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Isolation and Characterization of Vitamin K-Dependent Region of Bovine Blood Clotting Factor X

(protein sequence/homology/calcium binding/γ-carboxyglutamic acid/coagulation)

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ABSTRACT **A 39-residue peptide from the tryptic digestion of bovine blood clotting factor X has been isolated by specific adsorption on barium citrate. The amino- and carboxyl-terminal sequences of the peptide were determined and compared to the vitamin K-dependent Ca^{2+}-binding region from bovine prothrombin. The factor X peptide was found to contain γ-carboxyglutamic acid residues, and the results of independent analysis are consistent with all 14 glutamic acid residues as γ-carboxyglutamic acid. The similarity of the factor X peptide to the prothrombin peptide supports the hypothesis that the vitamin K-dependent blood clotting proteins are descended from a common ancestral gene.**

Four of the proteins of the blood clotting system are dependent upon vitamin K for synthesis of the physiologically active form. A current hypothesis states that at least three of the vitamin K-dependent blood clotting proteins (prothrombin, factor IX, and factor X) are derived from a common ancestral gene (1, 2). This hypothesis is based upon the facts that: (*a*) all three proteins are glycoproteins (3–6), (*b*) all three proteins require Ca^{2+} and phospholipid for physiological activation (7–11), (*c*) all three proteins have similar amino-terminal amino-acid sequences (2), and (*d*) all three proteins are serine esterases with common amino-acid sequences at the active site (1, 10). However, a number of differences exist between the three proteins: (*a*) different molecular weights (3–6), (*b*) different carbohydrate content and type of carbohydrate moiety (3–6, 12), and (*c*) little or no immunocross-reactivity between prothrombin (factor II) or factor X and factor IX (2).

Vitamin K has been established to be essential for the post-ribosomal modification of prothrombin, which results in the formation of the Ca^{2+}-binding site (13, 14). This modification is the γ-carboxylation of the first 10 glutamic acid residues in the amino-terminal sequence of bovine prothrombin (15–18,‡). The resulting amino acid, γ-carboxyglutamic acid, has also been identified in bovine factor X and rat prothrombin (18, 19). The amino-acid sequence of the vitamin K-dependent, Ca^{2+}-binding region of prothrombin§ has revealed that 8 of the 10 γ-carboxyglutamic acid residues are found in pairs (16, ‡). If the vitamin K-dependent blood clotting proteins are related proteins, then homology between these proteins would be expected for the Ca^{2+}-binding regions. We wish to report the isolation and characterization of a peptide from factor X which is similar to the vitamin K-dependent, Ca^{2+}-binding region of prothrombin. Preliminary accounts of some of this work have been presented (18, 20).

‡ J. Howard, M. Fausch, and G. Nelsestuen, submitted for publication.

§ Vitamin K-dependent peptide refers to the specific peptides from blood clotting factors II and X which adsorb on barium citrate. The peptide from factor II has been shown to contain part of the vitamin K-dependent modification, γ-carboxyglutamic acid (15). This paper demonstrates that there is an analogous region in factor X.

METHODS AND MATERIALS

Factor X was prepared as a by-product of a prothrombin purification procedure, which consists of barium citrate adsorption, $(NH_4)_2SO_4$ precipitation, and DEAE-Sephadex chromatography (12). Factor X, a minor component which elutes after prothrombin on DEAE-Sephadex chromatography, was pooled from several prothrombin preparations and rechromatographed on a 1.5 × 20 cm column of DEAE-Sephadex. The resin was equilibrated in 0.1 M potassium phosphate buffer at pH 6.0 and was eluted with a gradient of 0.1–0.55 M NaCl. The factor X prepared in this way was free of detectable contaminants by the following criteria: (*a*) the absence of prothrombin activity in the two-state assay for prothrombin, (*b*) a specific activity of 100 units of factor X per mg when assayed in factor VII/X deficient plasma (21), and (*c*) the presence of only the light and heavy chains of factor X on mercaptoethanol/sodium dodecyl sulfate/acrylamide gel electrophoresis (4). The barium citrate adsorbing peptide from factor X was isolated according to the procedure of Nelsestuen and Suttie (22) for the isolation of a similar peptide from prothrombin. Eighty milligrams of factor X were digested with 2 mg of trypsin for 18 hr at pH 9.0. Fifteen milliliters of 1 M $BaCl_2$ were added to the tryptic digest (125 ml of 0.04 M sodium citrate), and the barium citrate precipitate was collected. After unbound peptides were removed from the barium citrate by repeated washing, the adsorbed peptide was eluted with excess 1 M Na_2SO_4 and the peptide desalted by chromatography on Sephadex G-25. Based upon the amino-acid composition given in Table 1, this peptide was isolated in a 76% yield when the amount of factor X was estimated from a molecular weight of 55,000 and an $E^{1\%}_{280\ nm}$ of 12.4 (4).

Manual sequential Edman degradation, as described by Peterson *et al.* (23) was performed on 0.15 μmol of performic acid-oxidized (24), vitamin K-dependent peptide from bovine factor X. At the beginning of each cycle, approximately 5% of the remaining peptide was removed for dansylation (25).

TABLE 1. *Amino-acid analysis of the vitamin K-dependent peptide from factor X*

Amino acid	μmol[a]	Residues[b]	Vitamin K-dependent peptide from prothrombin (17)
Aspartic acid	0.0292	2.80 (3)	2
Threonine	0.0098[c]	0.93 (1)	1
Serine	0.0198[c]	1.88 (2)	2
Glutamic acid	0.1467	13.91 (14)	8
Proline	—	—	1
½ Cystine	0.0214[d]	2.03 (2)	2
Glycine	0.0102	0.97 (1)	1
Alanine	0.0321	3.04 (3)	4
Valine	0.0208[e]	1.97 (2)	—
Leucine	0.0436[e]	4.14 (4)	4
Phenylalanine	0.0223	2.12 (2)	2
Lysine	0.0169	1.61 (2)	1
Arginine	0.0209	1.98 (2)	2
Tryptophan	—	(1)[f]	1

[a] Average of duplicate analyses for 24- and 72-hr HCl hydrolysis at 110°.

[b] Numbers in parentheses indicate probable number of residues for each amino acid.

[c] Value determined for extrapolation to initial time of hydrolysis.

[d] Value determined as cysteic acid from duplicate samples that were oxidized with performic acid before acid hydrolysis (24).

[e] Value determined for release at 72 hr of hydrolysis.

[f] Value determined from UV spectrum.

The amino-acid residues removed were identified by amino-acid analysis after HI hydrolysis of the phenylthiazolinone amino acids (26). Dansyl amino acids were identified by thin-layer chromatography on polyamide plates. Vitamin K-dependent peptide from factor X (20 nmol) was digested for 5 and 18 hr with carboxypeptidases A and B (approximately 1 unit of each) at pH 8.0. The amino-acid residues were determined by amino-acid analysis of the enzyme digest. Enzyme blanks contained less than 1 nmol of any amino acid.

[^{3}H]Diborane reduction of the vitamin K-dependent peptide from factor X was performed as described by Zytkovicz and Nelsestuen (19). The reduced peptide was hydrolyzed, and the amino acid analysis was done by a modified single column methodology. After the ninhydrin reaction, the column effluent was collected and the fractions were assayed for tritium content by scintillation counting (1.0 ml of column effluent, 1.5 ml of water, and 10 ml of PCS scintillation solubilizer from Amersham/Searle Corp.).

Amino-acid analyses were performed on a model 119 amino-acid analyzer equipped with automatic sample injection, expanded scale, and single column methodology. Other methods are included as footnotes in the tables.

RESULTS AND DISCUSSION

A single peptide was isolated by barium citrate adsorption of the tryptic digestion of factor X. The peptide was homogeneous on Sephadex G-75 chromatography (K_d = 0.24) and DEAE-Sephadex ion exchange chromatography (pH 7.8, 50 mM Tris·HCl, elution at 0.65 M NaCl in a gradient of 0.1–1.0 M NaCl). In addition, a single amino- and carboxyl-terminal residue was found for the peptide (see below). The amino-acid composition of the barium citrate adsorbing peptide from factor X resembles the composition of the vitamin K-dependent, Ca^{2+}-binding peptide from prothrombin (Table 1). Both peptides are unusual tryptic peptides because they contain two trypsin-resistant arginine residues. These arginine residues have been shown to occur in -Arg-Glx-sequences in the prothrombin peptide, where the Glx is γ-carboxyglutamic acid‡. The factor X peptide has an additional trypsin-resistant lysine residue. Another distinguishing characteristic of both peptides is the high percentage of aspartic acid and glutamic acid residues (⅓ to ½ of the residues).

A logical progression of experimental evidence has demonstrated that the role of vitamin K in blood clotting is to mediate the post-ribosomal formation of γ-carboxyglutamic acid residues as part of the Ca^{2+}-binding region of prothrombin. Most of the γ-carboxyglutamic acid residues in prothrombin are found in the barium citrate adsorbing peptide. Therefore, to establish that the barium citrate adsorbing peptide of factor X is the vitamin K-dependent region of the protein, we must demonstrate the presence and number of γ-carboxyglutamic acid residues in the peptide.

TABLE 2. *Total carboxyl groups in the vitamin K-dependent peptide of factor X*

Sample	Expected carboxyl groups[a]	Glutamic acid residues	Glycine residues incorporated[b]	Extra carboxyl groups[c]	Extra carboxyl groups/glutamic acid residue
Insulin	6.0	4.0	5.8	—	—
Prothrombin vitamin K-dependent peptide (17)	10.0	8.0	17.9	7.9	0.99
Factor X vitamin K-dependent peptide	17.0	14.0	30.6	13.6	0.97

Peptides (0.05–0.10 μmol) were added to 1.0 ml of 1 M glycine ethyl ester containing 0.2 M 1-ethyl-3-dimethylaminopropylcarbodiimide at pH 4.8–5.0. After 2 hr at room temperature, an additional 20 mg of carbodiimide was added and the reaction was continued overnight. Peptides were purified by Sephadex G-25 chromatography and were demonstrated to be free of unreacted glycine ethyl ester by amino-acid analysis. Peptides were hydrolyzed and amino-acid analysis was performed.

[a] Total carboxyl groups calculated from the total glutamic acid and aspartic acid content of the peptides plus the terminal carboxyl groups. For insulin, asparagine and glutamine residues were excluded. For both the prothrombin and the factor X peptide, one asparagine has been found in the sequence.

[b] The glycine incorporated is calculated by subtracting the number of residues of glycine normally found in the peptide from the number of residues found in the peptide after reaction with the carbodiimide and glycine ethyl ester.

[c] The difference between the glycine incorporated and the number of expected carboxyl groups calculated from the amino-acid analysis.

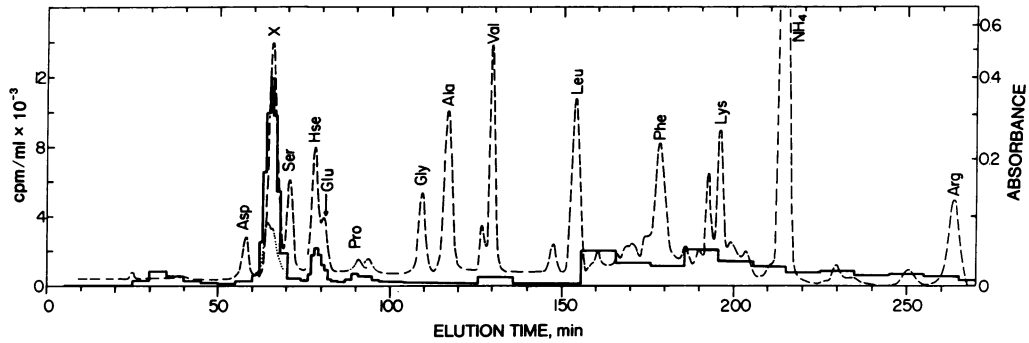

FIG. 1. Chromatogram of the amino-acid analysis of the [^{3}H]diborane-reduced factor X peptide. This analysis was performed on a Spinco 120 by single column methodology with 0.20 N sodium citrate, pH 3.25 (0–126 min), 0.20 N sodium citrate, pH 4.20 (127–160 min), and 1.6 N sodium citrate, pH 6.4 (161–270 min) as the eluting buffers. Amino-acid X, has been identified as 5,5′-dihydroxyleucine (19). Ninhydrin analysis is shown at 570 nm (*dashed curve*) and at 440 nm (*dotted curve*); ^{3}H (*solid curve*). The amino acid composition is Asp (0.57), Hse (1.90), X (5.9, based upon an average amino-acid color value for ninhydrin reaction), Ser (1.2), Glu + 5-hydroxynorvaline (0.5), Gly (1.23), Ala (3.0), Val (2.8), Leu (3.2), Phe (3.2), Lys (1.6), and Arg (1.8). A residue value of 1.0 corresponds to 0.030 μmol.

The presence of γ-carboxyglutamic acid residues in prothrombin can be determined by reducing the carboxylic acid groups to alcohols with diborane (19). Dihydroxyleucine, the product derived from γ-carboxyglutamic acid, was identified by both mass spectrometry and amino-acid analysis after HCl hydrolysis of the prothrombin vitamin K-dependent peptide (19). The barium citrate adsorbing peptide from factor X was reduced with [^{3}H]diborane, hydrolyzed with HCl, and analyzed as shown in Fig. 1. The identified products of the diborane reduction of the factor X peptide are homoserine (76 min), which is derived from aspartic acid residues, 5-hydroxynorvaline (82 min), which is derived from glutamic acid residues and elutes along with glutamic acid, and 5,5′-dihydroxyleucine (61 min; amino-acid X, Fig. 1), which is derived from γ-carboxyglutamic acid (19). As can be seen in Fig. 1, the primary product of [^{3}H]diborane reduction is 5,5′-dihydroxyleucine. The yield of all amino acids upon analysis was about 30% of theoretical yield. The low recovery has been attributed to the reduction of peptide bonds during the diborane reaction and limits the use of diborane reduction for direct quantitation of γ-carboxyglutamic acid residues (19). However, the very small amount of [^{3}H]5-hydroxynorvaline and glutamic acid (less than 0.5 residues per mole of peptide) in this analysis is consistent with a small and possibly insignificant amount of glutamic acid in the factor X peptide. The presence of [^{3}H]5,5′-dihydroxyleucine as the major product of reduction demonstrates that γ-carboxyglutamic acid is the predominant form of glutamic acid in the factor X peptide.

To determine more accurately the number of γ-carboxyglutamic acid residues in the factor X vitamin K-dependent peptide, we determined the number of extra carboxyl groups in the peptide by the method described for the prothrombin peptide (17). The carboxyl groups in the peptide were blocked with glycine ethyl ester, using 1-ethyl-3-dimethylaminopropylcarbodiimide to activate the carboxyl groups. The modified peptide was separated from the reagents by chromatography on Sephadex G-25, and the incorporation of glycine was determined by amino-acid analysis. The increase in glycine content was assumed to reflect the number of free carboxyl groups. For example, 95% of the free carboxyl groups in insulin could be blocked with glycine ethyl ester under our reaction conditions (Table 2). The vitamin K-dependent peptide from prothrombin had 7.9 carboxyl groups more than could be accounted by the side chain and carboxyl-terminal residues, which is one extra carboxyl group per glutamic acid residue. Therefore, all eight glutamic acid residues are actually γ-carboxyglutamic acid residues (17). The vitamin K-dependent peptide from factor X has 13.6 extra carboxyl groups (Table 2). As in the case of the prothrombin peptide, it would appear that most or all of the glutamic acid residues in the factor X vitamin K-dependent peptide are present as the γ-carboxyglutamic acid derivative. This is supported also by diborane reduction.

The partial amino-acid sequence of the factor X peptide is shown in Fig. 2. The first 10 amino-terminal residues were determined by sequential dansyl-Edman degradation. Eight of these residues correspond exactly to residues 6–15 of prothrombin and overlap the first three γ-carboxyglutamic acid residues of prothrombin (16, ‡). The first nine residues in this sequence have been reported as residues 5–13 of the factor X protein (2). Residues 7–10 of the factor X vitamin K-dependent peptide correspond to residues 1–4 of the barium citrate adsorbing vitamin K-dependent peptide from prothrombin‡. The carboxyl-terminal sequence of the factor X peptide is shown also in Fig. 2. The lysine and tryptophan residues of factor X correspond to the carboxyl-terminal sequence of the prothrombin vitamin K-dependent peptide if one assumes a deletion of an alanine residue in the factor X peptide. In contrast to the prothrombin peptide where the carboxyl-terminal ten residues could be quantitatively released with carboxypeptidases A and B digestion‡, only tryptophan and lysine were released upon exhaustive carboxypeptidase digestion of the factor X peptide. This would suggest a sequence difference in this region of the factor X peptide, e.g., the insertion of one or more of the γ-carboxyglutamic acid residues would inhibit carboxypeptidase digestion of the peptide¶. The unsequenced portion of the factor X peptide is shown in parentheses in Fig. 2 and is nearly identical with the same region of the prothrombin peptide. The only differences are the deletion of proline, the increase

¶ Our work on determination of the sequence of a number of small peptides containing γ-carboxyglutamic acid residues has shown that carboxyl-terminal or penultimate γ-carboxyglutamic acid residues prevent carboxypeptidase A digestion of such peptides at both pH 6.5 and 9.0.

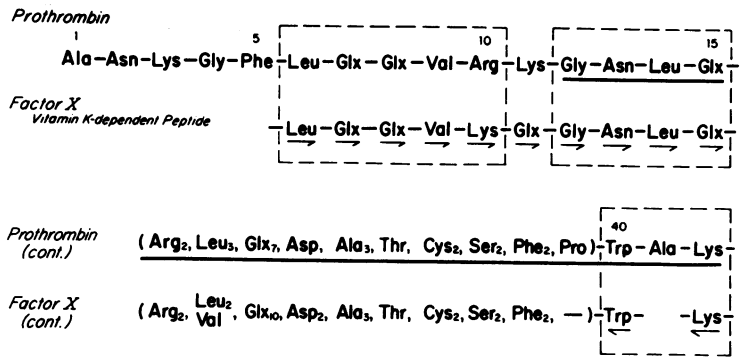

FIG. 2. Partial amino-acid sequence of the vitamin K-dependent, Ca^{2+}-binding region of factor X. The amino-terminal sequence was determined by sequential dansyl-Edman degradation (→) and the carboxyl-terminal region was determined by carboxypeptidase A + B digestion (←). Shown for comparison is the amino-terminal region of prothrombin (28). *The underlined portion* of the prothrombin sequence designates the barium citrate adsorbing peptide (vitamin K-dependent peptide) (22). The sequence for this peptide in prothrombin is -Gly-Asn-Leu-Glx-Arg-Glx-Glx-Leu-Glx-Glx-Pro-Cys-Cys-Arg-Glx-Glx-Ala-Phe-Leu-Glx-Ser-Leu-Ser-Ala-Asp-Thr-Ala-Phe-Trp-Ala-Lys-‡. All of the Glx in this portion of prothrombin and most or all of the Glx in this portion of factor X are γ-carboxyglutamic acid residues.

of three glutamic acid residues (probably as γ-carboxyglutamic acid) and one aspartic acid (or asparagine) residue, and the conservative replacement of one leucine by one valine residue.

The data presented here, comparing the vitamin K-dependent calcium-binding regions of prothrombin and factor X, lend support to the hypothesis that both proteins are descended from a common ancestral gene. Although there are several differences, it is evident that the calcium-binding region has been conserved. The noticeable difference between the two peptides is the larger size of the factor X tryptic peptide. This is a consequence of differences in the sequence at residues 10 and 11 of prothrombin, which results in a -Lys-Glx- sequence in factor X. From sequence studies on prothrombin vitamin K-dependent peptide, we know that such sequences are not susceptible to trypsin where Glx is γ-carboxyglutamic acid‡.

The fact that the calcium-binding region in factor X and prothrombin has been conserved suggests a structure-function role of this sequence. Although proline usually has a significant effect on the conformation of a peptide sequence, in the case of the vitamin K-dependent peptides, proline cannot be essential to the Ca^{2+}-binding conformation since the factor X peptide does not contain proline. In addition, the disulfide bridge is not essential for barium citrate adsorption (22), although it may be important for recognition of this region for the vitamin K-dependent carboxylation reaction. The most important structure-function relationship may be the presence of most of the γ-carboxyglutamic acid residues in pairs‡. Based upon model building, we have suggested that the γ-carboxyglutamic acids may not form an EDTA type of chelation of the Ca^{2+} ion, but possibly an extended cage involving up to three Ca^{2+} ions in the prothrombin peptide‡. This cage-like structure might explain the cooperative nature of Ca^{2+} binding (22, 27). The conservation of the amino-acid sequence in the region of the clustered γ-carboxyglutamic acid residues may provide further support for the extended cage structure of Ca^{2+} binding.

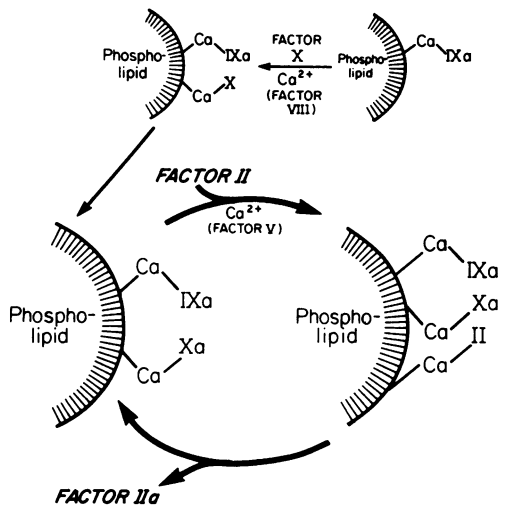

FIG. 3. Schematic representation of the role of Ca^{2+} binding and factors IX_a and X_a in the activation of prothrombin (factor II). Factors V and VIII are proteins whose exact roles in blood clotting are unknown.

Finally, the significance of the Ca^{2+} binding to blood coagulation should be emphasized (see Fig. 3). All three of the vitamin K-dependent blood clotting proteins from the intrinsic activation pathway have been studied with respect to mode of activation. Fujikawa *et al.* (10) recently reported that factor IX is converted to IX_a by the proteolytic removal of a peptide from the middle of the protein. This results in factor IX_a having two peptide chains linked by disulfide bond(s). Factor X has been shown to be activated in a similar way (4, 28). In both factor IX_a and X_a, the amino-terminal Ca^{2+}-binding region remains as an integral part of the activated protein (4, 10, 11, 28). Since Ca^{2+} binding is essential for binding the proteins to phospholipids, both coagulation factors are phospholipid-bound before and after activation. The activation of prothrombin (factor II) is different in that the active fragment of prothrombin (factor II_a or thrombin) is derived only from the carboxyl-terminal portion of prothrombin (29). The Ca^{2+}-binding amino-terminal region is removed during activation, which allows thrombin to leave the phospholipid surface (11, 29). Thus, these steps in the blood clotting process can be separated into two components: activation, which occurs on a phospholipid surface, and fibrin formation, which occurs in solution. Prothrombin conversion to thrombin appears to be the crossover point for the surface process to the solution process. The crossover point is de-

pendent on which bonds are cleaved during the activation process of the blood clotting factor, namely, whether the Ca^{2+}-binding region is retained as part of the activated protein or whether the Ca^{2+}-binding region is lost.

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