CHARACTERIZATION OF AMINO-TERMINAL FRAGMENT LIBERATED FROM BOVINE PROTHROMBIN BY ACTIVATED FACTOR X

Takashi MORITA, Sadaaki IWANAGA, Tomoji SUZUKI and Kazuo FUJIKAWA*

Division of Plasma Proteins, Institute for Protein Research, Osaka University, Suita, Osaka-565, Japan

and

**Department of Biochemistry, University of Washington, Seattle, Wash. 98195, USA*

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1. Introduction

Bovine prothrombin is a single chain polypeptide plasma glycoprotein having a molecular weight of approx. 72 000 [1]. Prothrombin seems to contain the thrombin part of the molecule in its *C*-terminal region [2] and the thrombin is released from the parent molecule by Factor Xa (thrombokinase) [3]. Because thrombin consists of two peptide chains with mol. wt of 5800 (A-chain) and 31 200 (B-chain), at least two proteolytic cleavages of the zymogen are required for the generation of thrombin from prothrombin, liberating one or more large fragments. Recently, several fragments, which appear accompanying the release of thrombin from the zymogen, have been detected by electrophoresis on SDS-polyacrylamide gel [4, 5]. However, knowledge regarding the exact origin of each of these fragments in the zymogen molecule is still limited. This paper describes the isolation and characterization of the *N*-terminal fragment liberated from prothrombin in the presence of Factor Xa.

2. Materials and methods

Bovine prothrombin was purified from citrated fresh plasma by a slight modification of the method of Ingwall and Scheraga [6]. The final purification step was chromatography on a Sephadex G-150 column under conditions similar to those used by Cox and Hanahan [7]. The specific activity of purified preparations measured according to the method of Magnusson [8] was 1050 NIH^+ units per mg protein. The purified material revealed a single component on SDS-gel electrophoresis and the *N*-terminal alanine was evidenced by the cyanate and PTC^+ methods. Bovine Factor X_1 was a purified preparation obtained by Fujikawa et al. [9] and the material was activated under the following conditions [10]. Fifty microlitres of Factor X_1 (0.14 mg/ml 0.15 M Tris–HCl buffer, pH 7.2) was incubated at 37°C with 50 μl of Russell's viper venom solution (10 μg per ml of 0.15 M NaCl) and 50 μl of 30 mM $CaCl_2$ for 20 min [10]. Sephadex G-150 was a product of Pharmacia, Uppsala, Sweden. SDS-polyacrylamide gel electrophoresis (8%) was done essentially by the method of Weber and Osborn [11]. Electrophoresis was performed at room temperature at a constant current of 7.0 mA per tube for 6 hr. During this time the marker dye, bromphenol blue migrated to within 50 mm from the bottom of the 60 mm-long gels. The gels were stained with Coomassie brilliant blue R-250. For amino acid analysis, the material (0.5 mg) was hydrolyzed for the indicated times in 1.0 ml of 5.7 N HCl at 110°C in an evacuated tube. Excess HCl was removed over KOH pellets in vacuo, and the residue was dissolved in 3 ml of 0.2 M sodium citrate buffer, pH 2.20. The analysis was performed with an amino acid analyzer, model JLC-5AH, Japan Electron Optics Lab. Ltd., by the method of Spackman et al. [12]. Hexoses were determined by the method of

* *Abbreviations*: RVV, *Russell's viper* venom; SDS, sodium dodecyl sulfate; PTC, phenyl*iso*thiocyanate; NIH, National Institutes of Health.

Dubois [13] and hexosamine was determined by the method of Gardell [14]. For the determination of sialic acid, the periodate–thiobarbituric acid method of Warren [15] and the resorcinol method of Svennerholm [16] were used; Amino-terminal analysis was performed by the cyanate [17] and phenyl*iso*thiocyanate [18] methods. PTH-amino acids were identified by thin layer chromatography [19]. Carboxyl-terminal residues were analyzed by the methods of hydrazinolysis [20] and carboxypeptidase digestion [21].

3. Results

3.1. *Preliminary studies on large fragments released from prothrombin during the activation by Factor Xa*

To 2.0 ml (4.16 mg) of prothrombin dissolved in 0.15 M NaCl was added 0.1 ml of Factor Xa solution and the mixture was incubated at 37°C for indicated times. These conditions have been arranged to retard a rapid activation of prothrombin by Factor Xa. At several intervals, 100 μl aliquots of the incubation mixture were taken and mixed with 100 μl of acetic acid. The mixtures were dialyzed overnight against 0.2 M acetic acid and subsequently lyophilized. The dried materials were dissolved in 1% SDS and subjected to SDS-gel electrophoresis. The results are shown in fig. 1. The molecular weights shown in the figure were estimated from the mobilities on 8% gels, referring the calibration curve prepared with bovine serum albumin, ovalbumin, α-chymotrypsinogen, myoglobin, cytochrome *c* and α-bungarotoxin. The gel at zero time, which was stained with Coomassie blue, showed a single prothrombin band with mol wt of 73 000 (±2000). After incubation for 30 min, prothrombin had been cleaved to an intermediate of 57 000 (±2000) daltons, liberating a definite amount of fragment with 28 000 (±1000) daltons. Within 4 hr, the prothrombin was completely degraded and the intermediate and the fragment with 28 000 daltons had accumulated in the reaction mixture. At this stage thrombin activity could scarcely be detected in the reaction mixture, suggesting no release of the thrombin molecule from precursor protein. Prolonged incubation, however, induced the degradation of the intermediate, resulting in generation of thrombin, presumably corresponding to the fragment with 39 000 daltons on the gels. In fact, strong thrombin activity was evidenced in the reaction mixture after incubation for 21 hr. It should be noted that the fragment with 28 000 daltons

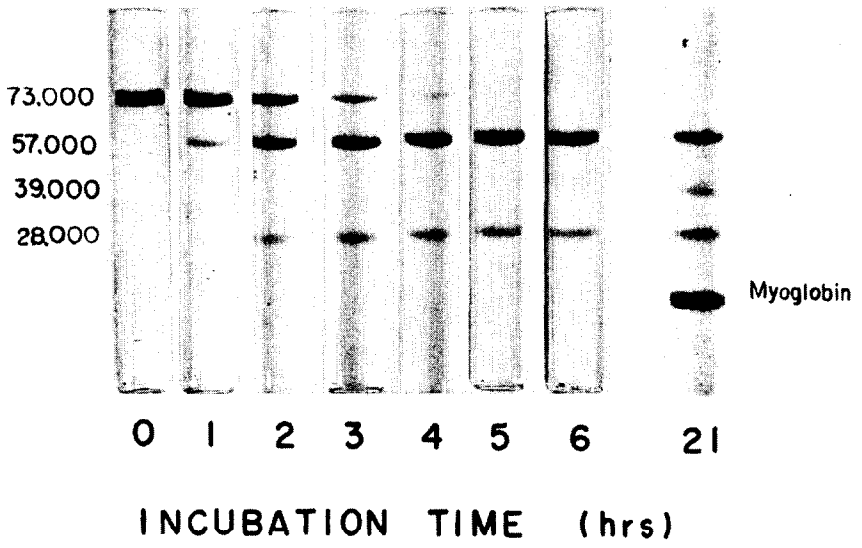

Fig. 1. SDS-gel electrophoresis of reaction products of bovine prothrombin with Factor Xa at various stages. The conditions and procedures used in this experiment were described in the text. Electrophoresis was done in 8% polyacrylamide gel for 6 hr using a current of 7 mA per tube.

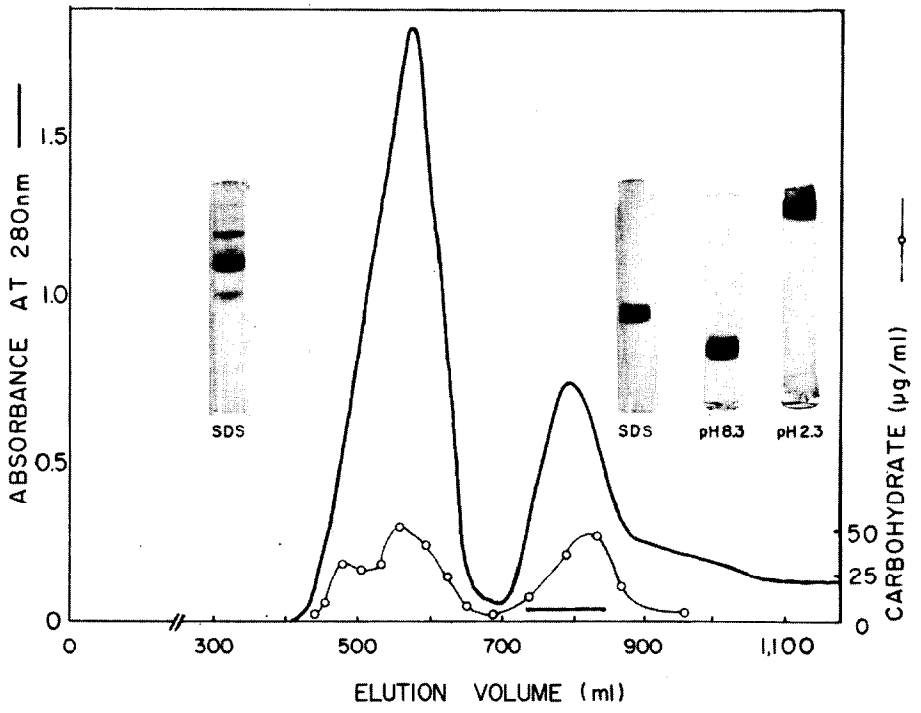

Fig. 2. Separation of reaction products of bovine prothrombin with Factor Xa on a Sephadex G-150 column. Bovine purified prothrombin (240.2 mg) dissolved in 120 ml of 0.15 M NaCl was incubated at 37°C with 6 ml of Factor Xa solution containing 280 μg of Factor X, 2 μg of RVV, 60 μmoles of $CaCl_2$ and 300 μmoles of Tris–HCl buffer, pH 7.2. After incubation for 4 hr, the reaction was terminated by adding 13.3 ml of acetic acid and the mixture was then lyophilized. The freeze-dried material was first dissolved in a small amount of 50% acetic acid and diluted to 30 ml with distilled water to give a final concentration of 30% acetic acid. The solution was applied to a column (4.0 × 127 cm) of Sephadex G-150, equilibrated with 30% acetic acid. The elution was performed with 30% acetic acid at a flow rate of 30 ml/hr. SDS-polyacrylamide gel (10%) electrophoresis on the components in peak 1 and 2 made with the same conditions as those of fig. 1, and the electrophoresis at pH 8.3 and 2.3 was performed according to the method of Davis [23].

was still detected on the gels even at later stages when the intermediate had disappeared completely. This fragmentation sequence of the prothrombin molecule with Factor Xa was very reproducible under the conditions used.

3.2. *Separation of the fragments from prothrombin during the activation by Factor Xa*

A large scale preparation of the fragments from prothrombin was performed under the same conditions as those used in the preliminary study. A weight ratio of prothrombin to Factor Xa was 900:1. After incubation for 4 hr, the incubation mixture was immediately lyophilized, dissolved in 30% acetic acid and applied to a column (4 × 127 cm) of Sephadex G-150, equilibrated with 30% acetic acid. The elution pattern is shown in fig. 2. Judging from the electrophoretic patterns on SDS-gels, the major components eluted in the first and second peaks should be the intermediate with 57 000 daltons and the fragment with 28 000 daltons, respectively. Especially the latter component seemed to be electrophoretically pure, as indicated by the patterns at pH 8.3 and 2.3. The fractions eluted later than 950 ml were ninhydrin negative, indicating the absence of peptide-like material. These results prompted us to characterize the fragment with 28 000 daltons.

Table 1
Amino acid and carbohydrate compositions of *N*-terminal fragment derived from bovine prothrombin.

Amino acid	24 hr	48 hr	72 hr	Av. or extrap. value	Nearest integer
	(Residues per mole)				
Aspartic acid	11.05	11.29	11.37	11.24	11
Threonine	7.22	7.12	6.90	7.08	7
Serine	7.62	7.04	6.52	8.00	8
Glutamic acid	17.24	17.48	17.56	17.42	17
Proline	7.86	7.88	7.72	7.82	8
Glycine	8.61	8.76	8.86	8.74	9
Alanine	7.79	8.19	8.12	8.03	8
Half-cystine*	6.85	6.53	6.06	6.48	6
Valine	7.35	8.31	7.74	7.80	8
Methionine	0.96	1.03	1.05	1.01	1
Isoleucine	2.91	3.01	3.06	2.99	3
Leucine	7.89	8.03	8.03	7.98	8
Tyrosine	2.95	2.93	2.91	2.93	3
Phenylalanine	3.05	3.07	3.09	3.07	3
Histidine	1.62	1.57	1.62	1.61	2
Lysine	3.91	3.95	4.05	3.97	4
Ammonia	15.27	18.86	19.24	17.79	(18)
Arginine	11.67	12.11	11.82	11.87	12
Tryptophan**				3.43	3
Total					121

Hexoses	8.3–9.9%
Glucosamine	5.2–6.1%
Sialic acid	3.2–3.8%***
	3.4–3.6%†

* This value was determined as cystine. The measurement of cysteic acid after performic oxidation of the material showed presence of 6 residues of half-cystine.
** Determined by ultraviolet analysis [25].
*** Determined by the methods of Warren [15] and Svennerholm [16], respectively.
†

3.3. *Chemical composition of the N-terminal fragment derived from prothrombin*

The terminal residues, amino acid, and carbohydrate compositions of the fragment with 28 000 daltons were determined. On Edman degradation, the fragment was found to have a single *N*-terminal alanine and subsequent analysis revealed the sequence, Ala–Asp–Lys–Gly–Phe–Leu–. These results indicated that the fragment must be derived from the *N*-terminal end of the prothrombin molecule, because the first six residues of prothrombin have recently been reported to be Ala–Asn–Lys–Gly–Phe–Leu– [22]. On hydrazinolysis the fragment liberated *C*-terminal arginine and also the main amino acids released in the initial stage of digestion with carboxypeptidases B was arginine. The amino acid composition of the fragment determined with 24, 48 and 72 hr hydrolyzates showed a total of 121 residues, indicating a minimum polypeptide molecular weight of 13 700. The material contained relatively large amounts of glutamic acid, arginine and aspartic acid, which together constituted nearly 35% of the total amino acids residues (table 1). Moreover, the fragment contained covalently linked sugar moieties consisting of hexoses (9.1%), glucosamine (5.7%) and sialic acid (3.7%). Since a total residue weight of those sugar moieties could be calculated to be approx. 5000, the probable total molecular weight of the fragment seems to be around 18 700.

4. Discussion

The experiments reported here are an attempt to identify the initial reaction products released in the generation of thrombin from prothrombin with Factor Xa. Thus, the reaction has been retarded using the conditions with absence of Factor V abd phospholipids which amplify the thrombin generation. As judged from the SDS-gel electrophoretic patterns obtained from the reaction mixture, the prothrombin molecule appears to be first degraded into two large fragments of 57 000 and 28 000 daltons. The former seems to include the thrombin molecule, since it disappears in the later stages when thrombin of 39 000 daltons appears on the SDS-gel patterns at the same time as strong clotting activity is observed in the reaction mixture. The latter fragment, on the other hand, must include the *N*-terminal portion of prothrombin molecule, since the *N*-terminal sequence of the first 6 residues was identical with that of the zymogen [22]. The *N*-terminal fragment consists of a total of 121 amino acid residues and 18.5% carbohydrate including hexoses, glucosamine and sialic acid. The molecular weight of fragment based on the chemical compositions could be calculated to be about 18 700, differing from the mol wt of 28 000 estimated by SDS-gel electrophoresis. Thus, polyacrylamide gel electrophoresis in the presence of SDS indicates a somewhat higher molecular weight, due to a glycoprotein nature of the *N*-terminal fragment.

These results led to the conclusion that the process of thrombin generation from prothrombin with Factor Xa could be initiated as a limited proteolysis at the *N*-terminal region of the molecule, liberating two large fragments of 57 000 and 18 700 daltons. The characterization of the 57 000 dalton intermediate released at the same time is now in process.

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