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A Comparison of Bovine Prothrombin, Factor IX (Christmas Factor), and Factor X (Stuart Factor)*

(blood coagulation/glycoprotein/homology/immunology)

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ABSTRACT **A comparison has been made of the electrophoretic behavior, chemical composition, amino-terminal sequence, and immunological properties of bovine prothrombin, factor IX (Christmas factor), and factor X (Stuart factor). Some immunological cross-reactivity was found between the antibody to prothrombin and factor X although prothrombin and factor X differ substantially in amino-acid and carbohydrate composition. Considerable amino-acid sequence homology was found in the amino-terminal portion of prothrombin, factor IX, and the light chain of factor X. These data provide further evidence to support the hypothesis that at least three of the vitamin K-dependent clotting factors have evolved from a common ancestral gene.**

Prothrombin, factor IX (Christmas factor), and factor X (Stuart factor) are three of the four coagulation factors that require vitamin K for their biosynthesis (1). Prothrombin and factor X have molecular weights of 70,000 (2) and 55,000 (3–5), respectively. Prothrombin is composed of a single polypeptide chain (6–8), while factor X contains a heavy and a light chain (3, 5). A recent report suggests that factor X may also be present as a single polypeptide chain (9). Factor IX has a molecular weight of 55,400 and consists of a single polypeptide chain (10). All three coagulation factors are glycoproteins.

During blood coagulation, factor IX, factor X, and prothrombin are converted to enzymes in a series of stepwise reactions (1). These enzymes then participate in coagulation as proteases of different protein substrate specificities. Thrombin and factor X_a (activated factor X) are serine esterases that are homologous in the amino-terminal and active-site regions of their heavy (or B) chains (11).

In the present communication, we compare some of the physical and chemical properties of prothrombin, factor IX, and factor X and show that the amino-terminal sequences of all three proteins are homologous.

MATERIALS AND METHODS

Bovine factor X_1 and the light chain of factor X_1 were prepared according to the method of Fujikawa *et al.* (3). Bovine factor IX was also prepared by the method of Fujikawa *et al.* (10). These two proteins were free of other known coagulation proteins. Bovine prothrombin was prepared by a modification of the procedure that Fujikawa *et al.* (10) developed for the isolation of factor IX (M. H. Coan, to be published). The prothrombin preparation was free of detectable impurities of factors VII, IX, and X. Chemicals used in these investigations were the same as those used in previous studies (3, 10, 11).

Disc-gel electrophoresis (pH 8.0) was performed by the method of Williams and Reisfeld (12). Zone electrophoresis was performed by the method of Williams and Chase, as described (3). Double-immunodiffusion studies were carried out by a modification of the method of Ouchterlony (13). *S*-pyridylethyl prothrombin was prepared by the method of Friedman *et al.* (14). Amino-acid analysis of prothrombin was performed according to the method of Spackman *et al.* (15) on samples that had been hydrolyzed for 24, 48, 72, and 96 hr at 110°. Half-cystine was estimated as *S*-pyridylethylcysteine. Tyrosine and tryptophan were determined spectrophotometrically (16). Hexose was assayed by the method of Dubois *et al.* (17), hexosamine by the procedure of Gardell (18), and sialic acid by the method of Warren (19). Amino-terminal sequences were determined by automated Edman degradation with a Beckman Sequencer model 890A. Sequenator analyses were carried out by the method of Edman and Begg (20) as modified by Hermodson *et al.* (21). In the quantitation of the terminal residues, protein concentration was determined in an amino-acid analyzer on a separate aliquot after hydrolysis. Norleucine was used as an internal standard to calculate protein recovery. Antisera to factor IX and to prothrombin were prepared by methods described for factor X (3). The protein concentrations of the antisera were 12 mg/ml for the antiserum against prothrombin, 8.0 mg/ml for antiserum against factor IX, and 8.5 mg/ml for antiserum against factor X. Assays for factors IX and X and prothrombin were carried out as described (3, 10).

RESULTS AND DISCUSSION

Disc-gel electrophoresis and zone electrophoresis patterns for prothrombin, factor IX, and factor X are shown in Figs. 1 and 2. Single sharp bands or spots are obtained for each of these proteins by both procedures. Under the conditions of these experiments, prothrombin migrates slowest; factor X migrates fastest and slightly ahead of factor IX.

The nomenclature for various coagulation factors is that recommended by an international nomenclature committee [Wright, I. (1959) *J. Amer. Med. Ass.* **170**, 325].

* A preliminary report of portions of this work has appeared elsewhere [Fujikawa, K., Legaz, M., Hermodson, M. & Davie, E. W. (1972) *Abstracts of IIIrd International Congress on Thrombosis and Haemostasis*, p. 90].

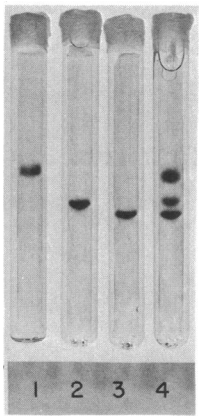

FIG. 1. Disc-gel electrophoresis of bovine prothrombin, factor IX, and factor X. Five micrograms of each purified protein was applied to the gels, and electrophoresis was carried out at pH 8.0. Protein was stained with Coomassie Brilliant Blue. *Sample 1* contained prothrombin; *sample 2*, factor IX; *sample 3*, factor X; and *sample 4*, prothrombin, factor IX, and factor X. The *anode* was at the bottom of the figure.

The amino-acid and carbohydrate compositions of these three proteins are shown in Table 1, calculated as residues per protein molecule. It is evident from these data that the three proteins differ markedly in amino-acid composition and in the approximate number of residues. The amino-acid composition of prothrombin is essentially identical to that reported by Cox and Hanahan (22) and to other data, recently summarized by Magnusson (23).

TABLE 1. *Amino-acid and carbohydrate compositions (residues per molecule*) of bovine prothrombin, factor IX, and factor X*

Components	Prothrombin (MW 70,000)	Factor IX† (MW 55,400)	Factor X‡ (MW 55,000)
Amino acid			
Lysine	29	28	22
Histidine	9	8	11
Arginine	44	17	26
Aspartic acid	56	36	40
Threonine	28	20	30
Serine	32	29	30
Glutamic acid	67	47	57
Proline	36	13	18
Glycine	45	30	38
Alanine	33	19	30
Half-cystine	23	17	19
Valine	35	25	27
Methionine	5–6	2–3	6–7
Isoleucine	20	19	12
Leucine	46	19	31
Tyrosine	14	9	9
Phenylalanine	20	15	20
Tryptophan	14	11	10
Total residues	556–557	364–365	436–437
Carbohydrate			
Hexose	15	33	9
N-acetylhexosamine	7	16	8
N-acetylneuraminic acid	9	16	6

* Rounded off to the nearest whole number.
† Calculated from the data of Fujikawa *et al.* (10).
‡ Calculated from the data of Fujikawa *et al.* (3).
MW, molecular weight.

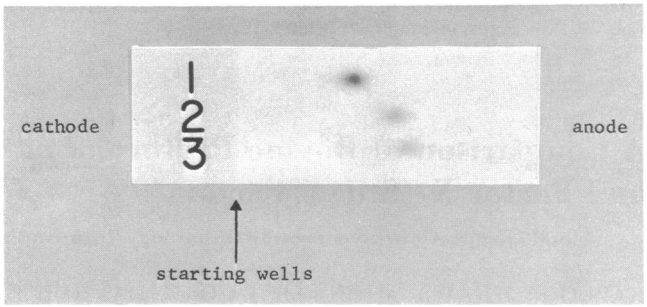

FIG. 2. Zone electrophoresis of bovine prothrombin (*1*), factor IX (*2*), and factor X (*3*). Three micrograms of each purified protein was placed in small wells on the agarose-coated slide, and electrophoresis was carried out at 50 V/6.5 cm for 2 hr. Protein was stained with Coomassie Brilliant Blue.

The three proteins differ also in carbohydrate content. Factor IX has a high content of hexose, hexosamine, and neuraminic acid which comprises approximately 26% of the glycoprotein. The carbohydrate content of prothrombin and factor X is approximately 10%.

Some similarity of prothrombin and factor X is apparent from their immunological properties (Fig. 3). The panel on the left shows an Ouchterlony double-immunodiffusion experiment in which rabbit antibody to prothrombin was added to the center well. Sharp precipitin lines developed against prothrombin, a light precipitin line against factor X, and no precipitin line against factor IX. It is possible that the cross-reaction of prothrombin antibody with factor X was due to contamination of the original prothrombin antigen by factor X. This seems unlikely, however, since the prothrombin antigen contained less than 0.0001% factor X, as measured by clotting activity. Trace contamination of the prothrombin by factor X, however, can never be completely ruled out. The prothrombin antibody used in these experiments was

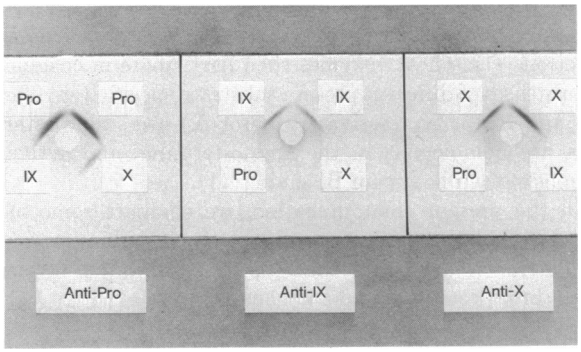

FIG. 3. Ouchterlony double-immunodiffusion of prothrombin, factor IX, and factor X against rabbit antibodies prepared from each of these proteins. The *center well* in the *left panel* contained 6 μl of rabbit antiserum to prothrombin. The *center wells* in the *middle* and *right panels* contained 6 μl of antiserum to factor IX and factor X, respectively. The *outer wells* contained 6 μl of antigen at a concentration of 0.5 mg/ml. After 10 hr, the non-precipitated protein was removed by washing the slides in 0.15 M NaCl overnight. The slides were then washed in water, allowed to dry, and stained with Coomassie Brilliant Blue. *Pro*, prothrombin; *IX*, factor IX; and *X*, factor X.

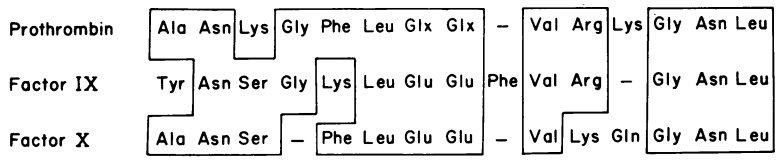

FIG. 4. Amino-terminal sequences of prothrombin, factor IX, and the light chain of factor X. Amino acids that are identical are shown in *blocks*. *Dashes* (—) refer to gaps or spaces that have been inserted to bring the three proteins into alignment for greater homology.

directed toward portions of the antigen molecule that are not directly involved in biological activity since the antibody did not inhibit prothrombin or factor X activity in regular clotting assays.

The middle panel in Fig. 3 shows similar double-diffusion experiments using antibody to factor IX. Sharp precipitin lines were observed against factor IX (top two wells), but no crossreactivity was evident against either prothrombin or factor X. Also, the antibody to factor IX had no effect on prothrombin or factor X activity, although it readily inactivated factor IX (10).

In the panel on the right, the center well contained rabbit antibody to factor X. Sharp precipitin lines formed against factor X (top two wells). A very faint precipitin line was occasionally observed against prothrombin and antibody to factor X, but no precipitin line formed against factor IX. Antibody to factor X readily inactivates factor X (3), but does not inactivate prothrombin or factor IX in the regular clotting assay.

The crossreactivity of antibodies to factors VII, IX, X, and prothrombin has been studied in detail by Denson (24). Although some crossreactivity was observed between these proteins, he concluded that it was due to mixed antibodies and not an antibody crossreaction. The present data suggest that the crossreaction observed between factor X and antibody to prothrombin was not due to contamination of the prothrombin antigen by factor X.

The amino-terminal residues for prothrombin, factor IX, and the light chain of factor X were also determined. Prothrombin contains an amino-terminal alanine (0.94 equivalents per mol), factor IX contains tyrosine (0.77 equivalents per mol), and the light chain of factor X contains alanine (0.80 equivalents per mol). Factor IX also contains 0.2 equivalents of amino-terminal asparagine, which is present in the second position of the major sequence. As shown in Fig. 4, six of the first 14 amino acids are identical in all three proteins. Two amino acids (residues 7 and 8) are similar (or probably identical) in all three proteins. In addition, five of the first 14 amino acids are identical in two of the three proteins. Gaps or spaces have been inserted in positions 4, 9, and 12 to bring the three proteins into better alignment. The first six residues of prothrombin are identical to those recently found by Stenflo (25). This sequence is also identical to that found by Magnusson (26) and Heldebrant *et al.* (27) for prothrombin.

It is clear from these experiments that these three proteins, which participate in different parts of the coagulation reaction, have homologous amino-terminal regions. The amino acids that are different require two base changes in the DNA genome. This is evident for the Ala/Tyr conversion in position 1, the Ser/Lys conversion in position 3, and the Phe/Lys conversion in position 5. Nevertheless, these data provide further evidence to support the hypothesis that at least three of the four vitamin K-dependent clotting factors have evolved from a common ancestral gene. Whether factor VII, the fourth clotting factor requiring vitamin K for its biosynthesis, has similar traits of homology remains to be tested.

The active sites of thrombin and factor X_a occur in the carboxyl-terminal region of the molecules (23, 11), which are homologous (11). Homology also occurs in the amino-terminal regions of the precursors of these two enzymes and factor IX. Thus, the molecular weight changes that have apparently occurred during evolution of these three proteins are due to the removal or addition of polypeptide segments in the middle portion of the polypeptide chain of prothrombin. This is evident from the fact that prothrombin contains approximately 560-amino-acid residues per 70,000 daltons of glycoprotein, factor X contains approximately 440 residues per 55,000 daltons of glycoprotein, and factor IX, approximately 370 residues per 55,400 daltons of glycoprotein (Table I).

The sequence homology of factor X, prothrombin, and factor IX also suggests that factor X was initially synthesized as a single polypeptide chain which contained the light chain at the amino-terminus. Thus, limited proteolysis of a single-chain factor X *in vivo* or during its isolation may have resulted in the formation of a two-chain structure. This hypothesis is further supported by the recent publication of Mattock and Esnouf which reported the presence of single-chain factor X in bovine plasma (9). Further experiments are required to clarify these observations, however, since the single-chain component was not activated by Russell's viper venom or by the extrinsic clotting system (28).

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