Thrombospondin fragmentation by α -thrombin and resistance to γ -thrombin

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1. In the presence of 2 mM- Ca^{2+} , α -thrombin slowly cleaved thrombospondin (M_r 180 000) into 150 000- M_r and 30 000- M_r fragments.
2. In the absence of Ca^{2+} , the platelet glycoprotein was progressively and completely hydrolysed by 3 units of the enzyme/ml to 130 000- M_r , 95 000- M_r and 65 000- M_r fragments.
3. In contrast, the non-clotting enzyme form, γ -thrombin, did not hydrolyse the platelet protein either in the presence or in the absence of Ca^{2+} , even at 10-fold higher concentrations of enzyme.
4. Protein-interacting regions removed from the catalytic site, like those required for fibrinogen recognition, are necessary for thrombin proteolysis of thrombospondin.

Thrombospondin (thrombin-sensitive protein, glycoprotein G) is a high- M_r protein secreted from platelet α -granules on stimulation with such agonists as thrombin, collagen, ADP/fibrinogen and the Ca^{2+} ionophore A23187 (Baenziger *et al.*, 1971; Lawler *et al.*, 1978; Phillips *et al.*, 1980). Such stimulated platelets furthermore express a lectin-like agglutinin that is not expressed in the resting cell (Gartner *et al.*, 1978). This endogenous lectin is also an α -granule component (Gartner *et al.*, 1981) and is thought to be the same protein as thrombospondin (Jaffe *et al.*, 1982).

Isolated thrombospondin has been shown to bind to washed platelets in the presence of Ca^{2+} , causing agglutination, and also to bind in the absence of the bivalent cation in a non-agglutinating (univalent) manner (Gartner & Dochter, 1983). It has further been found to interact or bind with histidine-rich glycoprotein (Leung *et al.*, 1984) and with fibrinogen in a specific and saturable manner. The formation of thrombospondin–fibrinogen complex can be partially blocked by glucosamine or arginine, suggestive of carbohydrate–protein involvement in lectin binding (Leung & Nachman, 1982).

Thrombospondin consists of three protein subunits, which on reduction sediment as a species of M_r approx. 150 000 (Margossian *et al.*, 1981) or electrophorese as an 180 000- M_r component on discontinuous SDS/polyacrylamide gels (Lawler *et al.*, 1982). The subunit is proteolytically degraded

by thrombin, plasmin, trypsin and elastase. Depending on the enzyme and hydrolytic conditions, high- M_r components of M_r approx. 130 000–150 000 are formed (M_r 390 000–450 000 non-reduced), as well as 30 000- M_r heparin-binding fragments (reduced and non-reduced) (Lawler & Slayter, 1981). The presence of 2 mM- Ca^{2+} has been shown to restrict the proteolytic degradation of thrombospondin, where primarily the larger 150 000- M_r fragments are formed in the presence of Ca^{2+} . Since the 30 000- M_r fragments and the parent protein share the same *N*-terminal sequence, thrombospondin appears to be a trimer of similar, if not identical, subunits (Coligan & Slayter, 1984).

Human α -thrombin (Fenton *et al.*, 1977b) with high fibrinogen-clotting and all other thrombin-ascribed activities can be converted, autoproteolytically or by controlled tryptic digestion, almost entirely into γ -thrombin. This enzymic form essentially lacks clotting activity yet retains high synthetic-substrate estero-/amido-lytic activities, and is an equivalent or only slightly weaker proteinase with many thrombin-susceptible proteins (Fenton, 1981; Fenton *et al.*, 1977a, 1979). The present study shows that the non-clotting thrombin form does not hydrolyse thrombospondin under conditions where α -thrombin fragments thrombospondin slowly in the presence of and more rapidly in the absence of 2 mM- Ca^{2+} . This observation suggests that the thrombospondin cleavage sites are restricted by protein structural considerations and require protein-binding regions removed from the catalytic site of α -thrombin.

Abbreviation used: SDS, sodium dodecyl sulphate.

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Experimental

Thrombin preparations

Human α -thrombin was prepared from fraction III paste (gift from Armour Pharmaceutical Co., Kankakee, IL, U.S.A.) and evaluated as previously described (Fenton *et al.*, 1977b). The γ -thrombin form was made by controlled passage of α -thrombin at pH 6.2 through trypsin immobilized on agarose (Fenton *et al.*, 1979). An M_r of 36 500 was assumed in calculations for both enzyme forms.

Thrombospondin preparation

Thrombospondin was isolated from activated bovine platelets by a modification of the method of Lawler *et al.* (1978). Briefly, 30–50 litres of blood was collected into a final concentration of 0.36% sodium citrate and 0.2% glucose at pH 7.8 and was centrifuged at 2000g for 17 min at 4°C. The buffy coats were gently collected with a Teflon aspirator; the pooled platelets were then washed in buffer containing 0.102M-NaCl, 3.9mM-K₂HPO₄, 3.9mM-Na₂HPO₄, 22mM-NaH₂PO₄ and 5.5mM-glucose at pH 6.5. Washing was performed three times in order to concentrate the plasma to 1% of the platelet volume. Washed platelets were resuspended in buffer containing 0.14M-NaCl, 15mM-Tris, 5mM-glucose and 2mM-CaCl₂ at pH 7.6 at a final volume of 1/300th the initial blood volume. These platelets were incubated for 15 min at 37°C with 3 clotting units of bovine thrombin (Armour Pharmaceutical Co.)/ml; 75mg of di-isopropyl phosphorofluoridate was then added to inhibit further serine-proteinase activity. The incubation mixture was then centrifuged at 4000g for 15 min, and the supernatant was applied to a column (1.6cm $\times$ 10cm) of heparin-agarose (Bio-Rad Laboratories, Richmond, CA, U.S.A.) equilibrated with the resuspension buffer. The column was washed successively with 0.14M-, 0.20M-, 0.25M-, 0.55M- and 1.0M-NaCl, where thrombospondin was eluted predominantly in the 0.55M salt

fraction. This fraction was pooled, concentrated and gel-filtered through a column (5cm $\times$ 95cm) of agarose A-1.5m equilibrated with buffer containing 0.55M-NaCl, 15mM-Tris and 2mM-CaCl₂ at pH 7.6. The final product was concentrated to 1mg/ml by evaporation and stored frozen at -50°C.

Experimental conditions

Thrombin proteolysis of thrombospondin was carried out in the presence of either 2mM-CaCl₂ or 10mM-EDTA with, separately, α - or γ -thrombin. Reactions were terminated by the addition of electrophoresis buffer containing 0.125M-Tris, 4% (w/v) SDS, 20% (v/v) glycerol and 0.1% (v/v) 2-mercaptoethanol, pH 6.8. Electrophoresis was carried out by the technique of Laemmli (1970) on 10%-acrylamide polyacrylamide slabs cast with a 3.3%-acrylamide polyacrylamide stacking gel.

Results and discussion

The human thrombin preparations used in the present studies met criteria for high functional purity (Table 1). On radiolabelling the catalytic or functional serine residue (Ser-195 homologue in chymotrypsin), the α -thrombin preparation electrophoresed in 0.1%-SDS/10%-cross-linked-polyacrylamide gels with over 99% of the label migrating as single protein component (M_r approx. 37 000). The γ -thrombin preparation, treated in the same manner, electrophoresed with over 97% of the label migrating with a 12 000- M_r species corresponding to the serine-containing B-chain fragment (Fenton *et al.*, 1979). The α -thrombin preparation had a high specific clotting activity of more than 3000 U.S. N.I.H. clotting units/mg of protein, whereas the γ -thrombin preparation had an activity of less than 1 unit/mg (Table 1). On the other hand, both preparations possessed high esterolytic activities, where the α -thrombin titrated to 100% active with *p*-nitrophenyl *p'*-guanidinobenzoate and the γ -thrombin to 81.9% active

Table 1. Specifications of thrombin preparations utilized in the present studies

Property	α -Thrombin	γ -Thrombin
Thrombin form*	99.7% α 0.2% β 0.1% γ	0.1% α 2.4% β 97.5% γ
Fibrinogen-clotting specific activity	3122 units/mg	0.3 unit/mg
Esterolytic activities		
<i>p</i> -Nitrophenyl <i>p'</i> -guanidinobenzoate active-site titration†	100% active	81.9% active
<i>N</i> - α -Tosyl-L-arginine methyl ester active-site titration‡	—	79.7% active

* Relative percentage of [¹⁴C]di-isopropyl phosphorofluoridate detected after electrophoresis.

† Determined in Albany before performing the thrombospondin-digestion experiments.

‡ Determined in Detroit after performing these experiments.

(determined at the time of preparation). Independently, the γ -thrombin relative to the α -thrombin preparation was found to be 79.7% as active by *N*- α -tosyl-L-arginine methyl ester hydrolysis (determined after performing thrombospondin-digestion experiments). Hence either preparation was essentially only one enzymic form with high synthetic-substrate activity.

The α -thrombin preparation caused maximal thrombospondin secretion at approx. 25 nM (1 μ g of enzyme/ml or 3 clotting units/ml). Contrastingly, the γ -thrombin preparation required 12-fold higher molar concentration (approx. 300 nM or 12 μ g of enzyme/ml) to achieve the same degree of secretion. Since the γ -thrombin preparation was only 80% active (Table 1), γ -thrombin was 10-fold diminished in this stimulatory activity, as reported by Detwiler and associates for other secretory activities (Tam *et al.*, 1980).

The thrombospondin isolated from platelets treated with the above α -thrombin and γ -thrombin concentrations electrophoresed in an identical manner, as also did the thrombospondin isolated from ionophore A23187-stimulated platelets (results not shown). The fact that each of the three isolates exhibited similar trace amounts of

components of M_r less than 180 000 suggests that endogenous platelet proteinases are degrading thrombospondin during its storage in α -granules or upon secretion. Thus the trace fragments cannot be attributed to degradation by α -thrombin employed in the conventional isolation procedure.

When the isolated thrombospondin was incubated in the presence of 2 mM- Ca^{2+} with 3 clotting units of α -thrombin/ml, it was slowly degraded to the 30 000- M_r and 150 000- M_r fragments (Fig. 1). During the course of 24 h, the protein was only partially consumed, and only a slight intensification of the 130 000- M_r fragment doublet was observed. In marked contrast, when thrombospondin was incubated in the same manner, except in the absence of Ca^{2+} (presence of 10 mM-EDTA), then fragmentation occurred much more rapidly; the protein was completely consumed within 24 h. This fragmentation resulted in progressively increasing amounts of the 130 000- M_r , 95 000- M_r and 65 000- M_r fragments. On the other hand, an equivalent weight or molar concentration of γ -thrombin did not cause the fragmentation of thrombospondin in either the presence or the absence of Ca^{2+} (Fig. 1). Even at a 10-fold increased concentration of this enzyme form, no

Fig. 1. Electrophoresis of thrombin-incubated thrombospondin in 0.1% SDS/1% 2-mercaptoethanol/10%-cross-linked polyacrylamide gels

Thrombospondin (lane G) at 1 mg/ml was incubated with 3 units of α -thrombin/ml (25 nM) (lanes A–F) or 25 nM- γ -thrombin (lanes H–M) in the presence of 2 mM- CaCl_2 (lanes A–C and H–J) or 10 mM-EDTA (lanes D–F and K–M). Incubation times were 4 h (lanes A, D, H and K), 8 h (lanes B, E, I and L) and 24 h (lanes C, F, J and M). All samples, including the starting sample (lane G), were incubated at 37°C.

significant degradation of the protein was apparent within 24h (results not shown), implying that γ -thrombin cannot proteolytically fragment thrombospondin. This presumably is analogous to γ -thrombin's inability to clot fibrinogen, despite its high esterolytic activities (Table 1).

Like the parent α -thrombin form, γ -thrombin is capable of carrying out the proteolytic activation of Factor XIII (Lorand & Credo, 1977). It can cleave Fragment I from prothrombin (Seegers *et al.*, 1975). Complexing with antithrombin III involves a proteolytic cleavage (Jörnvald *et al.*, 1979), and γ -thrombin is inactivated by this inhibitor almost as rapidly as the native enzyme in the presence and in the absence of heparin (Chang *et al.*, 1979). Both thrombin forms also complex with α_2 -macroglobulin. Although γ -thrombin stimulates platelets, approx. 10-fold higher concentrations are required than for the equivalent response initiated by α -thrombin (Tam *et al.*, 1980). The non-clotting enzyme form also has other biological activities, which include induction of smooth-muscle contracture (D. A. Walz & G. F. Anderson, unpublished work), mitogenic stimulation of certain cultured cell types (Cunningham *et al.*, 1979), initiation of ion fluxes in fibroblasts (Stiernberg *et al.*, 1984) and the activation of metabolic pathways in neuroblastoma cells (Snider & Richelson, 1983).

In contrast, like with fibrinogen, γ -thrombin does not appear to be capable of activating blood coagulation Factor V or Factor VIII. Both of these factors are very large plasma proteins, and their activations involve proteolytic cleavage (Nesheim & Mann, 1979), perhaps analogous to fibrinopeptide release by α -thrombin. Thrombospondin thus behaves like another large protein, which requires protein-binding regions removed from the catalytic site for protein binding/recognition found in the native enzyme form but not in the non-clotting derivative enzyme forms.

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