terminal sequence is an Ala-Asx bond. The fragment contains about 16% carbohydrate, binds phospholipids in the presence of Ca^{2+} and is adsorbed to $BaSO_4$. The pK_a of its $BaSO_4$-binding group(s) is 3.1–3.5. 3. By CNBr cleavage of fragment PF-I two peptides (C-1 and C-2) were obtained with molecular weights of about 5900 (C-2) and 12400 (C-1) on the basis of amino acid and carbohydrate analyses. Only the smaller (*N*-terminal) peptide is adsorbed to $BaSO_4$ and, since the ability of the whole protein to bind to $BaSO_4$ is known to be absent in samples obtained from patients treated with vitamin K antagonists, this peptide probably contains the site of a modification to the structure of the protein which occurs during biosynthesis and depends on vitamin K. This peptide does not contain hexosamine or sialic acid.

The normal coagulation factors II, VII, IX and X require vitamin K for their biosynthesis. In the absence of vitamin K, inactive factor molecules are formed (Nilehn & Ganrot, 1968; Josso *et al.*, 1968; Prydz, 1970; Prydz & Gladhaug, 1971; Larrieu & Mayer, 1970) which, though being very similar to the active factors immunologically and with regard to amino acid and carbohydrate composition, differ from them in being (1) unadsorbable to $BaSO_4$ up to 70mg/ml, (2) unable to bind the same amount of Ca^{2+} and (3) slightly less negatively charged. These inactive factors have been called proteins induced by vitamin K absence or antagonists, or PIVKAs (Hemker *et al.*, 1968).

The Ca^{2+}-dependent binding of prothrombin to phospholipids is a necessary step in the normal coagulation process (Barton & Hanahan, 1969). The activation of bovine prothrombin to thrombin proceeds through a sequence of proteolytic reactions (Mann *et al.*, 1971*a*,*b*; Stenn & Blout, 1972; Heldebrant *et al.*, 1973*a*,*b*; Fass & Mann, 1973). A fragment of molecular weight about 24500–27000 (Stenn & Blout, 1972; Heldebrant *et al.*, 1973*b*; Stenflo, 1973; Benson *et al.*, 1973) is released from the *N*-terminal end of prothrombin, and the rest of the prothrombin molecule gives rise to thrombin after further proteolysis. This fragment binds to phospholipids in the presence of Ca^{2+} (Gitel *et al.*, 1973).

Several groups have isolated such a fragment from bovine prothrombin after activation by thrombin or factor Xa and studied the properties of the purified fragment (Stenflo, 1973; Benson *et al.*, 1973; Morita *et al.*, 1973; Heldebrant *et al.*, 1973*a*,*b*).

We report data on a similar fragment (prothrombin fragment I; fragment PF-I) purified to homogeneity from human serum and demonstrate that the groups responsible for the characteristic vitamin K-induced adsorbability to $BaSO_4$ are located near to the *N*-terminal end on one of the two peptides obtained from fragment PF-I by CNBr cleavage.

Materials and Methods

Human serum

Blood, drawn from healthy fasting donors, was immediately shaken with 80 glass beads/450ml of blood for 5min to activate coagulation and then left to clot for 5h at 20–22°C. Serum was removed by suction after centrifugation for 30min at 1100*g* at 2°C and stored at −22°C.

Coagulation tests

Reagents and procedures for testing factors II, VII, X and Xa have been described (Gladhaug & Prydz, 1970; Prydz & Gladhaug, 1970). The thrombin–fibrinogen system had a final concentration of 0.5mg of fibrinogen/ml and 0.6 N.I.H. unit of thrombin/ml. Cephalin was prepared from human brain (Hjort *et al.*, 1955) and diluted to about 1mg/ml in veronal buffered saline. Veronal buffered saline consisted of

0.9 vol. of 0.15M-NaCl and 0.1 vol. of a buffer made by mixing sodium diethylbarbiturate (5.875g), NaCl (7.335g), 0.1M-HCl to pH7.3 and distilled water to 1000ml. Factor Xa was purified as described by Gladhaug Berre *et al.* (1973).

Immunological methods

Immunodiffusion (Prydz, 1963), immunoelectrophoresis (Scheidegger, 1955) and immunoprecipitations and immunoabsorptions (Prydz & Gladhaug, 1971) were carried out by standard methods. Antisera were raised in rabbits by immunization with purified fragment PF-I at monthly intervals. The purification procedure is described in the Results section. On each occasion 200–350μg of protein with Freund's complete or incomplete adjuvant was given subcutaneously at multiple sites. Blood was drawn 8 and 9 days after the third and subsequent injections. The immunoglobulin G fraction was isolated by precipitation with $(NH_4)_2SO_4$ [50% (w/v) saturation] and purified by DEAE-Sephadex chromatography or batch absorption by using gels equilibrated to 0.05M-Tris–HCl buffer, pH7.0.

N-Terminal and C-terminal amino acids

These were determined by the dansyl method (Deyl & Rosmus, 1965; Gros & Labouesse, 1969; Woods & Wang, 1967) and by digestion with carboxypeptidase B (Sigma Chemical Co., St. Louis, Mo., U.S.A.) and C (Röhm, Darmstadt, Germany), as described by Ambler (1972) and Tschesche & Kupfer (1972) respectively. The protein was oxidized with performic acid (Hirs, 1967) before carboxypeptidase digestion. The liberated amino acids were determined in a Jeol JLC-6AH amino acid analyser. The manual dansyl–Edman method (Bruton & Hartley, 1970) was used to determine the second amino acid from the *N*-terminal end.

Reduction and alkylation

Fragment PF-I (before or after CNBr cleavage) at a concentration of 2mg/ml in 8M-urea–0.002M-EDTA–0.1M-Tris–HCl, pH8.8, was reduced with a 50-fold excess of dithiothreitol at 50°C for 4h under N_2 (Königsberg, 1972) and alkylated with a 50% excess of iodoacetamide at 20–22°C for 2h (Parr *et al.*, 1972). When carried out before CNBr cleavage, the preparations were dialysed against distilled water and freeze-dried.

CNBr cleavage

Fragment PF-I (before or after reduction and alkylation) was dissolved in 70% (v/v) formic acid and treated with a 200–300-fold molar excess of CNBr for 24h at 20–22°C in the dark. The preparations were dried in a vacuum oven and then submitted to reduction and alkylation. When the fragment PF-I had been reduced and alkylated before CNBr cleavage, the fragments were separated by filtration through Sephadex G-50 (gel bed 2cm×40cm) in 0.1M-acetic acid.

Ca^{2+} binding

The prothrombin fragment was dialysed against 1 litre of 5mM-EDTA–0.5M-Tris–HCl, pH7.0, and then against four changes (each 1 litre) of 0.05M-Tris–HCl buffer, pH8.0. The binding of ^{45}Ca was studied by Amicon filtration (Nelsestuen & Suttie, 1972) by using 50–100μg of fragment PF-I, $CaCl_2$ and $^{45}CaCl_2$ (sp. radioactivity 0.88mCi/mmol; IFA, Kjeller, Norway).

Gel electrophoresis

Analytical polyacrylamide-gel electrophoresis was carried out as described by Ornstein (1964) and Davis (1964) by using 2–35μg of fragment PF-I in 0.1–0.2ml of the upper electrophoresis buffer. Sodium dodecyl sulphate–polyacrylamide-gel electrophoresis was carried out by the method of Weber & Osborn (1969) or Neville (1971) by using 5–160μg of fragment PF-I in 0.1–0.2ml of the respective upper electrophoresis buffers. The gels were stained as described by Bjørklid *et al.* (1973). Standard proteins for the determination of molecular weight (with the values of the molecular weights in parentheses) were bovine serum albumin (68000), γ-globulin H chain (51500), ovalbumin (45000) and γ-globulin L chain (23500) (all from Sigma Chemical Co.). The proteins were dissolved in the respective upper electrophoresis buffers containing 0.1% sodium dodecyl sulphate and reduced by boiling for 3min with 10% (v/v) 2-mercaptoethanol (Fluka, A. G., Chemische Fabrik, Buchs, Switzerland). Preparative polyacrylamide-gel electrophoresis (Buchler Instruments, Fort Lee, N.J., U.S.A.) was carried out as described by the manufacturers by using 7.5 or 10% (w/v) separating gels. Electrofocusing in polyacrylamide gel was done as described by Catsimpoolas (1968).

Amino acid analysis

Samples (1–2nmol) of purified fragment PF-I or its isolated CNBr-cleaved fragments were submitted to hydrolysis in redistilled 6M-HCl at 110°C for 18, 24, 48 and 72h under N_2, dried *in vacuo*, washed and dried once more and analysed in a Jeol JLC-6AH amino acid analyser. Norleucine (Sigma Chemical Co.) and *S*-β-(4-pyridylethyl)-DL-penicillamide (Pierce Chemical Co., Rockford, Ill., U.S.A.) were used as internal standards (Friedman *et al.*, 1973). Trypto-

phan was determined after hydrolysis in 4M-methanesulphonic acid containing 0.2% 3-(2-aminoethyl) indole (Pierce Chemical Co.) (Moore, 1972). The recoveries for the various amino acids were determined by assuming that the losses of internal standards were approximately equal to the losses of the amino acids in the polypeptide chain. Known amounts (2 or 4nmol) of each amino acid were added to samples of fragment PF-I before hydrolysis. The average recoveries from three such determinations (Table 1) were used to correct the various amino acid values from hydrolysates of fragment PF-I.

Carbohydrate analysis

The carbohydrate composition was analysed by enzymic and colour reactions (Gladhaug Berre *et al.*, 1973) and, in the case of the hexosamines, by the amino acid analyser. Total hexoses were measured by the phenol–H_2SO_4 method (Briggs *et al.*, 1956) and the orcinol reaction (Winzler, 1955) with an equimolar mixture of galactose and mannose as standard. Sialic acid was liberated by 0.05M-H_2SO_4 for 1h at 80°C. Galactose and hexosamines were analysed after hydrolysis in 3M-HCl for 5h at 105°C in ampoules sealed under N_2.

Other methods

Protein was measured by the method of Lowry *et al.* (1951), or by the method of Böhlen *et al.* (1973), by using fluorescamine (Hoffmann–LaRoche and Co. Ltd., Basel, Switzerland). Bovine serum albumin was used as the standard in both cases. Thiols were determined by the Ellman (1959) method and disulphides as described by Zahler & Cleland (1968). Sulphate was measured by the method of Antonopoulos (1962) and phosphate as described by Chen *et al.* (1956) after digestion with H_2SO_4 and $HClO_4$. The contents of tyrosine and tryptophan were measured as described by Edelhoch (1967). Adsorption with $BaSO_4$ was carried out as described by Prydz (1964).

Table 1. *Recovery of amino acids added as internal standards before hydrolysis of fragment PF-I*

Hydrolysis was in 6M-HCl for 24h at 110°C. For further details see the text.

	% recovered
Lysine	92
Histidine	96
Arginine	90
Aspartic acid	94
Threonine	99
Serine	63
Glutamic acid	74
Proline	100
Glycine	100
Alanine	77
Valine	82
Isoleucine	84
Leucine	86
Tyrosine	86
Phenylalanine	99

Results

Purification and characteristics of fragment PF-I

Human serum was treated with $BaSO_4$ and submitted to DEAE-Sephadex chromatography as described (Gladhaug Berre *et al.*, 1973; Gladhaug & Prydz, 1970). Fragment PF-I was eluted at about the same ionic strength (about 0.35–0.40mol/l) as factor Xa (Fig. 1) and further purified by repeated preparative polyacrylamide-gel electrophoresis. The final product was homogeneous both in analytical polyacrylamide-gel electrophoresis and in sodium dodecyl sulphate–polyacrylamide-gel electrophoresis (Fig. 2*a* and 2*b*) and gave rise to a monospecific antiserum when injected into rabbits.

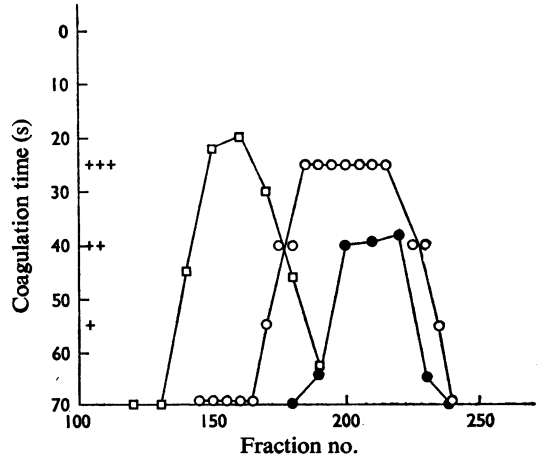

Fig. 1. *Elution diagram of factor VII* (□), *factor X* (●) *and prothrombin fragment I* (○) *from a column* (4cm×55cm) *of DEAE-Sephadex*

The column was equilibrated to 0.01M-sodium phosphate buffer, pH8.0, and eluted with a linear gradient of 0–0.4M-NaCl at a rate of about 100ml/h. Fractions (10–20ml) were collected. The eluate was diluted 1:10 for assay of factors VII and X. (The values for factor X are from the test system without Russell's-viper venom.) Fragment PF-I was estimated by visual grading of immunodiffusion precipitation lines as 0, +, ++ and +++.

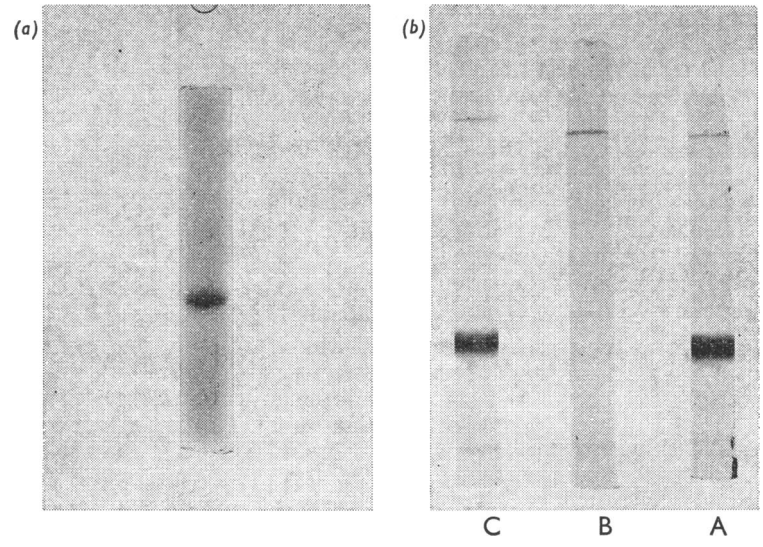

Fig. 2. *Polyacrylamide-gel electrophoresis of fragment PF-I*

(*a*) Sodium dodecyl sulphate–polyacrylamide-disc-gel electrophoresis of fragment PF-I (5μg). Electrophoresis was done as described by Neville (1971). The anode is at the bottom. The protein was reduced with 2-mercaptoethanol (for details see the Materials and Methods section). (*b*) Analytical polyacrylamide-disc-gel electrophoresis of purified fragment PF-I (10μg). The anode is at the bottom. Gel A, fragment PF-I before $BaSO_4$ treatment; gel B, after $BaSO_4$ treatment showing that fragment PF-I has been removed; gel C, fragment PF-I in the eluate from $BaSO_4$.

Fragment PF-I had a mobility relative to Bromophenol Blue of 0.80 in analytical polyacrylamide-gel electrophoresis; an apparent molecular weight of 27000±2000 was estimated by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis. A pI of 4.82 was found by electrofocusing. The molar extinction coefficient at 280nm was about 19700 (calculated on the basis of protein determinations and assumed mol.wt. of 19600). No alteration in the mobility of fragment PF-I in sodium dodecyl sulphate–polyacrylamide-gel electrophoresis was seen after reduction and alkylation. Coupled with the finding of only one *N*-terminal amino acid, this indicated that fragment PF-I consisted of one polypeptide chain. During storage fragment PF-I was broken down to fragments which could be seen in analytical polyacrylamide-gel electrophoresis. It is not known at present whether this proteolytic activity is an intrinsic property of fragment PF-I or results from traces of a proteolytic contaminant. The proteolysis was inhibited by di-isopropyl phosphorofluoridate (Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.) and toluene-*p*-sulphonyl fluoride (Pierce Chemical Co.). Thrombin did not have any detectable proteolytic effect on fragment PF-I.

Amino acid composition

The amino acid composition of fragment PF-I (Table 2) revealed nothing unusual. About 11% of the residues were arginine and lysine. Glutamic acid and aspartic acid accounted for about 24%: no attempt was made to determine how many of these residues were present as glutamine and asparagine before hydrolysis.

The values for serine and glycine in fragment PF-I are rounded up to the nearest higher integer in view of the results of the analyses of the CNBr fragments.

The finding of 3.1 tryptophan residues by the spectrophotometric method agreed quite well with the amino acid analysis. Only 2 (1.5) tyrosine residues and no free thiol groups were found. By the method of Zahler & Cleland (1968) an average of 7.1 half-cystine residues was found, in reasonable agreement with the 7.7 residues found by amino acid analysis. Thus a total of four internal disulphide bridges is likely. One methionine residue was found per molecule of fragment PF-I.

The amino acid content/mol of protein was calculated on the basis of measurements of protein by the method of Lowry *et al.* (1951) and corrected for recovery. The data obtained (Table 2) suggested

Table 2. *Amino acid analysis of hydrolysates of fragment PF-I and its CNBr-cleaved framents corrected for loss during hydrolysis*

For details of hydrolysis see the Materials and Methods section. Results for fragment PF-I, large and small CNBr-cleaved fragments are means of nine, two and four analyses respectively. N.D., Not detected (less than 0.1 mol/mol for methionine). Values in parentheses are nearest whole numbers of residues.

	Amino acid content (mol/mol of protein)		
	Fragment PF-I	Large CNBr fragment (C-1)	Small CNBr fragment (C-2)
Lysine	8.0 (8)	3.2 (4)	3.3 (4)
Histidine	3.5 (4)	2.1 (2)	1.5 (2)
Arginine	9.3 (9)	6.3 (7)	1.2 (2)
Aspartic acid	12.5 (13)	8.3 (9)	3.3 (4)
Threonine	10.9 (11)	7.2 (8)	2.4 (3)
Serine	11.2 (12)	7.2 (7)	5.8 (6)
Glutamic acid	23.8 (24)	10.7 (11)	5.8 (6)
Proline	7.2 (6)	6.4 (6)	1.2 (1)
Glycine	15.1 (16)	8.7 (9)	6.6 (7)
Alanine	11.3 (11)	5.2 (5)	6.0 (6)
Cystine (half)*	7.7 (8)	3.6 (4)	3.9 (4)
Valine	8.2† (8)	3.4 (4)	1.8 (2)
Methionine	0.5 (1)	N.D. (0)	Homoserine
Isoleucine	4.3† (5)	2.3 (3)	1.1 (2)
Leucine	8.2† (8)	3.3 (4)	1.6 (2)
Tyrosine	2.7 (3)	1.2 (2)	0.8 (1)
Phenylalanine	3.6 (4)	1.2 (2)	1.2 (2)
Tryptophan	3.5‡ (3)	2.1§ (2)	0.9§ (1)

* Determined as cysteic acid.

† Means of three analyses of 72h hydrolysates.

‡ Determined after hydrolysis with methanesulphonic acid.

§ Determined by the method of Edelhoch (1967).

a total of 155 amino acid residues in fragment PF-I, resulting in a calculated molecular weight of 17100 for the polypeptide chain. Addition of the molecular weight of the carbohydrate component (Table 3) gave a total molecular weight of about 19600, and this value has been used for all calculations.

The *N*-terminal amino acids of fragment PF-I were an Ala-Asx bond. No *C*-terminal amino acid was found in carboxypeptidase C digests, whereas in carboxypeptidase B digests, arginine appeared. We conclude that arginine is the *C*-terminal amino acid of fragment PF-I.

CNBr-cleaved fragments

In accordance with the finding of one methionine residue per molecule of fragment PF-I by amino acid analysis, we obtained two fragments after CNBr cleavage (fragments C-1 and C-2). These fragments were separated by gel filtration. Their electrophoretic mobilities in sodium dodecyl sulphate–polyacrylamide-gel electrophoresis as well as their elution volumes in gel filtration were smaller than expected. The anomalous behaviour of both fragments is probably caused by their denaturation and aggregation and for fragment C-1 also by its carbohydrate content. No molecular weight estimation was therefore possible by sodium dodecyl sulphate–polyacrylamide-gel electrophoresis. Fragment C-2 had the highest electrophoretic mobility and the highest elution volume and was therefore probably the smaller of the two fragments. The amino acid composition of the two CNBr fragments is in reasonable agreement with that of fragment PF-I except for serine (one or two residues more when the CNBr fragments are added), and valine and leucine (two residues less than expected when the CNBr fragments are added). The difference between the number of glutamic acid residues detected in fragment PF-I and the sum of the same residues in the CNBr-cleaved fragments is probably too great to be due to experimental error. Altogether 29% of the glutamic acid residues were not recovered after CNBr cleavage and subsequent acid hydrolysis. This amounts to seven residues per molecule of fragment PF-I.

Fragments C-1 and C-2 contained about 89 and 56 amino acid residues respectively. The *N*-terminal amino acids were alanine for the C-2 fragment and threonine for the C-1 fragment. The C-2 fragment contained homoserine. The *N*-terminal alanine of fragment C-2 as well as the *C*-terminal homoserine indicated that this subfragment was the *N*-terminal part of fragment PF-I.

Carbohydrate composition

By enzymic and colorimetric reactions fragment PF-I was found to contain sialic acid, galactose and hexosamine (Table 3). The latter was identified as glucosamine by using the amino acid analyser. No glucose was found. Total hexoses were 9.4% by the orcinol method and 12.8% with the phenol–H_2SO_4 method. The difference between total hexoses (9.4%) and the content of galactose (2.5%) is probably due largely to mannose. Our data suggest that there are 2mol of sialic acid, 4mol of glucosamine, 2–3mol of galactose and about 7mol of mannose per mol of fragment PF-I of mol.wt. 19600.

Preliminary studies of the carbohydrate content of the CNBr fragments (Table 3) showed that no sialic acid (i.e. less than 0.1%) and no glucosamine (less than 0.2%) was present in the C-2 fragment. The hexoses were not measured. The amount of sialic acid was decreased also in the C-1 fragment, probably owing to the exposure to low pH values during CNBr cleavage and subsequent peptide

Table 3. *Carbohydrate composition of fragment PF-I and its CNBr-cleaved fragments C-1 and C-2*

For details see the text. N.D., Not detected (i.e. less than 0.1% for sialic acid, less than 0.2% for glucosamine).

	Fragment PF-I		Large CNBr fragment (C-1)		
	(%)	(mol/mol of protein)	(%)	(mol/mol of protein)	Small CNBr fragment (C-2)
Sialic acid	3.2	2.0	1.2	—	N.D.
Glucosamine	3.5	3.8	4.7	2.9	N.D.
Galactose	2.5	2.7			
Total hexoses	9.4	10.0			
Total	16.1				

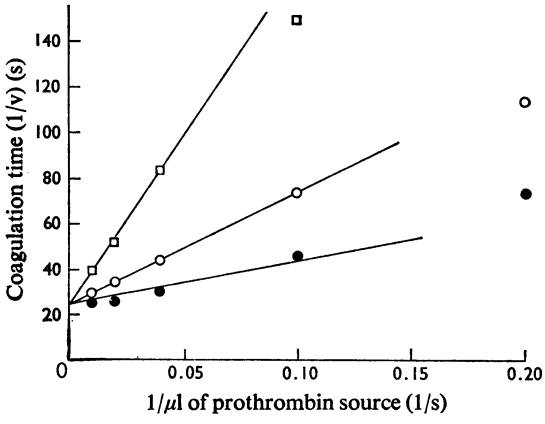

Fig. 3. *Double-reciprocal plot of the activation of prothrombin by prothrombinase in the presence of various fixed concentrations of fragment PF-I*

The prothrombinase consisted of 0.1ml of $BaSO_4$-adsorbed bovine plasma, 0.1ml of cephalin (1mg/ml), 0.05ml of factor Xa (12μg/ml) and $CaCl_2$ (final concn. 10mM). □, With 0.05ml of fragment PF-I (0.5mg/ml) added; ○, with 0.025ml of fragment PF-I (0.5mg/ml) added; ●, with 0.02ml of albumin (1mg/ml) added. The prothrombin source is bentonite-adsorbed human plasma.

separation. The glucosamine content of the C-1 fragment was nearly sufficient to account for all of the glucosamine in fragment PF-I. Thus it seems that the C-1 fragment contains all of the carbohydrate chains from fragment PF-I.

Coagulation activity

Purified preparations of fragment PF-I had no detectable procoagulant activity in test systems for factors II, VII, X or thrombin. No inhibition was found when 20–25μg of fragment PF-I (in 0.05ml) was added to the thrombin–fibrinogen system. Neither did treatment with *Echis carinatus* venom [0.1ml of venom (1–10μg/ml) was incubated with 0.3ml of fragment PF-I (0.5mg/ml) for 15 and 30min at 37°C and portions (0.2ml) were withdrawn for testing with 0.2ml of fibrinogen] induce any procoagulant activity in preparations of fragment PF-I. In the prothrombin test system 6–8μg of fragment PF-I (in 10μl) had a pronounced effect. Kinetic studies revealed that the inhibition was competitive with respect to prothrombin (Fig. 3). The source of prothrombin was bentonite-treated plasma (Hougie, 1962) containing about 50 units of prothrombin/ml when normal human plasma is taken to contain 100 units/ml. The K_m value for prothrombin was equivalent to about 8μl of the bentonite-treated plasma or about $1–2\times10^{-8}$M and the K_i was 3×10^{-7}M.

Binding to $BaSO_4$, Ca^{2+} and phospholipids

Purified fragment PF-I (50μg/ml) was adsorbed completely to $BaSO_4$ (10mg/ml) in the presence of 0.10M-sodium oxalate, since only traces of protein were left in the supernatant after adsorption. Fragment PF-I could be eluted from the $BaSO_4$ by 0.15M-trisodium citrate (Fig. 2*b*).

With cephalin as a source of phospholipids, fragment PF-I was bound in the presence of 5mM-Ca^{2+} and sedimented at 2×10^5*g*-min. The sediments were washed and fragment PF-I was eluted by resuspending them in 3mM-trisodium citrate. Fragment PF-I was not bound in the absence of Ca^{2+}.

The binding of $^{45}Ca^{2+}$ to fragment PF-I was conveniently demonstrated by the method of Nelsestuen & Suttie (1972). Dialysis bags were boiled in 10mM-EDTA for 10min and washed several times in distilled water. Fragment PF-I (50μg/ml) was dialysed for 24h at 4°C against 0.1M-Tris–HCl buffer, pH 8.0–0.15-M-NaCl to which various amounts of $^{40}Ca^{2+}$ and $^{45}Ca^{2+}$ up to a concentration of 1μM were added.

Portions (1ml) were withdrawn from the contents of the bag and from the surrounding buffer and dried in an Amicon cell with a UM-10 filter. The filters

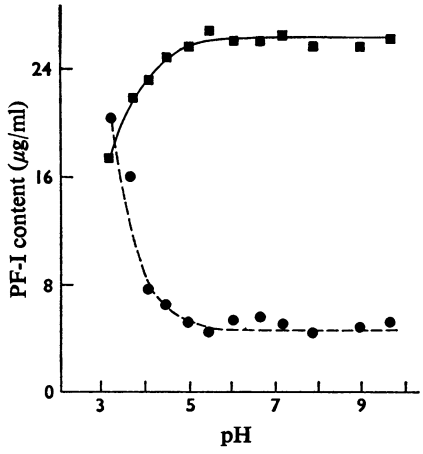

Fig. 4. *$BaSO_4$ adsorption of fragment PF-I from solutions of different pH values*

The pH of solutions of fragment PF-I (70μg/ml) in veronal buffered saline containing 0.01M-potassium oxalate was adjusted to the desired value by addition of 0.1M-NaOH or 0.1M-HCl; 6mg of $BaSO_4$/ml was used. ■, Fragment PF-I eluted from $BaSO_4$; ●, fragment PF-I left unadsorbed.

were washed with ethylene glycol on the reverse side and counted for radioactivity in 10ml of toluene with 6g of 2,5-diphenyloxazole and 1.2g of 1,4-bis-(5-phenyloxazol-2-yl)benzene (both Koch–Light Laboratories Ltd.) per litre of toluene. An exact quantitative study was, however, difficult with this method. The results suggested a binding of about 6–10mol of Ca^{2+}/mol of fragment PF-I at a concentration of 1mM-Ca^{2+}.

A study of the adsorption of fragment PF-I to $BaSO_4$ at different H^+ concentrations revealed that the adsorption was essentially complete between pH9.6 and 4.8 (Fig. 4). Below pH4.8 the amount adsorbed declined. If the small (and constant) amount of non-adsorbed material found between pH9.6 and 4.8 is neglected, an apparent p*K* of 3.1–3.5 for the group(s) responsible for the adsorption may be calculated.

We next submitted the two CNBr-cleaved fragments to $BaSO_4$ treatment. The smaller, *N*-terminal C-2 fragment was almost completely adsorbed to $BaSO_4$ in the presence of 0.01M-potassium oxalate. The C-1 fragment was left unadsorbed in the solution. No sulphate groups and fewer than one phosphate group per 2.2 molecules of fragment PF-I were detected.

Immunology

The antiserum raised against fragment PF-I was monospecific, giving one precipitation line with normal serum or plasma as well as with purified fragment PF-I in gel diffusion and immunoelectrophoresis. Factor II in plasma was completely neutralized. The antiserum did not react with factor X in plasma, nor with $BaSO_4$-treated plasma and serum, nor with thrombin. Neither did the antiserum react with the factor PIVKA-II in plasma from patients under continuous warfarin treatment. A weak precipitation line was given by the small amount of normal prothrombin present in such plasma. No precipitation line was found with the plasma of warfarin-treated patients, from which normal prothrombin had been removed by $BaSO_4$ treatment and where only PIVKA-II factor was present.

Discussion

Fragments of bovine prothrombin apparently corresponding to fragment PF-I have been isolated and characterized by Heldebrant *et al.* (1973*a*,*b*), Stenflo (1973), Benson *et al.* (1973) and Morita *et al.* (1973). The present report is to our knowledge the first one dealing with the purification and characterization of such a fragment from human serum.

Fragment PF-I has a calculated molecular weight of about 19600. The much higher value derived from the gel-electrophoretic experiments may be caused by the irregular behaviour of glycoproteins in such experiments as well as in protein determinations. There is also marked difference between the apparent molecular weights derived when the system of Weber & Osborn (1969) is compared with that of Neville (1971), the same standard proteins being used. We consider that the chemical analyses provide a better basis for the estimation of the molecular weight and all calculations are based on a molecular weight of fragment PF-I of 19600.

Fragment PF-I is the *N*-terminal fragment of the prothrombin molecule, corresponding to the Intermediate 3 of Heldebrant *et al.* (1973*a*,*b*). It is probably split off by factor Xa or thrombin, cleaving an arginine–serine bond. This part of the molecule is responsible for the binding of prothrombin to phospholipid membranes by Ca^{2+}-bridges as well as for the adsorption to $BaSO_4$ characteristic of the four vitamin K-dependent coagulation factors. It exerts a competitive inhibition of the activation of prothrombin, probably by competing with the whole molecule for limited binding sites. The appearance of fragment PF-I during coagulation may therefore contribute to the limitation of the conversion of prothrombin into thrombin *in vivo*. The inhibitor observed in bovine prothrombin preparations treated with thrombin (Marciniak *et al.*, 1967), and the corresponding activity obtained from human blood (Marciniak, 1972), might well be similar to or identical with fragment PF-I.

The similarities between fragment PF-I and the

corresponding bovine fragment are quite marked. The bovine fragment binds to phospholipid particles under the same conditions as prothrombin and fragment PF-I. The amino acid composition of fragment PF-I is quite similar to that of the corresponding bovine fragment. When the data of Stenflo (1973), Morita *et al.* (1973) and Heldebrant *et al.* (1973*b*) are recalculated on the basis of an assumed mol.wt. of 17100 to allow comparison, the main differences are: fragment PF-I contains 8 lysine, 4 histidine, 7 proline and 16 glycine residues, whereas the bovine fragment probably contains 5 lysine, 2–3 histidine, 8–9 proline and 11 glycine residues per 16700-mol.wt. polypeptide chain. There is no immunological cross-reaction between the antiserum to fragment PF-I and bovine prothrombin, indicating that certain differences must be present.

The carbohydrate content of the bovine fragment (Morita *et al.*, 1973), comprising sialic acid, hexoses and glucosamine adding up to about 18%, is very similar to that observed by us (Table 2).

The purity of the fragment PF-I preparations was demonstrated by their appearance as a single band in analytical polyacrylamide-gel electrophoresis and the monospecific antiserum resulting from immunization of rabbits. This antiserum did not react with PIVKA-II (the abnormal prothrombin appearing during vitamin K deficiency or dicoumarol treatment) and must therefore be largely directed against the structures representing the vitamin K-induced modifications in the molecule. This is a further indication that these structures are located in the PF-I fragment. One of the few detectable differences between the normal coagulation factors and their respective PIVKA factors is the absence of Ca^{2+}-binding and $BaSO_4$-adsorbable groups in the PIVKA factors (Ganrot & Nilehn, 1968; Prydz & Gladhaug, 1971). The attachment or demasking of these groups must represent the result of the vitamin K-dependent post-synthetic modification of the coagulation factors. We have therefore investigated which of the two CNBr-cleaved fragments of fragment PF-I carried the groups responsible for $BaSO_4$-adsorbability and demonstrated that they are located to a *N*-terminal fragment of molecular weight of about 5900.

Nelsestuen & Suttie (1973) reported using the same strategy to select a relevant peptide from a tryptic digest of bovine prothrombin. They describe a peptide of molecular weight about 3000–4000 without detectable sugar. The peptide does not comprise the *N*-terminal end of prothrombin (at least not the first three residues). No carbohydrate groups are apparently attached to the peptide; this agrees with our finding. This peptide was digested further by a neutral proteinase which, however, left a peptide of molecular weight about 900–1900 undigested. Nelsestuen & Suttie (1973) suggest the existence of a covalently attached prosthetic group with a molecular weight of about 1000.

What then are the possible groups involved in the $BaSO_4$- and Ca^{2+}-binding? We can exclude sulphate or phosphate groups and the usual carbohydrate groups, in agreement with our own earlier studies of factor Xa (Gladhaug Berre *et al.*, 1973) and Nelsestuen & Suttie (1973). The pK_a of the binding groups (3.1–3.5) points to the importance of carboxyl groups. The abundance of glutamic acid in the peptide isolated by Nelsestuen & Suttie (1973), as well as the seven glutamic acid residues missing when the contents of the two CNBr fragments are added, suggest that the γ-carboxyl group of glutamic acid may be involved.

Human factors VII and X are both adsorbed to $BaSO_4$ in their activated form (Gladhaug & Prydz, 1970; Gladhaug Berre *et al.*, 1973), whereas the activated form of prothrombin (i.e. thrombin) is not adsorbed. The $BaSO_4$-binding structures must therefore be connected with the procoagulant part of the factors VII and X molecules. This suggests that the structural analogy between factors VII, X and prothrombin is not complete in the sense that the vitamin K-induced modification is located in a different part of the molecule in factors VII and X from that in prothrombin. Fragment PF-I decreased the anti-(factor X) titre when added to certain anti-(factor X) sera, but factor Xa did not react with anti-(fragment PF-I) in this way (T. Holm & H. Prydz, unpublished work). This immunological cross-reaction suggests some structural similarity between fragment PF-I and the factor Xa used to raise the antisera. This might further point to the presence of structures in fragment PF-I and in factor Xa that might be similar and which might possibly be vitamin K dependent.

B. Ø. was a Research Fellow of the Norwegian Council for Science and the Humanities. This research was supported by Norsk Medisinaldepot. *Echis carinatus* venom was kindly donated by F. Kornalik, Institute of Pathophysiology, Prague, Czechoslovakia.

References

Ambler, R. P. (1972) *Methods Enzymol.* **35**, 143–154
Antonopoulos, C. A. (1962) *Acta Chem. Scand.* **16**, 1521–1522
Barton, P. G. & Hanahan, D. J. (1969) *Biochem. Biophys. Acta* **187**, 319–327
Benson, B. J., Kisiel, W. & Hanahan, D. J. (1973) *Biochim. Biophys. Acta* **329**, 81–87
Bjørklid, E., Storm, E. & Prydz, H. (1973) *Biochem. Biophys. Res. Commun.* **55**, 969–976
Böhlen, P., Stein, S., Dairman, W. & Udenfriend, S. (1973) *Arch. Biochem. Biophys.* **155**, 213–220
Briggs, D. R., Garner, E. F., Montgomerey, R. & Smith, F. (1956) *Anal. Chem.* **28**, 1333–1335

Bruton, C. J. & Hartley, B. S. (1970) *J. Mol. Biol.* **52**, 165–178
Catsimpoolas, N. (1968) *Anal. Biochem.* **26**, 480–482
Chen, P. S., Toribara, T. Y. & Warner, H. (1956) *Anal. Chem.* **28**, 1756–1758
Davis, B. J. (1964) *Ann. N.Y. Acad. Sci.* **121**, 404–427
Deyl, Z. & Rosmus, J. (1965) *J. Chromatog.* **20**, 514–520
Edelhoch, H. (1967) *Biochemistry* **6**, 1948–1954
Ellman, G. L. (1959) *Arch. Biochem. Biophys.* **82**, 70–77
Fass, D. N. & Mann, K. G. (1973) *J. Biol. Chem.* **248**, 3280–3287
Friedman, M., Norna, A. T. & Masri, M. S. (1973) *Anal. Biochem.* **51**, 280–287
Ganrot, P. O. & Nilehn, J. E. (1968) *Scand. J. Clin. Lab. Invest.* **22**, 23–28
Gitel, S. N., Owen, W. G., Esmon, C. T. & Jackson, C. M. (1973) *Proc. Nat. Acad. Sci. U.S.* **70**, 1344–1348
Gladhaug, Å. & Prydz, H. (1970) *Biochim. Biophys. Acta* **215**, 105–111
Gladhaug Berre, Å., Østerud, B., Christensen, T. B., Holm, T. & Prydz, H. (1973) *Biochem. J.* **135**, 791–795
Gros, C. & Labouesse, B. (1959) *Eur. J. Biochem.* **7**, 463–470
Heldebrant, C. M., Noyes, C., Kingdom, H. S. & Mann, K. G. (1973*a*) *Biochem. Biophys. Res. Commun.* **54**, 155–160
Heldebrant, C. M., Butkowski, R. J., Bajaj, S. P. & Mann, K. G. (1973*b*) *J. Biol. Chem.* **248**, 7149–7163
Hemker, H. C., Veltkamp, J. J. & Loeliger, E. A. (1968) *Thromb. Diath. Haemorrh.* **19**, 346–363
Hirs, C. H. W. (1967) *Methods Enzymol.* **11**, 197–199
Hjort, P. F., Rapaport, S. I. & Owren, P. A. (1955) *J. Lab. Clin. Med.* **46**, 89–97
Hougie, C. (1962) *Proc. Soc. Exp. Biol. Med.* **109**, 754–756
Josso, F., Lavergne, J. M., Gouault, M., Prou-Wartelle, O. & Soulier, J. P. (1968) *Thromb. Diath. Haemorrh.* **20**, 88–98
Königsberg, W. (1972) *Methods Enzymol.* **25**, 185–188
Larrieu, M. J. & Mayer, D. (1970) *Lancet* **2**, 1085
Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
Mann, K. G., Heldebrant, C. M. & Fass, D. N. (1971*a*) *J. Biol. Chem.* **246**, 5594–6001
Mann, K. G., Heldebrant, C. M. & Fass, D. N. (1971*b*) *J. Biol. Chem.* **246**, 6106–6114
Marciniak, E. (1972) *J. Lab. Clin. Med.* **79**, 924–934
Marciniak, E., Murano, G. & Seegers, W. H. (1967) *Thromb. Diath. Haemorrh.* **18**, 161–166
Moore, S. (1972) in *Chemistry and Biology of Peptides* (Meienhofer, J., ed.), pp. 626–653, Ann. Arbor Publishers, Ann Arbor
Morita, T., Iwanaga, S., Suzuki, T. & Fujikawa, K. (1973) *FEBS Lett.* **36**, 313–317
Nelsestuen, G. L. & Suttie, J. W. (1972) *Biochemistry* **11**, 4961–4964
Nelsestuen, G. L. & Suttie, J. W. (1973) *Proc. Nat. Acad. Sci. U.S.* **70**, 3366–3370
Neville, D. M., Jr. (1971) *J. Biol. Chem.* **246**, 6328–6334
Nilehn, J. E. & Ganrot, P. O. (1968) *Scand. J. Clin. Lab. Invest.* **22**, 17–22
Ornstein, L. (1964) *Ann. N.Y. Acad. Sci.* **121**, 321–349
Parr, D. M., Percy, M. E. & Connell, G. E. (1972) *Immunochemistry* **9**, 51–63
Prydz, H. (1963) *Scand. J. Clin. Lab. Invest.* **15**, 450–456
Prydz, H. (1964) *Scand. J. Clin. Lab. Invest.* **16**, 409–414
Prydz, H. (1970) in *Immunological Mechanisms in Blood Coagulation, Thrombosis and Hemostasis* (Duckert, F., Brinkhous, K. M. & Hinnom, S., eds.), p. 296–299, F. K. Schattauer Verlag, Stuttgart and New York
Prydz, H. & Gladhaug, Å. (1970) *Thromb. Diath. Haemorrh.* **24**, 601–602
Prydz, H. & Gladhaug, Å. (1971) *Thromb. Diath. Haemorrh.* **25**, 157–165
Scheidegger, J. J. (1955) *Int. Arch. Allergy* **7**, 103–110
Stenflo, J. (1973) *J. Biol. Chem.* **248**, 6325–6332
Stenn, K. S. & Blout, E. R. (1972) *Biochemistry* **11**, 4502–4515
Tschesche, H. & Kupfer, S. (1972) *Eur. J. Biochem.* **26**, 33–36
Weber, K. & Osborn, M. (1969) *J. Biol. Chem.* **244**, 4406–4412
Winzler, R. J. (1955) *Methods Biochem. Anal.* **2**, 279–311
Woods, K. R. & Wang, K. T. (1967) *Biochim. Biophys. Acta* **133**, 369–370
Zahler, W. L. & Cleland, W. W. (1968) *J. Biol. Chem.* **243**, 716–719