Some Characteristics of the Coagulation Factor Xa purified from Human Serum

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1. Coagulation Factor X was purified from human serum to apparent homogeneity in disc gel electrophoresis, sodium dodecyl sulphate–polyacrylamide-gel electrophoresis, immunoelectrophoresis and analytical ultracentrifugation. The method used was a modification of that described by Gladhaug & Prydz (1970). 2. The method permits the isolation of an activated form of Factor X (Xa) which has a molecular weight of about 25000. 3. Factor Xa is a glycoprotein containing about 14% carbohydrate. A preliminary report of the amino acid composition is given.

A method for the purification of the coagulation Factors VII and X from human serum has been described (Gladhaug & Prydz, 1970). The yield of both Factors was small (0.2–4%) and the Factor X preparations gave two or three bands in disc gel electrophoresis. We describe here a modified purification procedure which permits the isolation of activated Factor X (Xa) in somewhat higher yield. Factor Xa is a form of Factor X which can participate in the formation of prothrombinase without further activation by the intrinsic or extrinsic coagulation systems or Russel-viper venom. The activation of Factor X to Xa took place during the purification. The preparations obtained were homogeneous in immunoelectrophoresis, disc gel electrophoresis, sodium dodecyl sulphate–polyacrylamide-gel electrophoresis and analytical ultracentrifugation. Some characteristics of purified Factor Xa are reported.

Materials and Methods

Materials

Human serum. This was obtained, prepared and stored as described by Gladhaug & Prydz (1970).

Coagulation tests

Reagents and procedures for coagulation tests have been described by Gladhaug & Prydz (1970).

Factor Xa activity was tested by omission of Russell's-viper venom (Stypven; Wellcome Reagents Ltd., Beckenham, Kent, U.K.) from the Factor X test system (Gladhaug & Prydz, 1970).

Electrophoretic methods

Analytical polyacrylamide-gel disc electrophoresis, sodium dodecyl sulphate–polyacrylamide-gel electrophoresis and immunoelectrophoresis were carried out

as described by Grossbach (1965), Weber & Osborn (1969) and Scheidegger (1955), respectively. The anti-serum was raised against purified Factor X as described by Prydz & Gladhaug (1971). Ovalbumin, myoglobin, bovine serum albumin (Sigma Chemical Co., St. Louis, Mo., U.S.A.) and human γ -globulin (Kabi, Stockholm, Sweden) were used as markers. Preparative polyacrylamide-gel electrophoresis (Buchler Instruments, Fort Lee, N.J., U.S.A.) was carried out as described by the manufacturers.

Analytical ultracentrifugation

A Beckman Model E centrifuge equipped with double-beam photoelectric scanner and the An-E rotor in conjunction with a 30mm double-sector cell was used.

The short-column technique and overspeeding (Richards *et al.*, 1968) was used for sedimentation-equilibrium experiments at 2°C. The molecular weight was calculated from the slope of a plot of $\ln c$ (c = concentration measured by E_{280}) versus the square of the radius.

The sedimentation coefficient was determined at 19°C and 40000 rev./min. The 30mm double-sector and capillary-type synthetic-boundary cell was used to obtain a well-defined initial boundary.

G.l.c. for carbohydrates (Bolton *et al.*, 1965)

Protein samples (of known weight, about 150 μ g) with 2.5 μ g of D-mannitol in 10 μ l of methanol added were carefully dried and hydrolysed in 2ml of methanolic 1M-HCl in sealed tubes at 100°C for 7h and the hydrolysates evaporated to dryness over NaOH pellets.

Methanol (150 μ l) and acetic anhydride (50 μ l) were added and the mixture was left for 24h at 37°C

to reacetylate the amino sugars. After evaporation to dryness, first over NaOH pellets and then over P_2O_5 , 20 μ l of the silylation mixture [1 vol. of trimethylsilyl chloride (Pierce Chemical Co., Rockford, Ill., U.S.A.), 1 vol. of hexamethyl disilazane (Pierce Chemical Co.) and 5 vol. of dry pyridine] was added and the reaction left for 10 min at room temperature.

The mixture was evaporated to dryness and 10 μ l of chloroform added. Two 5 μ l portions were analysed in a F & M model 400 gas-liquid chromatograph with a 150 cm column (internal diam. 3 mm) in isothermal operation at 175° and 220°C (the latter for the determination of sialic acid) and He at 70 ml/min as carrier gas. The stationary phase was Diatoport S (80/100 mesh) coated with 3.1% SE-30 (Hewlett-Packard, Avondale, Pa., U.S.A.).

Enzymic and colour reactions for carbohydrates

Glucose oxidase (Boehringer, Mannheim, Germany) and galactose oxidase reagents (Kabi, Stockholm, Sweden) were used as indicated by the manufacturers. Sialic acid, fucose, hexosamines and hexuronic acid were determined as described by Warren (1959), Gibbons (1955), Kabat & Mayer (1961) and Dische (1947) respectively. Sialic acid was liberated by neuraminidase at pH 5.5 or by hydrolysis with 0.05 M- H_2SO_4 for 1 h at 80°C. The other carbohydrates were analysed after hydrolysis with 50 ml of 3 M-HCl in a 100 ml flask for 5 h at 100°C under air. HCl was removed in vacuo, the residue dissolved in a small volume of water and dried again. Corrections for loss during hydrolysis were made for fucose (15%), glucosamine (15%), galactose (13%) and glucose (13%) based on experiments with internal standards.

Amino acid analysis

Two samples of about 10 nmol and two of about 5 nmol of Factor Xa were hydrolysed at 110°C in a great excess of 6 M-HCl in evacuated tubes for 24 h. The hydrolysates were analysed in a Jeol JLC-6AH amino acid analyser. Norleucine was used as internal standard. The relative colour coefficients of the various amino acids were determined in standard runs. Corrections for loss during hydrolysis were made for serine (7%), threonine (10%) and cysteine (5%) based on earlier model experiments. The absolute amount of each amino acid was calculated on this basis.

Other methods

Sulphate was determined by the method of Antonopoulos (1962). Tyrosine and tryptophan were determined spectrophotometrically in 6 M-urea as described by Edelhoch (1967). Thiols were determined by the

method of Ellman (1959) with a 200-fold excess of 5,5'-dithiobis-(2-nitrobenzoic acid).

Reduction was carried out in 8 M-urea with 50 mM-2-mercaptoethanol in 50 mM-Tris-HCl buffer, pH 8.5, for 5 h at room temperature and alkylation was at pH 11 for 10 min with 0.2 M-sodium monoiodoacetate; subsequently 1 M-HCl was added to give pH 8.5.

Protein was measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard.

Immunodiffusion was carried out as described (Prydz, 1963). All chemicals were of analytical grade.

Experimental and Results

Purification of Factor Xa

Human serum (500 ml) with 50 ml of 0.1 M-potassium oxalate added was treated with 50 g of $BaSO_4$ (Merck, Darmstadt, Germany) and the $BaSO_4$ was washed and eluted with 50 ml of 0.15 M-sodium citrate as described by Hjort (1957). The eluate was submitted to DEAE-Sephadex chromatography as described by Gladhaug & Prydz (1970). The appropriate fractions were reabsorbed with $BaSO_4$ (5 mg/ml) after addition of 0.1 vol. of oxalate, and eluted with 0.01 vol. of 0.15 M-sodium citrate (Hjort, 1957). The eluate was submitted to filtration through polyacrylamide gel P-100 (Bio-Rad, Richmond, Calif., U.S.A.) (gel bed 2.5 cm $\times$ 40 cm), equilibrated and eluted with the upper buffer for preparative disc electrophoresis.

Sucrose (final concentration 10%, w/v) was added to the fractions containing Factor Xa before preparative polyacrylamide-gel disc electrophoresis. The volume of the stacking gel was always equal to the volume of sample (usually 130 ml, containing about 5 mg of protein). After the first preparative electrophoresis, analytical disc gel electrophoresis showed two or three bands (Gladhaug & Prydz, 1970). The appropriate fractions were pooled, concentrated with Sephadex G-25 (Pharmacia, Uppsala, Sweden) or Aquacide (Calbiochem, Calif., U.S.A.) outside dialysis bags and submitted to the various tests for purity. Preparations used for carbohydrate analysis were concentrated by evaporation. The yield was regularly 200–500 μ g of protein, compared with 100–200 μ g with the former procedure (Gladhaug & Prydz, 1970). The specific activity of the product was about 9000 units/mg. Normal plasma is taken to contain 100 units/ml.

Electrophoresis

Analytical disc gel electrophoresis gave one band with a mobility relative to the marker (Bromophenol Blue) $R_m = 0.86$ –0.90. Sodium dodecyl sulphate-polyacrylamide-gel electrophoresis gave a molecular weight of 25000 ± 2000 . Reduced and alkylated

Table 1. *Composition of the carbohydrate portion of Factor Xa*

Results are means of three or more determinations. N.D., Not detected.

Carbohydrate	Content (% of total weight)		Content (mol/mol of protein)
	By g.l.c.	By colour-enzymic reactions	
Galactose	1.6	1.3	2-3
Fucose	N.D.	3.4	5-6
Glucose	2.8	0.9	1-4
Glucosamine	4.2	4.7	6-7
N-Acetylneuraminic acid	4.2	3.6	3-4
Hexuronic acid	N.D.	0.8	0.1
Total	12.8	14.7	

Factor Xa gave also only one band of apparent molecular weight 24000-29000. An antiserum raised against purified Factor X (Prydz & Gladhaug, 1971) gave one band in immunoelectrophoresis against normal human plasma and against purified Factor Xa.

Analytical ultracentrifugation

Plots of $\ln c$ versus the square of the radius were straight lines at 12000 rev./min and 18000 rev./min, thus indicating homogeneity of the protein samples. Molecular weights calculated from the slopes of such plots were 24500 at 18000 rev./min and 25000 at 12000 rev./min. The sedimentation coefficient $s_{20,w} = 2.1$ S was determined separately. A partial specific volume of 0.72 ml/g for Factor Xa was assumed. The protein concentration used was 0.25 mg/ml.

Carbohydrates

The composition of the carbohydrate part of Factor Xa was determined by colour reactions, enzymic methods and g.l.c. (Table 1).

The amount of material reacting as glucose varied between preparations and may have been a contaminant, although every effort was taken to exclude this possibility. Added glucose was completely removed during the purification, suggesting that the remaining glucose was covalently bound to the macromolecules. However, since glucose is a rather uncommon component of serum glycoproteins, this observation deserves further study. Fucose was not detected in several g.l.c. runs, but the colour reaction for methylpentoses was clearly positive, with a spectrum like that given by the fucose standard. Fucose is therefore likely to be a true component of Factor Xa.

Hexuronic acid was detected in a small and varying amount by colorimetric tests, but was not seen on g.l.c. The colorimetric and g.l.c. analyses for the other

Table 2. *Amino acid analyses of Factor Xa hydrolysates*

Results are means of four analyses. The values in parentheses are the nearest whole numbers.

Amino acid	Content (mol/mol of protein)*
Lysine	6.4 (7)
Histidine	3.1 (3)
Arginine	11.6 (12)
Aspartic acid	17.6 (18)
Threonine	18.1† (18)
Serine	10.8† (11)
Glutamic acid	24.9 (25)
Proline	7.8 (8)
Glycine	12.8 (13)
Alanine	13.8 (14)
Cystine (half)	5.1‡ (6)
Valine	10.5 (11 or more)
Methionine	1.4 (2)
Isoleucine	6.6 (7 or more)
Leucine	11.5 (12)
Tyrosine	4.8 (5)
Phenylalanine	6.1 (6)
Tryptophan*	3.3 (3)

* Determined spectrophotometrically (Edelhoch, 1967).

† Corrected for destruction during hydrolysis.

‡ Given as the sum of cystine and cysteic acid, corrected for destruction during hydrolysis.

carbohydrate molecules were in reasonable agreement.

Sialic acid was not necessary for the procoagulant nor for the antigenic activity of Factor Xa, since removal of about 75% of the sialic acid by neuraminidase did not influence the coagulation time of the Factor Xa test system, and Factor Xa still reacted

with the antiserum against purified Factor X. Hydrolysis in 0.05 M-H₂SO₄ released slightly more sialic acid than did neuraminidase.

Thiol groups

Incubation in 200-fold excess of 5,5'-dithiobis-(2-nitrobenzoic acid) for 1 h did not inhibit the activity of Factor Xa and differential spectrophotometry with this reagent indicated less than 0.1 thiol group per protein molecule even when the protein was dissolved in 6 M-guanidinium chloride. No sulphate groups were detected.

Amino acid composition

The molecular weight of the polypeptide chain of Factor Xa (total molecular weight ÷ molecular weight of carbohydrate) is about 21 500. Determination of the absolute amounts of amino acids recovered (Table 2) gave about 180 mol of amino acid residues per mol of Factor Xa, adding up to a total molecular weight of about 20 000. Therecovery of the amino acid residues was therefore about 90–95%. The amino acid composition was unremarkable. Since there were no free thiols detectable, the protein probably contains three disulphide bridges.

Tyrosine and tryptophan in Factor Xa dissolved in 6 M-urea were determined spectrophotometrically (Edelhoch, 1967) and found to be 3.2 mol and 3.3 mol per mol of protein respectively.

Discussion

Factor X is one of a group of coagulation factors which circulate in plasma as inactive molecules and become activated at various stages in the coagulation process. Factor X is known to be activated by both the extrinsic and the intrinsic coagulation systems as well as by a component of Russel-viper venom. Prothrombin (Factor II), a close analogue of Factor X, is activated to thrombin in a series of proteolytic reactions (Aronson & Ménaché, 1966; Lanchantin *et al.*, 1967; Seegers *et al.*, 1968; Mann *et al.*, 1971; Stenn & Blout, 1972).

There is now general agreement that bovine Factor X consists of two chains of molecular weights 38 000–39 000 and 15 000–17 000 (Fujikawa *et al.*, 1972a,b; Jackson, 1972). An activation sequence for bovine Factor X has been described (Fujikawa *et al.*, 1972a,b). During activation with Russel-viper venom, a peptide of molecular weight 11 000 is split off from the N-terminal end of the heavy chain. This split may be followed by loss of a peptide of molecular weight 3000 from the C-terminal end. The resulting heavy chain has a molecular weight of 24 000.

Seegers *et al.* (1963) reported a bovine preparation with autoprotease C activity (now believed to be

identical with Factor Xa activity) of molecular weight 24 200 and a sedimentation coefficient $s_{20,w} = 2.27S$, consisting of about 195 amino acid residues. We may have isolated an analogous form of Factor Xa from human serum.

The molecular weight of unactivated Factor X purified from serum was about 72 000 as estimated (without correction for the anomalous behaviour of glycoproteins) by gel filtration on a calibrated Sephadex G-200 column (Å. G. Berre, T. Holm & H. Prydz, unpublished work). By carrying out the purification process more rapidly, Factor Xa with a molecular weight of about 46 000–48 000 as estimated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis was obtained. More than one form of Factor Xa must therefore exist. It is not known whether the various ways of activation (extrinsic, intrinsic or Russel-viper venom) produce the same sequence of reactions. Factor Xa as described here is probably a result of both extrinsic and intrinsic activation.

Our present results suggest that the present form of Factor Xa consists of one polypeptide chain. The addition of about 14% of carbohydrate to the sum of the amino acids recovered (about 180 mol/mol of protein) (Table 2) allows one to calculate a total molecular weight between 23 000 and 24 000, which is in reasonable agreement with the molecular weight observed in sodium dodecyl sulphate–polyacrylamide gel electrophoresis and in the analytical ultracentrifuge. Since there were probably six half-cystine residues and no free thiol groups, Factor Xa in the present form most likely contained three internal disulphide bridges. The lower amount of tyrosine in the spectrophotometric titration, compared with the amino acid analysis, was most probably due to incomplete ionization.

Compared with the amino acid analyses given by Radcliffe & Barton (1973) and Fujikawa *et al.* (1972a) there are several obvious differences, for instance a much higher content of half-cystine. These analyses do, however, refer to bovine samples of higher molecular weight and are therefore not strictly comparable.

Our analyses are quite similar to the data of Seegers *et al.* (1967) for 10 of the 18 amino acids reported. There is less agreement with the data in Seegers *et al.* (1969).

The value for the molecular weight of Factor Xa reported here agrees closely with that calculated by Seegers *et al.* (1969) for bovine autoprotease C when the carbohydrate portion of about 11% is taken into consideration (Mammen, 1971). The absence of detectable differences in the apparent molecular weight after reduction and alkylation of the present Factor Xa molecule and the absence of detectable subunits mark definite differences from the structure of thrombin, where two or three polypeptide chains

connected by disulphide bridges are found (Magnusson, 1969), and from bovine Factor Xa which has two polypeptide chains (Fujikawa *et al.*, 1972a). Mattock & Esnouf (1973) recently described a single-chain form of Factor X from bovine plasma with a molecular weight of about 56000. On activation the molecular weight decreased to about 35000. The presence of more than one polypeptide chain therefore appears unnecessary for both bovine and human Factor Xa activity.

The coagulation factors II, VII, IX and X depend on vitamin K for their biosynthesis and they adsorb quantitatively or nearly so on BaSO₄ in the presence of oxalate ions. So far no common structural feature has been revealed which distinguishes these four factors from other glycoproteins not dependent on vitamin K or adsorbed to BaSO₄.

The presence of glucose might have been such a feature, but no glucose has been reported in factor II (prothrombin). It is uncertain at present if glucose is a contaminant or a true constituent of the carbohydrate portion of Factor Xa.

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