Isolation and Characterization of an Active Three-Chain Molecular Species of Bovine Thrombin

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Partially purified bovine prothrombin was activated in half-saturated trisodium citrate seeded with thrombin, and the resulting thrombin was chromatographed on Amberlite IRC-50, followed by rechromatography on DEAE-Sephadex A-50. Five fractions, possessing both esterase and clotting activities, were partially isolated, but fraction VI was shown to be a pure three-chain active species with threonine, isoleucine and lysine, in 1:1:1 molar proportions as *N*-termini. The amino acid composition and *C*-termini of fraction VI were determined. The molecular weights of the isolated chains, as determined by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, were 7300, 12000 and 19500 respectively. These data, when taken together with the amino acid sequence of the two-chain thrombin reported by Magnusson *et al.* (1975) [in *Prothrombin and Related Coagulation Factors* (Hember, H. C. & Veltkamp, J. J., eds.), pp. 25–46, Leiden University Press, Leiden], indicated that proteolysis occurred at the Arg₍₇₈₎–Lys₍₇₉₎ peptide bond of the B chain of a precursor molecular species, thus converting this two-chain species into the three-chain active form described here.

Thrombin (EC 3.4.21.5) is a serine proteinase which plays a multiple role in blood clotting. It functions in the activation of factor XIII (Lorand & Konishi, 1964) and probably also of factors V and VIII, as well as in platelet aggregation (Shermer *et al.*, 1961) and in cleaving fibrinogen. It consists basically of two disulphide-linked chains (Magnusson, 1971), although one of these may undergo cleavage at the peptide bond after arginine-75 or arginine-78 (Glover & Shaw, 1971; see also Magnusson *et al.*, 1975) to give an active three-chain form of thrombin, the isolation of which has not yet been reported.

The identification of lysine as a third *N*-terminal residue of our thrombin preparations has been briefly reported (Rossi & Giglio, 1969). The present paper describes the isolation of an electrophoretically pure three-chain form of bovine thrombin, possessing threonine, isoleucine and lysine as *N*-termini, from partially purified prothrombin, and reports on some of its properties.

Experimental

Materials

The following were purchased from the sources indicated: protamine sulphate (grade 1), trypsin (type 1, twice crystallized, approx. 10000 Bz-Arg-

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OEt[†] units/mg of protein), phenylmethanesulphonyl fluoride, Tos-Arg-OMe, HCl, bovine serum albumin (crystalline, catalogue number 850–100), 2,4-dinitrofluorobenzene (grade 1), dinitrophenyl-amino acids, ovalbumin (crystalline, grade VI), carboxypeptidase A (lot number 49B-2321), α -chymotrypsinogen A (crystallized, type II) and cytochrome *c* (from horse heart, type VI) were from Sigma Chemical Co., St. Louis, MO, U.S.A.; toluene-*p*-sulphonic acid was from Baker Chemical Co., Phillipsburg, NJ, U.S.A.; 3-(2-aminoethyl)indole was from Eastman Organic Chemicals, Rochester, NY, U.S.A.; Amberlite IRC-50 was from Rohme and Haas, Philadelphia, PA, U.S.A.; DEAE-Sephadex A-50 (3.5 meq./g, bead size 40–120 μ m) was from Pharmacia Fine Chemicals, Uppsala, Sweden; carboxypeptidase B (198 units/mg) was from Worthington Biochemical Corp., Freehold, NJ, U.S.A. All other reagents used without previous purification were of analytical grade.

Methods

Purification of reagents. Fibrinogen (95% clottable protein) from bovine plasma, was prepared by the method of Blombäck & Blombäck (1957), and hydrazine, 98–100% (w/v) purity, by redistilling a commercial 85% product (Carlo Erba, Milan, Italy) as described by Smith & Howard (1944), titration being performed with standard KBrO₃ solution (Kolthoff & Belcher, 1957).

† Abbreviations: Bz-Arg-OEt, α -*N*-benzoyl-L-arginine ethyl ester; Tos-Arg-OMe, *N*-tosyl-L-arginine methyl ester.

Enzyme purification. Partially purified non-chromatographed bovine prothrombin was prepared as previously described for pig prothrombin (Leone *et al.*, 1976), except that isoelectric precipitations at pH 5.30 and 4.55 (Malhotra & Carter, 1968) were carried out after fractionation with $(\text{NH}_4)_2\text{SO}_4$ and dialysis, at 2°C, against water, until precipitation of BaSO_4 by 1M- BaCl_2 in the dialysis solution was no longer visible (approx. 15h). The final orange-yellow sticking mass was dissolved in a few millilitres (5ml for a batch of 10 litres of original plasma) of 2.5M- NH_3/HCl buffer, pH 8.0. All further steps of enzyme purification, unless otherwise stated, were carried out at 2°C.

Activation. The above solution was immediately mixed with the same volume of saturated (at 22°C) trisodium citrate, and 0.1–0.5ml of a thrombin solution, containing 500 Tos-Arg-OMe units/litre of original plasma, was added. For a batch of 10 litres of plasma, the final activation mixture was about 12ml. Activation was performed at 22°C and was followed by measuring the esterase activity at 37°C as previously described (Rossi & Giglio, 1974). The activated mixture was then dialysed against a 0.05M-phosphate buffer, pH 7.0, and processed for chromatography, with or without previous inhibition. Inhibition was performed with phenylmethanesulphonyl fluoride (Lundblad, 1971) in batches of about 2×10^6 Tos-Arg-OMe units to which 520mg of inhibitor, in 10ml of anhydrous propan-2-ol, was slowly added, the pH being maintained at 9.0 by titration with 1M-KOH at room temperature (25°C) for 1h. Inhibition was taken to a level of 99–100%. If not, more inhibitor was added.

Chromatography. Amberlite IRC-50 was washed and equilibrated in 0.05M-potassium phosphate buffer, pH 7.0, as previously described (Rossi & Giglio, 1974). Elution was started with the same buffer, followed by 0.20M and 0.30M buffers, both at pH 8.0. Alternatively, the resin and sample were equilibrated in 0.01M-Tris/HCl, pH 8.0, and elution was started with this buffer, followed by two others containing 0.20M- and 0.45M-KCl respectively, in 0.01M-Tris/HCl, pH 8.0. In both cases, each buffer was replaced after eluting a corresponding fraction. The thrombin fraction (peak III) was precipitated with 1 vol. of acetone at -20°C, centrifuged at 23500g for 10min in a refrigerated RC2-B Sorvall centrifuge, dissolved in the smallest volume of water, dialysed against 0.01M-potassium phosphate, pH 7.0, centrifuged and rechromatographed on DEAE-Sephadex A-50.

Manufacturers' recommended procedures were followed for preparation of DEAE-Sephadex A-50 and packing of columns. Initial equilibration was in 0.01M-potassium phosphate buffer, pH 7.0, against which the sample was dialysed and cleared as above, followed by 0.02M and 0.15M buffer, pH 7.0. Fraction

VI was eluted by this last buffer and then concentrated and desalted by ultrafiltration. The final solution (5–10ml) was cleared by centrifugation, and the protein precipitated by 1 vol. of acetone at -20°C. For N-terminal analysis, fraction VI was simply precipitated, without previous concentration, with 0.5 vol. of 20mM- HgCl_2 at 0°C, centrifuged at 3000g for 10min, dissolved in a few millilitres of 0.4M-EDTA, pH 8.0, dialysed for 15h at 2°C against water and freeze-dried. Thrombin so obtained is water-soluble, but is precipitated immediately in the presence of Tos-Arg-OMe or fibrinogen. Therefore the clotting assay (see below) could not be performed, yet no esterase activity was found in the similarly turbid Tos-Arg-OMe/thrombin assay mix.

Enzyme assays, protein determination, N-terminal analysis, disc electrophoresis in polyacrylamide gel and amino acid analysis were performed essentially as previously described (Rossi & Giglio, 1974). In cases where the biuret reaction could not be used owing to interferences, such as in Tris buffers, thrombin was measured directly as E_{280} , assuming an $E_{1\%}^{1\text{cm}} = 19.5$ for the thrombin fraction from the Amberlite column (Winzor & Scheraga, 1964).

Amino acid analysis was carried out with the aid of the autoanalyser described by Alonzo & Hirs (1968). Tryptophan was determined independently after hydrolysis of the sample with toluene-*p*-sulphonic acid, previously recrystallized from concentrated HCl and dried until completely freed of the last traces of HCl (m.p. 103–104.5°C; expected 104–105°C), hydrolysis being performed in the presence of 3-(2-aminoethyl)indole at 110°C for 24h (Liu, 1972).

C-Terminal analysis was carried out by hydrazinolysis (Narita, 1970), and by the action of carboxypeptidase B (Ambler, 1972).

Molecular weights were determined by disc gel electrophoresis in the presence of 2-mercaptoethanol and sodium dodecyl sulphate (Weber & Osborn, 1969). Bovine serum albumin (68 000), ovalbumin (43 000), carboxypeptidase A (34 600), α -chymotrypsinogen (25 700) and cytochrome *c* (12 000) were used as standards. All runs were performed in gels 10% (w/v) in acrylamide.

Results and Discussion

Activation of the partially purified prothrombin thus prepared gave rise to 25000 or more Tos-Arg-OMe units/litre of plasma after 4–6h of incubation, at 22°C, in the citrate/thrombin medium. At 37°C, this time could be decreased, but lower yields resulted. Activations by thrombokinase (Aronson & Ménaché, 1968) and by poly-L-lysine (Miller, 1960) are similarly reported as maximal at 22°C. Highly purified prothrombin, obtained by column chromatography as previously described (Leone

Fig. 1. Rechromatography of fraction III from the Amberlite column on DEAE-Sephadex A-50

The column (1.3 cm $\times$ 140 cm) was equilibrated with 0.01 M-potassium phosphate buffer, pH 7.0 (buffer A); 700480 Tos-Arg-OMe units were loaded, and elution was started with the same buffer, followed by 0.02 M- (buffer B) and 0.15 M- (buffer C) potassium phosphate buffers, pH 7.0. Clotting (Δ) and esterase ($\square$) specific activities were determined for the pooled fractions of peak VI, but only for the fractions of highest absorbance ($\bullet$) for peaks I-V. Flow rate was 23 ml/h, and the temperature was 2°C.

et al., 1976) was poorly activated in the citrate medium even after several days.

Chromatography of the activated sample on Amberlite IRC-50, performed with either the phosphate or the Tris buffer system, resulted always in a chromatographic pattern consisting essentially of three fractions. Fraction III contained all the clotting activity that emerged from the column and about 35% of the total protein. Heterogeneity of fraction III might be shown, however, by polyacrylamide-gel electrophoresis (five to seven bands being detected), by measurement of the specific activity along the peak or by *N*-terminal analysis. All samples of fraction III from the Amberlite column which were analysed by us showed threonine, isoleucine and small amounts of lysine as *N*-terminal residues. The molar ratio was always variable from sample to sample and far from integral. Treatment of fraction III with 4M-urea to remove possible adsorbed peptides (Landaburu *et al.*, 1961a), rechromatography on Amberlite IRC-50 as above or previously saturated with rivanol (6,9-diamino-2-ethoxyacridine lactate) as described by Miller (1959), and on Sephadex G-50, G-75 and G-100, interruption of the activation process at different times, before, after and at the point of maximum activity, inhibition of thrombin with phenylmethylsulphonyl fluoride immediately after activation, and, finally, use of different media to activate prothrombin, namely trypsin (Landaburu *et al.*, 1961b), protamine sulphate (Landaburu & Seegers, 1958) and brain thromboplastin + Ca^{2+} (Magnusson, 1965), failed to provide a homogeneous active fraction. Clotting activity of the electrophoretic bands could easily be demonstrated by simply dip-

ping slices of the gel in fibrinogen solution at pH 7.4 (Rossi & Giglio, 1974). Rechromatography of the Amberlite fraction III on the DEAE-Sephadex A-50 column, however, resolved it into seven others, at least six of them with thrombin activity (Fig. 1). Fraction VI could thus be isolated as an electrophoretically pure form (Fig. 2), but only after complete elution of fraction V with the 0.02 M buffer. Otherwise the tail of fraction V contaminates fraction VI and a second band becomes visible on the electrophoretic pattern.

The *N*-terminal analysis of fraction VI revealed again threonine, isoleucine and lysine, but at molar proportions of 1.00:1.05:1.07 respectively, strongly suggesting a three-chain molecular species. Analysis of the free *C*-terminal residues after hydrazinolysis revealed 0.017 μmol of serine, 0.004 μmol of glycine and 0.031 μmol of lysine and/or ornithine per 1.63 mg of protein. Ornithine, which originates from *C*-terminal arginine during hydrazinolysis (Niu & Fraenkel-Conrat, 1955), could not be separated from lysine under the conditions of our autoanalyser programme. Carboxypeptidase B, however, split only arginine from fraction VI. The only neutral amino acid split by hydrazine in significant amounts was therefore serine (about 0.38 mol/mol of protein). The recovery of 38%, although apparently low, is comparable with that obtained for other proteins in similar conditions (Niu & Fraenkel-Conrat, 1955). The small amount of glycine (about 0.09 mol/mol of protein) was due to the low stability of the glycine hydrazide (Narita *et al.*, 1966) and cannot be considered here. The exclusion of lysine as *C*-terminal indicated that hydrazine split 0.70 mol of arginine/

mol of protein. The ratio of serine to arginine, 0.38/0.70, when taken with the unitary molar ratio for the *N*-terminus, suggested that two chains of the thrombin molecule should end in arginine and one in serine.

The amino acid composition of fraction VI is reported in Table 1, which shows that DEAE-Sephadex fraction VI has apparently a composition similar to but not identical with that of the two-chain thrombin reported by Magnusson *et al.* (1975), thus indicating that the cleavage of one of the chains may have occurred in a common precursor with mol.wt. higher than or equal to 36775, rather than in the referred two-chain thrombin, to originate the three-chain species.

Fig. 2. Polyacrylamide-gel electrophoresis of phenylmethane-sulphonyl fluoride-inhibited fraction VI from the DEAE-Sephadex column

Conditions were as follows: gel concentration 7% (w/v); gel dimensions 0.6cm × 9cm; 4mA/tube, constant; temperature 2°C; marker Bromophenol Blue; stain Amido Schwarz 10B, 1% (w/v) in 7% (v/v) acetic acid. Direction of migration was downward to the anode.

Definitive evidence for the presence of three constituent polypeptide chains in the DEAE-Sephadex fraction VI was gained by sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the reduced enzyme (Plate 1). Table 2 shows the value of the molecular weight found for each chain. The fact that

Table 1. Amino acid composition of fraction III from the Amberlite column and fraction VI from the DEAE-Sephadex column

The values in parentheses represent the nearest integers to the experimental values. The results for the A+B chains are from Magnusson *et al.* (1975).

Residues	Amino acid composition (mol/mol)	
	DEAE-Sephadex	
	A-50 fraction VI	A+B chains
Asx	29.165 (29)	28
Thr	15.005 (15)	14
Ser	15.883 (16)	16
Glx	34.026 (34)	33
Pro	19.000 (19)	16
Gly	26.954 (27)	25
Ala	15.466 (15)	14
Cys	7.954 (8)	8
Val	22.095 (22)	21
Met	4.861 (5)	5
Ile	15.908 (16)	15
Leu	29.165 (29)	27
Tyr	11.926 (12)	11
Phe	14.140 (14)	13
Lys	26.066 (26)	24
His	6.186 (6)	7
Arg	21.211 (21)	22
Trp	7.512 (8)	9
Total	322	308
Molecular weight (amino acids only)	36775	35404
Molecular weight (+5% carbohydrates)	38710	37267

Table 2. Molecular weights of chains α , β and γ from DEAE-Sephadex fraction VI compared with those of chains A and B from the two-chain thrombin

For details, see the text. Values for the two-chain thrombin were calculated from the amino acid sequence (Magnusson *et al.*, 1975).

DEAE-Sephadex fraction VI		Two-chain thrombin	
Chain	Mol.wt.	Chain	Mol.wt.
α (fast moving band)	7330	A	5721
β (diffuse band)	12030	B	29683
γ (slow moving band)	19500	Total	35404
$\beta + \gamma$	31530	+5% carbohydrates	37267
Total	38860	Carbohydrates	1863
		B+carbohydrates	31546

EXPLANATION OF PLATE I

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of the 2-mercaptoethanol-reduced phenylmethanesulphonyl-inhibited fraction VI

Conditions were as follows: gel concentration 10% (w/v); gel dimensions 0.6 cm × 9 cm; 8 mA/tube, constant; temperature 25°C; stain Coomassie Brilliant Blue R, 1.25% (w/v) in methanol/acetic acid/water (2:3:40, by vol.). Direction of migration was downward to the anode.

the molecular weight of the $\beta+\gamma$ chains is practically the same as that of the B chain plus carbohydrate strongly suggests that the B chain was split into $\beta+\gamma$ chains. As the carbohydrate moiety of the B chain is linked to asparagine-65B (Magnusson *et al.*, 1975) (therefore within the first quarter of the sequence), the diffuse intermediate band in Plate 1, here called β chain, is the one that carries this carbohydrate moiety. On the other hand, the α chain is heavier than the A chain and so contributes additional residues to the three-chain species, as we have discussed above when analysing Table 1, again obtaining evidence for a common precursor for the two- and three-chain thrombin.

The appearance of a three-chain active thrombin during the activation of prothrombin is apparently an unavoidable event, at least when the described procedure is followed. The finding of a fractional molar ratio for the *N*-termini of the two-chain species, such as 1 mol of isoleucine to 0.5 mol of threonine (Magnusson, 1965), by other laboratories, indicates that the three-chain species may be omnipresent and that the third *N*-terminus may not have been properly detected. The physiological involvement of this event has, however, to be investigated.

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