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SPECIFIC FRAGMENTATION OF NATURAL INHIBITOR OF MITOCHONDRIAL ATPase BY THROMBIN

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Summary : Cleavage of natural inhibitor of mitochondrial ATPase by thrombin occurs at two specific sites. First an Arg-Ser bond is split giving two peptides. The main peptide which retains integral biological activity is further cleaved at two successive Arg-Ala bonds, none of the products is able to inhibit ATPase. The isolation of these peptides and their characterization are described.

INTRODUCTION

The high proteolytic specificity of thrombin (1) makes it a potentially interesting tool to study proteins especially when only a few cleavages are required. The action of thrombin was tested upon the natural inhibitor (IF_1) of mitochondrial ATPase, in order to identify region(s) of the molecule involved in the interaction with the enzyme (2).

IF_1 is a low molecular weight protein (about 10,000) which plays an important role in the regulation of oxidative phosphorylation (3). IF_1 inhibits ATPase activity on binding specifically to β, one of the 5 subunits of the soluble F_1 part of the enzyme (4).

Studies of IF_1 digestion by thrombin have shown the presence of two cleavage sites which were further characterized as Arg-Ser and Arg-Ala bonds.

ABBREVIATIONS

MW : Molecular weight.
SDS-PAGE : Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate.
HPLC : High performance liquid chromatography.
IF_1 : Natural inhibitor of mitochondrial ATPase.

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When compared to other known substrates being cleaved at the same sites by thrombin, the surrounding primary sequences do not exhibit any obvious similarity.

MATERIALS AND METHODS

Horse thrombin was purchased from Laboratoire ISH as a lyophilized powder in vials of 100 NIH units. 20 NIH units/ml solutions were used without further purification after stabilization of the thrombin activity overnight at 4°C. Purified human α-thrombin was prepared by the activation of pure human prothrombin by the crude venom of the taipan snake (*Oxyuranus scutellatus scutellatus*) as described in (5). The venom was obtained from Sigma and human prothrombin was prepared according to (6). Aliquots of the α-thrombin preparation (2,500 NIH units/mg of protein, 2-3 mg/ml in 0.75 M NaCl) were stored at - 80°C as recommended in (7).

IF_1 was prepared according to (8, 9) from isolated beef heart mitochondria (10). Purity controls were (a) a single band (apparent MW ∿ 10,000) on SDS-PAGE (b) assay of inhibitory capacity on ATPase activity as described in (4). Protein concentrations were determined according to (11) using bovine serum albumin as standard. Action of thrombin on IF_1 was carried out at various enzyme/substrate ratios and different incubation times at 37°C in a medium made of 50 mM (NH_4) CH_3COO and 1 % (NH_4) HCO_3, pH 8.0. IF_1 digestion by thrombin was followed by electrophoretic separation of the incubation products on 20 % polyacrylamide slab gel in the presence of SDS (12) and by measuring the residual inhibitory capacity towards ATPase.

Preparative separation of the digestion products was achieved by high performance liquid chromatography using the instrument from Waters Associates S.A. Amino-acid analysis were performed using a Durrum D-500 Amino-Acid Analyzer after total acid hydrolysis. Dansylation and identification of N-terminal residues were carried out according to (13).

RESULTS

Human α-thrombin prepared from its proenzyme form gave a single band (apparent MW ∿ 36,000) on SDS-PAGE while horse thrombin commercial preparation appeared to be a mixture of several proteins. However both preparations released identical peptide patterns when incubated with IF_1. Figure 1 illustrates the incubation of IF_1 with thrombin for a 24 hours period. Thrombin action occurred in two successive steps. Firstly the protease released a fragment, Thr 1 which migrated on SDS-PAGE with an apparent MW ∿ 8,000 and was still able to inhibit ATPase activity ; the other peptide Thr 2 did not stain with Coomassie blue. Secondly, Thr 1 was further digested into inactive smaller peptides which migrated on SDS-PAGE and were stained as a broad spot of MW ranging between 2,500 and 4,500.

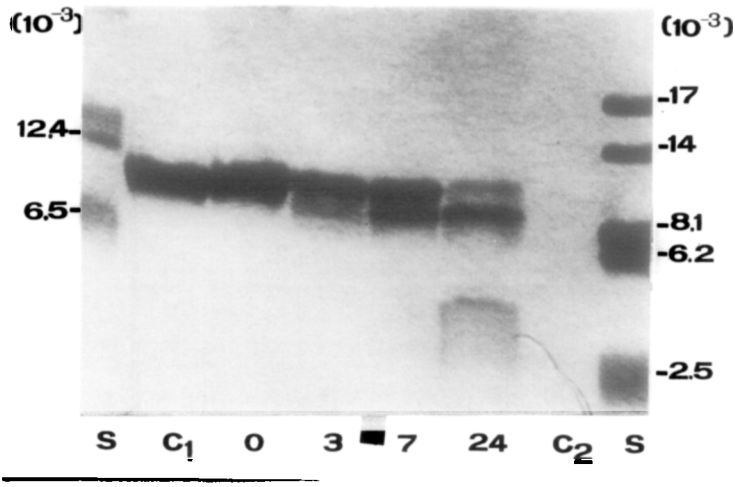

Figure 1. Separation by SDS-PAGE of the digestion products of IF_1 by thrombin.
E/S = 10 units/mg - slots S : MW markers, slot C_1 : IF_1 incubated for 24 hours without thrombin, slot C_2 : thrombin incubated 24 hours without substrate. Numbers under other slots indicate incubation time in hours.

The separation of the different peptides obtained after 24 hours incubation of 2 mg of IF_1 with 20 NIH units of thrombin was performed by high performance liquid chromatography. The elution profile is given on figure 2. The four peaks were characterized by their MW from SDS-PAGE, their respective amino-acid compositions and N-and C-terminal residues identification. These results are summarized in table I. Peak I did not stain on SDS-PAGE but from its amino-acid composition it was identified as the N-terminal peptide Thr 2. Peaks II and III contained one peptide each of respective apparent MW of < 2,500 and 3,800. These peptides were produced by the cleavage of Thr 1. Peak IV contained Thr 1 and a small amount of non-digested IF_1. The established IF_1 amino-acid sequence (14, 15), the amino-acid compositions, MW and N-and C-terminal residues identification of the different fragments allowed us to localize them in the whole protein IF_1. A schematic representation of the action of thrombin on IF_1 is given on figure 3. These results show that the first cleavage site of thrombin on IF_1 was an Arg-Ser bond giving Thr 1 and Thr 2. Thr 1 was further cleaved into Thr 1a and Thr 1b. However, more complete characterization using carboxypeptidases A&B and amino-acid analysis has revealed that peaks II and III contained peptides presenting

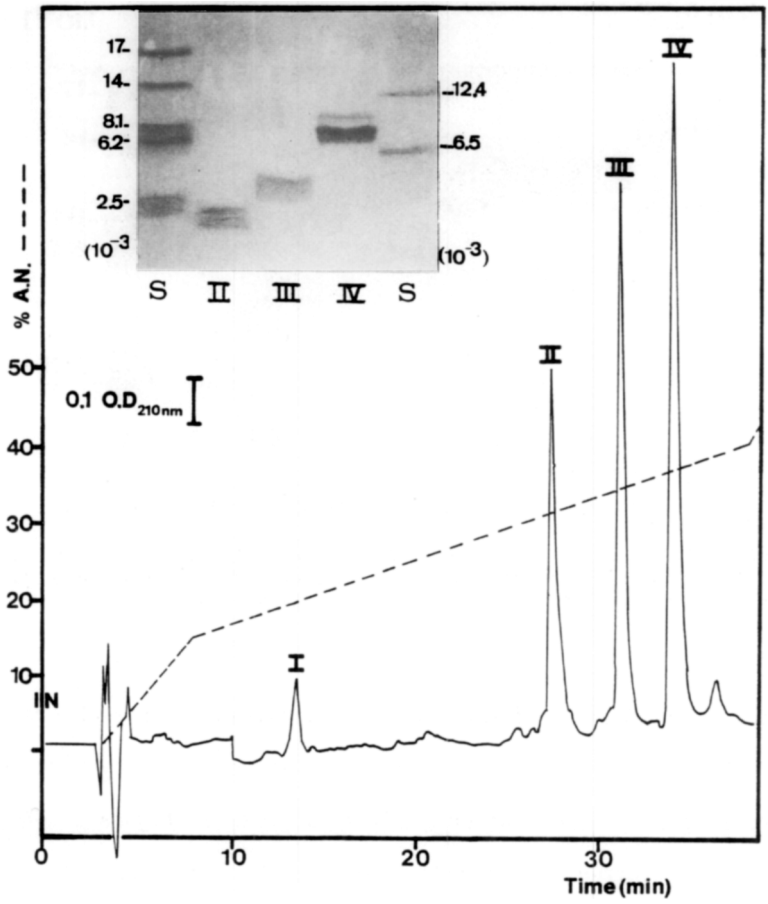

Figure 2. Separation of the peptides obtained after 24 hours incubation of IF_1 with thrombin by HPLC. At IN point 30 nmoles of hydrolysate were injected. Elution was achieved by a linear gradient of acetonitrile (0 to 50 %) in 10 mM trifluoroacetic acid. Protein was detected by absorbance at 210 nm. Column (30 x 0,6 cm) μ Bondapak C_{18}, 10 μm particles, flow rate 1 ml/min. Numbers under the inserted gel pattern refer to the corresponding peaks. S : MW markers.

slight heterogeneity at their extremities. This is due to the repetitive Arg-Ala bond in the IF_1 sequence : -Tyr-Phe-Arg-Ala-Arg-Ala-Lys-. Peak II contained 2/3 of peptide having Tyr-Phe-Arg as C-terminal sequence (Thr 1a) and 1/3 of peptide having Tyr-Phe-Arg-Ala-Arg as C-terminal sequence (Thr 1a'). As a consequence peak III contained a mixture of peptides Thr 1b and Thr 1b'.

DISCUSSION

This study demonstrates that the natural inhibitor of mitochondrial ATPase is a substrate for the serine-proteinase thrombin. Three cleavage sites due

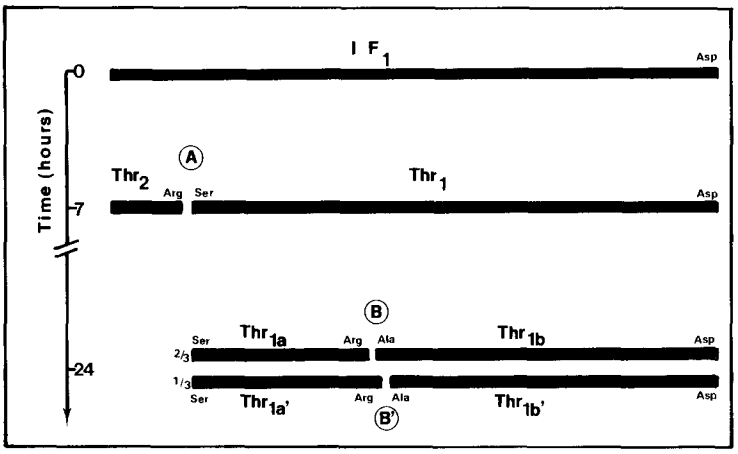

Figure 3. Schematic representation of the action of thrombin on IF_1 (E/S = 10 NIH units/mg).

to this blood clotting enzyme have been identified on IF_1. Thrombin first splits an Arg-Ser bond. The main peptide bearing integral biological activity is further cleaved at two successive Arg-Ala bonds. At this stage none of the products retains inhibitory capacity towards ATPase. Under the above conditions no further action of thrombin is detected although IF_1 contains 4 other

Table I. Characterization of the contents of peaks I, II, III, IV obtained by HPLC.

PRODUCTS	PEAK I	PEAK II	PEAK III	PEAK IV
	Thr 2	Thr 1a Thr 1a'	Thr 1b Thr 1b'	Thr 1 IF_1
APPARENT Mw FROM SDS-PAGE	Not stained by Coomassie Blue	< 2500	3800	8200 9700
NUMBER OF RESIDUES	9	26 28	49 47	75 84
MW FROM AA COMPOSITION	921	2855 3082	5892 5665	8729 9631
N-TERMINAL RESIDUE :				
- AMINOPEPTIDASE M	0	0	0	0
- DNS + TLC	n.d.	Ser	Ala	Ser
C-TERMINAL SEQUENCE :				
- CARBOXYPEPTIDASES A&B		Tyr-Phe-Arg (2/3) Tyr-Phe-Arg-Ala-Arg (1/3)	0	0
- CARBOXYPEPTIDASE P	n.d.	0	Ser-Glu-Asp-Asp-Asp	Ser-Glu-Asp-Asp-Asp
INHIBITORY CAPACITY OF ATPase ACTIVITY	0	0	0	+

Table II. Comparison of amino-acid sequences around certain bonds cleaved by thrombin in various substrates (see ref. 1 for substrates other than IF_1).

	Thrombin ↓	
Human Fibrinogen :		
Chain (Aα)	Phe-Leu-Ala-Glu-Gly-Gly-Gly-Val-Arg^{16}	Gly-Pro-Arg-Val-Val-Glu-Arg-His-
Chain (Aα)	Gly-Gly-Gly-Gly-Val-Arg-Gly-Pro-Arg^{19}	Val-Val-Glu-Arg-His-Gln-Ser-Ala-
Chain (Bβ)	Asn-Glu-Glu-Gly-Phe-Phe-Ser-Ala-Arg^{14}	Gly-His-Arg-Pro-Leu-Asp-Lys-Lys-
Human Plasma F XIII α-chain	Val-Glu-Leu-Gln-Gly.....Val-Pro-Arg^{36}	Gly-Val-Asx-Leu-Glx-Glx-
Bovine prothrombin	Arg-Val-Thr-Val-Glu-Val-Ile-Pro-Arg^{156}	Ser-Gly-Gly-Ser-Thr-Thr-Ser-Gln-
Actin	Gly-Phe-Ala-Gly-Asp-Asp-Ala-Pro-Arg^{28}	Ala-Val-Phe-Pro-Ser-Ile-Val-Gly-
Growth hormone :		
Ovine	Arg-Glu-Leu-Glu-Asp-Val-Thr-Pro-Arg^{52}	Ala-Gly-Gln-Ile-Leu-Lys-Gln-Thr-
Bovine	Arg-Glu-Leu-Glu-Asp-Gly-Thr-Pro-Arg^{131}	Ala-Gly-Gln-Ile-Leu-Lys-Gln-Thr-
ATPase inhibitor from beef-heart mitochondria (IF_1)	Gly-Ser-Glu-Ser-Gly-Asp-Asn-Val-Arg^{9}	Ser-Ser-Ala-Gly-Ala-Val-Arg-Asp-
	Gln-Ala-Glu-Gln-Glu-Arg-Tyr-Phe-Arg^{35}	Ala-Arg-Ala-Lys-Glu-Gln-Leu-Ala-
	Glu-Gln-Glu-Arg-Tyr-Phe-Arg-Ala-Arg^{37}	Ala-Lys-Glu-Gln-Leu-Ala-Ala-Leu-

Arg residues and 10 Lys residues (14, 15). The early cleavage at Arg-Ser bond was also observed with trypsin and clostripain (2), this suggests that this part of the IF_1 molecule is an easily accessible region.

The cleavage by thrombin of IF_1 molecule and of other substrates other than blood clotting factors is obtained at much higher enzyme/substrate ratio and with much longer incubation times than that of fibrinogen and factor XIII. Table II summarizes the action of thrombin on various substrates, the comparison of surrounding sequences of the cleavage sites Arg-Ser and Arg-Ala do not exhibit any obvious similarity.

In vivo thrombin is produced from its proenzyme form, prothrombin, at the platelet membrane interface. The concentrating effect of the membrane receptors of the various blood clotting factors involved in prothrombin activation (16) implies that high thrombin concentration may be available locally. To our knowledge it is not known whether an isomer of IF_1 exists in platelets, but if it does and since IF_1 is able to inhibit ATPase activity of actomyosin (17) this would suggest a supplementary role for thrombin in the regulation of platelet aggregation.

Our results also prove that thrombin can be a useful tool to study protein structure. In some cases it is able to release only a small number of fragments which can be directly sequenced or assayed for biological activity. Furthermore its proteolytic action can be easily controlled using a powerful and highly specific inhibitor hirudin (18).

ACKNOWLEDGMENTS

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