THE ACTIVATION OF PROTHROMBIN III. THE PARTIAL AMINO ACID SEQUENCES AT THE AMINO TERMINAL OF PROTHROMBIN AND THE INTERMEDIATES OF ACTIVATION*

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Summary

The partial amino acid sequences at the amino terminal of prothrombin and the intermediates of activation have been determined. These data indicate that the products of the first step of activation, whether derived from the action of factor Xa or thrombin, are identical. The data also show that the activation of prothrombin proceeds by the sequential cleavage of the amino terminal region of prothrombin and the intermediates, and confirm the mechanism of prothrombin activation as: NH_2 - Prothrombin-COOH $\xrightarrow{\text{Xa or thrombin}}$ NH_2-Intermediate 3 + Intermediate 1 - COOH; NH_2-Intermediate 1-COOH $\xrightarrow{Xa}$ NH_2-Intermediate 4 + Intermediate 2-COOH; NH_2-Intermediate 2-COOH $\xrightarrow{Xa}$ NH_2-A chain α-thrombin -S-S-B chain α-thrombin-COOH.

Previous reports from this laboratory have demonstrated that the activation of prothrombin proceeds through several single-chain intermediates prior to the appearance of thrombin activity. (1) Subsequent studies have

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demonstrated that the action of factor Xa on prothrombin produced these intermediates (intermediates 1, 2, 3, and 4) and have reported their isolation and partial characterization (2,3). The action of thrombin on prothrombin results in the production of only two products, intermediates 1_t and 3_t [6], which are physically and chemically identical to their factor Xa produced counterparts, intermediates 1 and 3 respectively (3-6). Analysis of the partial activation reactions, and of physical and chemical studies of the intermediates, have permitted the formulation of a structural model for the prothrombin molecule and the prothrombin activation process (3-6).

Prothrombin (NH_2 terminal-Alanine) is cleaved by either factor Xa or thrombin to yield intermediate 3 (NH_2 terminal-Alanine) from the amino terminal, and intermediate 1 (NH_2 terminal-Serine) from the carboxyl terminal portion of the prothrombin molecule. Subsequent activation steps are effected only by factor Xa. Intermediate 1 is cleaved by factor Xa to yield intermediate 4 (NH_2-terminal-Serine) from its amino terminal, and intermediate 2 (NH_2 terminal-Threonine) from its carboxyl terminal segment. Intermediate 2, the single chain immediate thrombin precursor, is then cleaved by factor Xa to yield the disulfide-linked two chain α-thrombin[7] molecule, with the 6,000 dalton A chain (NH_2 terminal-Threonine (7)) comprising the amino terminal segment and the 33,000 dalton B chain (NH_2 terminal-Isoleucine (7)) the carboxyl terminal segment of the intermediate 2 molecule. The mechanism may be schematically written as follows:

		NH_2 Terminal		COOH Terminal
Prothrombin	$\xrightarrow{\text{Xa or thrombin}}$	Intermediate 3	+	Intermediate 1

6. The subscript 't' is used to denote the catalytic origin of the thrombin produced intermediate 1 and intermediate 3.

7. The nomenclature of thrombin structural forms is as summarized by Mann, et al.(11) The term thrombin when used without a Greek letter prefix denotes an intermediate mixture of all thrombin structural forms, α-, β-, and γ-.

TABLE 1

PARTIAL AMINO ACID SEQUENCES AT THE AMINO TERMINALS OF PROTHROMBIN AND INTERMEDIATES

Residue	II	3	3t	1	1_t	4	2	A^1 α-IIa
1	Ala	Ala	Ala	Ser	Ser	Ser	Thr	Thr
2	-	-	Asn	Gly	-	Gly	Ser	Ser
3	-	Lys	Lys	Gly	-	Gly	Glu	Glu
4	Gly	Gly	Gly	Ser	Ser	Ser	Asp	Asn
5	Phe	Phe	Phe	-	-	Thr	His	His
6	Leu	Leu	Leu	Thr	-	Thr	Phe	Phe
7	-	-	Glu	Ser	Ser	Ser	Glu	Glu
8	-	-	Glu	Glu	Glu	Glu	Pro	Pro
9	Val	Val	Val	-	Ser	Ser	Phe	Phe
10	-	-	-	Pro	Pro	Pro	Phe	Phe
11	-	Lys	Lys	Leu	Leu	Leu	Asn	Asn
12	Gly	Gly	Gly	Leu	Leu	Leu	Glu	Glu
13	Asn	Asn	Asn	-	Glu	Glu	-	Lys
14	Leu	Leu	Leu	-	Thr	Thr	Thr	Thr
15	-	-	Glu	-	-	-	Phe	Phe
16	-	-	-	Val	Val	Val	Gly	Gly
17	-	-	Glu	Pro	-	Pro	Ala	Ala
18	-	Asp	-	-	-	-	-	Gly
19	Leu	Leu	-	Arg	Arg	-	-	Glu
20	-	-	-	-	Gly	Gly	Ala	Ala
21	Glu	Glu	Glu	-	-	-	Asn	Asp
22	Pro	Pro	Pro	-	Glu	-	-	Cys
23	-	-	-	Tyr	Tyr	Tyr	-	Gly

1. Sequence is from Magnusson (4).

Intermediate 1 $\xrightarrow{Xa}$ Intermediate 4 + Intermediate 2

Intermediate 2 $\xrightarrow{Xa}$ A chain α-thrombin B chain α-thrombin (S–S)

We have determined the amino acid sequences at the amino terminal of prothrombin, the factor Xa produced intermediates (1, 2, 3, and 4) and the thrombin produced intermediates (1_t and 3_t) in order to establish clearly the coidentity of the factor Xa and thrombin produced intermediates, and to confirm the alignment of the intermediates within the prothrombin molecule.

Materials and Methods

Bovine prothrombin was purified by the method of Bajaj and Mann (8).

The intermediates of activation were purified as described by Heldebrant and Mann (2), and Heldebrant et al. (3).

Automated Edman degradations (9) were performed on a Beckman model 890 B Sequencer, using the Quadrol program supplied by the manufacturer. The phenylthiohydantoin amino acids liberated after each cycle of the degradation were identified as such or as the trimethylsilyl derivative by gas chromatography (10) on a Beckman GC-45 gas chromatograph.

Results

The partial amino terminal amino acid sequences of prothrombin (II) and the intermediates of activation - 1, 1_t, 2, 3, 3_t, and 4, and of the A chain of α-thrombin (4) are shown in Table 1.

It is apparent that the thrombin produced intermediates, 1_t and 3_t, are identical to their factor Xa produced counterparts, 1 and 3, in terms of amino terminal amino acid sequence. These data are in agreement with the previously reported coidentity of amino acid compositions, molecular weights and amino terminal amino acids for intermediates 1 and 1_t and intermediates 3 and 3_t (3-6).

The data indicate that prothrombin, intermediate 3, and intermediate 3_t have the same amino terminal sequences, and thus intermediate 3 (3_t), as predicted, occupies the amino terminal portion of the prothrombin molecule. Intermediate 1, 1_t, and intermediate 4 have the same amino terminal sequences, and hence as predicted, intermediate 4 occupies the amino terminal portion of the intermediate 1 (1_t) molecule. Intermediate 2 has the same sequence as the A chain of α-thrombin (4) and hence the A chain of α-thrombin comprises the amino terminal portion of intermediate 2. These data are in full agreement with and confirm the proposed mechanism.

The only discrepancies between the data reported here for intermediate 2 and the data reported for the A-chain of thrombin by Magnusson (7) are the amide assignments at positions 4, 7, and 21. Since such assignments are known to be difficult when attempted on small piptides subjected to

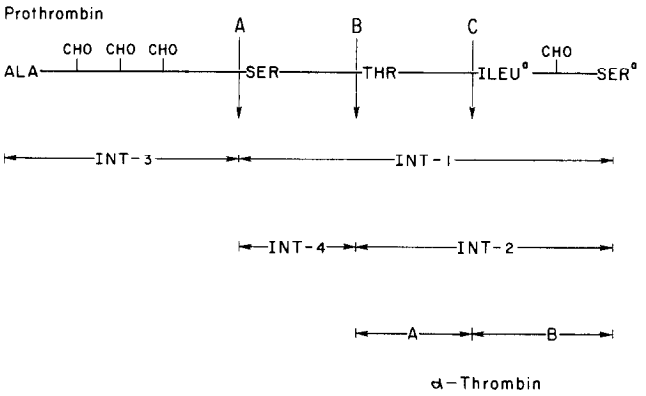

Figure 1: Schematic structural model of prothrombin. CHO represents a carbohydrate side chain. A, B, and C indicate the order in which cleavages occur during the activation of prothrombin.

extensive purification and sequenced by subtractive Edman, and since we have had much better results using automated Edman and direct identification of PTH-amino acids by gas-liquid chromatography, we feel that our data, although preliminary, are probably correct.

The amino terminal data presented in Table 1 for prothrombin and the activation intermediates permit the schematic diagram presented in figure 1 for prothrombin activation to be drawn.

It should be noted again that neither intermediate 3 nor intermediate 4 contribute substance to the thrombin molecule; all of the amino acids in α-thrombin are derived directly from intermediate 2.

It should also be noted that the mechanism shown above is different from that originally suggested by our laboratory (1-2). Our initial hypothesis, which relied heavily upon the kinetics of intermediate production and sodium dodecysulphate molecular weight analysis, suggested that intermediate 3 was derived from either prothrombin directly or from intermediate 1, and did not include the subsequently observed intermediate 4. These studies confirm the mechanism of prothrombin activation and orientation of intermediates within the prothrombin molecule which we have recently proposed (3-6). The results indicate that the total amino acid

sequence of the prothrombin molecule can be deduced from the sequences of its activation intermediates and we are continuing our studies toward this goal.

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