162. Activation Mechanism of Bovine Prothrombin to Thrombin by Activated Factor X

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Bovine prothrombin of 72,000 daltons is a zymogen of the clotting enzyme, thrombin. During the activation of prothrombin by Factor Xa, the thrombin molecule is released from the C-terminal region of parent molecule together with other large fragments. As reported previously, the process of thrombin generation from prothrombin with Factor Xa is initiated by a limited proteolysis at the N-terminal portion of the parent molecule, liberating two large fragments of 57,000 and 18,700 daltons. This paper describes subsequent studies on the chemical characterizations of fragments produced when prothrombin is converted to α-thrombin by Factor Xa. In addition, the overall activation mechanism of prothrombin to α-thrombin will be proposed.

Materials and methods. Bovine prothrombin purified from citrated fresh plasma was used. The specific activity of the preparation measured by the method of Magnusson was 1,200 NIH units per mg protein. Purified bovine Factor Xa was a generous gift from Dr. K. Fujikawa, Dept. of Biochemistry, University of Washington, Seattle. The material was activated with the venom of Vipera russelli under the conditions described previously. Sephadex G-150 was a product of Pharmacia, Uppsala, Sweden. Disc polyacrylamide gel electrophoresis in the presence or absence of sodium dodecyl sulfate (SDS) was made essentially by the methods of Weber and Osborn and Davis. The gels were stained with Coomassie brilliant blue R-250. Amino acid analysis was performed by the method of Spackman et al. with an amino acid analyzer, Model JLC-5AH from Japan Electron Optics Lab., Ltd. Hexose and hexosamine were determined, respectively, by the methods of Dubois and Gardell. For determination of sialic acid, the periodate-thiobarbituric acid method of Warren was used. N-Terminal analysis was performed by the cyanate, 2, 4-dinitrofluorobenzene and phenylisothiocyanate

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methods, and the phenylthiohydantoin-derivatives were estimated quantitatively from their U.V. absorption at 269 m\(\nu\) and identified by thin layer chromatography. Molecular weights were estimated from electrophoretic mobilities on 10% SDS-gels, referring to calibration curves prepared with bovine serum albumin, ovalbumin, chymotrypsinogen A, myoglobin, cytochrome c and \(\alpha\)-bungarotoxin.

**Results.** Prothrombin (0.6 mg) dissolved in 0.15 M NaCl was incubated at pH 7.2 at 37° with Factor Xa (5 \(\mu\)g) in a total volume of 0.35 ml. Aliquots of the mixture were taken at intervals. Half of each was used to estimate of thrombin and the other half was subjected to SDS-gel electrophoresis after dialysis and lyophilization. In parallel with the rapid formation of thrombin activity, prothrombin was fragmented into four fragments of 57,000, 39,000, 28,000 and 14,000 daltons. The fragment of 57,000 disappered in course of time. The maximum formation of thrombin under the conditions used was achieved after incubation for 30 hrs. Based on these results, large scale fragmentation of prothrombin (200 mg) with Factor Xa (0.94 mg) was performed and the resulting products were separated by chromatography on a Sephadex G-150 column. Through this procedure, three major fragments having the molecular weights of 39,000, 28,000 and 14,000 were isolated. The characterizations of these fragments were performed by analyses on the N-terminal sequences and amino acid and carbohydrate compositions as follows.

**Fragment of 39,000 daltons (±2,000).** This fragment gave a single band on SDS-gel electrophoresis, but the SDS-gel pattern after reduction of the sample with \(\beta\)-mercaptoethanol showed the presence of three major fragments, which were estimated to have molecular weights of 39,000, 30,000 and 6,000. Among them, the fragment of 39,000 daltons, which was the same as that of the unreduced sample, appeared to be \(\alpha\)-thrombin precursor consisting of a single polypeptide chain (intermediate 2 in Fig. 1), and the other two seemed to correspond to the A-chain of 5,800 daltons and B-chain of 31,200 daltons of the \(\alpha\)-thrombin molecule. The amino acid composition of the unreduced sample was indistinguishable from that of \(\alpha\)-thrombin (Table I). Moreover, N-terminal analysis of the unreduced sample revealed the presence of threonine and isoleucine (Table II), which must be derived, respectively, from the N-terminal ends of the A- and B-chains of \(\alpha\)-thrombin.

**Fragment of 28,000 daltons (±2,000).** This fragment gave a single band on electrophoresis on SDS-gel and disc-gel at pH 8.3. The N-terminal residue was alanine and further analysis revealed the sequence Ala-Asn-Lys- (Table II). Moreover, the amino acid and carbohydrate compositions (Table I) were identical to those pre-
Table I. Amino acid and carbohydrate compositions of bovine prothrombin and its fragments

| Amino acid and carbohydrates | Prothrombin | Present authors\(^a\) | \(\alpha\)-Thrombin Mann et al.\(^21\) |
|-----------------------------|-------------|------------------------|----------------------------------|
|                             | Man et al.\(^21\) | Cox and Hanahan\(^20\) | Prothrombin | Sum of three fragments | N-terminal fragment | Inner fragment | Intermediate 2+ \(\alpha\)-thrombin |
| Aspartic acid               | 59          | 55                     | 57         | 59                | 11               | 16          | 32          | 30          |
| Threonine                   | 27          | 26                     | 26         | 26                | 7                | 4           | 15          | 15          |
| Serine                      | 32          | 32                     | 32         | 32                | 8                | 8           | 16          | 14          |
| Glutamic acid               | 71          | 67                     | 67         | 67                | 17               | 13          | 37          | 41          |
| Proline                     | 34          | 32                     | 35         | 33                | 8                | 8           | 17          | 19          |
| Glycine                     | 46          | 44                     | 47         | 47                | 9                | 10          | 28          | 27          |
| Alanine                     | 33          | 31                     | 33         | 33                | 8                | 9           | 16          | 15          |
| 1/2 Cystine                 | 17          | 17                     | 18         | 18                | 6                | 4           | 8           | 6           |
| Valine                      | 35          | 32                     | 33         | 35                | 7                | 5           | 23          | 21          |
| Methionine                  | 6           | 5                      | 6          | 7                | 1                | trace       | 6           | 4           |
| Isoleucine                  | 18          | 18                     | 18         | 20                | 3                | 1           | 16          | 14          |
| Leucine                     | 46          | 42                     | 45         | 46                | 8                | 9           | 29          | 28          |
| Tyrosine                    | 19          | 17                     | 19         | 17                | 3                | 3           | 11          | 11          |
| Phenylalanine               | 19          | 18                     | 19         | 20                | 3                | 3           | 14          | 14          |
| Histidine                   | 9           | 8                      | 9          | 10                | 2                | 1           | 7           | 8           |
| Lysine                      | 29          | 29                     | 32         | 30                | 4                | 3           | 23          | 25          |
| Arginine                    | 42          | 43                     | 42         | 40                | 12               | 7           | 21          | 20          |
| Tryptophan                  | 11          | 11                     | (11)       | (11)              | (8)              |             |             |             |
| **Total**                   | 553         | 527                    | 552        | 551               | 120              | 104         | 327         | 322         |

\(\text{a) The amino acid compositions of the fragments estimated by us were calculated from extrapolated or average values estimated on samples of 24, 48 and 72 hrs hydrolyzates, except for that of prothrombin which was calculated from the values analyzed on a sample of the 24 hr hydrolyzate. b) Taken from the data of Nelsestuen and Suttie.}\(^{19}\) \(c)\) The molecular weights based on chemical analyses were obtained from the sum of the total amino acid and carbohydrate residues. d) Taken from the data of Magnusson.\(^{11}\)
Previously found in the N-terminal fragment of prothrombin.5)

Fragment of 14,000 daltons (±2,000). This fragment gave a single band on disc-gel electrophoresis at pH 8.3, and on electrophoresis on SDS-gel. However, the electrophoretic pattern on disc-gel at pH 4.0 indicated the existence of three components with slightly different mobilities. These components were partially separated by an isoelectric focusing method and their amino acid compositions were found to be the same. Thus, the difference in their electrophoretic mobilities may be due to microheterogeneity in their carbohydrate moieties. The fragment contained N-terminal serine, as identified by the DNP method, while stepwise Edman degradation revealed the sequence Ser-Gly-Gly (Table II). Its amino acid and carbohydrate compositions were found to be quite different from those of the two fragments described above (Table I).

Table I also shows the amino acid and carbohydrate compositions of bovine prothrombin and α-thrombin obtained by two other groups. The data indicate that the sum of the total amino acid residues of the three-fragments is in good agreement with those of the parent molecule analyzed by the three groups.

Discussion. The results presented here indicate that three major fragments are produced when prothrombin is converted to α-thrombin by Factor Xa. One of these fragments, which has a molecular weight of 39,000, must be α-thrombin, as judged from its N-terminal residues and amino acid composition. The fragment contains an additional component, consisting of a single polypeptide chain of the same size.
as that of \(\alpha\)-thrombin. This component could be a precursor molecule of \(\alpha\)-thrombin (intermediate 2 in Fig. 1), as its S-carboxymethyl derivative has threonine as the single N-terminal (Table II).

The second fragment of 28,000 daltons must be derived from the N-terminal region of prothrombin, because the first three amino acids in its N-terminal sequence and its amino acid and carbohydrate compositions are identical with those of the N-terminal fragment of prothrombin characterized previously.\(^5\)

The third fragment of 14,000 daltons with N-terminal serine seems to be derived from the N-terminal portion of the intermediate of 57,000 daltons (intermediate 1 in Fig. 1) reported previously.\(^5\) This was concluded because production of the fragment was accompanied by disappearance of the intermediate 1 and because the N-terminal sequence of Ser-Gly-Gly- of the fragment was identical to that of the intermediate 1 (Table II). The fragment had a relatively high carbohydrate content, suggesting the presence of one oligosaccharide chain per mole of fragment.

In conclusion there should be at least three peptide-bond cleavages associated with the activation of prothrombin yielding \(\alpha\)-thrombin, an N-terminal fragment and an inner fragment. The results also indicate that there is no disulfide bridge linking these three fragments, and that of the four oligosaccharide chains in the prothrombin molecule,\(^19\) two must be located in the N-terminal fragment and the other two in the inner fragment and \(\alpha\)-thrombin, respectively. Thus, based on the results described above and previous studies,\(^5\) the processes of prothrombin activation with Factor Xa in the absence of Factor V and phospholipids may be deduced as shown in Fig. 1.

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