# BINDING OF FITC-LABELED α-THROMBIN BY PERITONEAL MAST CELLS

**S. M. Strukova, S. V. Khlgatyan, and S. V. Zaitsev**

UDC 612.112.93.015.36:612.115.35

**KEY WORDS:** α-thrombin; mast cells; protein-cell interaction

α-Thrombin (EC 3.4.21.5), a key enzyme of the hemostasis system, interacts with proteins and cells to regulate the mechanisms of blood clotting and anticlotting, induces atherogenesis, and is involved in inflammation and the development of neoplasms [4].

The polyfunctional nature of α-thrombin is linked with high specificity in the selection of substrates and cell receptors, due to the existence of an additional recognition center for high-molecular-weight compounds in the structure of the enzyme, independent of its active center. This recognition center consists of subcenters and is responsible for realization of the enzymic and hormonelike properties of thrombin in its reactions with cells [1, 2, 4].

Thrombin binds with and activates blood cells (except erythrocytes), and also endothelial cells, fibroblasts, etc. [9]. According to data in the literature [7], thrombin binds with mast cells in the bone marrow (the parameters of binding have not been determined), and stimulates histamine secretion by them. The writers showed previously that α-thrombin provokes more intensive secretion of heparin (but not histamine) by peritoneal mast cells (MC) [3]. However, nothing is known about whether this reaction of thrombin with MC is of the agonist – receptor type.

The aim of this investigation was to study interaction of thrombin with peritoneal MC.

## EXPERIMENTAL METHOD

Heparin, fluorescein isothiocyanate (FITC), 2,7-bis-carboxyethyl-5(6)-carboxyfluorescein (BCECF), and 4-acetamido-4′-isothiocyano-2,2′-stilbenesulfonate (SITS) were obtained from "Sigma." Bovine α-thrombin, obtained by purification of the commercial product by the method in [9], was homogeneous on polyacrylamide gel electrophoresis in the presence of SDS and had clotting activity of 2000-2500 NIH units/mg protein.

FITC-labeled α-thrombin with intact anion-binding subcenter of the recognition center was obtained by our modification of the method in [6] as follows. An aliquot of 6 μM α-thrombin with heparin (molar ratio 1:4) was incubated for 15 min at 4°C in 0.05 M bicarbonate buffer, pH 8.85. The complex thus formed was conjugated with 4 μM FITC and incubated during mixing for 1 h. Free FITC and heparin were removed by dialysis for 10 h against the same buffer. The FITC-labeled α-thrombin was subjected to further purification by gel-filtration on Sephadex G-50 at high ionic strength (0.5 M bicarbonate buffer, pH 8.9, containing 1 M NaCl) or by high-pressure gel-filtration (HPLC) on the TSK 6-2000 SW apparatus.

The protein concentration in the samples was determined by Lowry's method, the heparin concentration on the basis of its ability to block the amidase activity of standard thrombin (relative to chromozyme TH) in the test to determine low doses of heparin according to the "Boehringer Mannheim GmbH Diagnostica" formula. Amidase activity of FITC-labeled α-thrombin was determined relative to the chromogenic substrate Phe-Pip-Arg-p-NA.

MC were isolated and purified in a Ficoll density gradient by the method in [10]. To determine total, specific, and nonspecific binding the cells were incubated with FITC-labeled α-thrombin in a concentration of 0.01-10 nM both in the presence and in the absence of 0.1 μM di-isophosphoryl (DIP)-α-thrombin, which has no clotting or proteolytic activity.

M. V. Lomonosov Moscow State University. (Presented by Academician of the Academy of Medical Sciences of the USSR I. P. Ashmarin.) Translated from Byulleten' Éksperimental'noi Biologii i Meditsiny, Vol. 112, No. 10, pp. 385387, October, 1991. Original article submitted March 29, 1991.

 0007-4888/91/0010-1438$12.50 

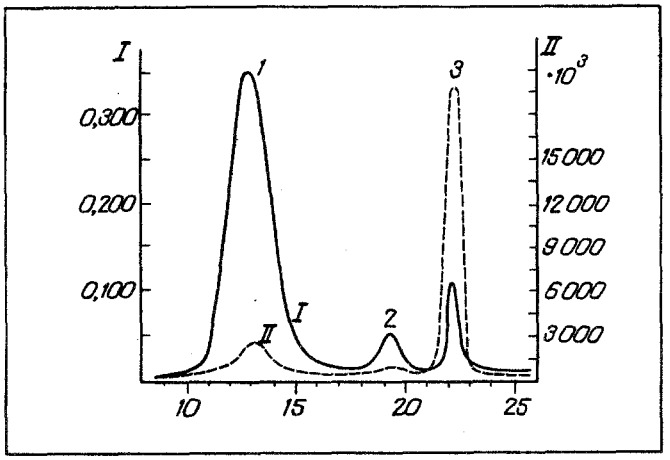



Fig. 1. Elution profiles of FITC-labeled $\alpha$-thrombin obtained by high-pressure gel-filtration (HPLC) on the TSK 6-2000 SW apparatus. Abscissa, sample No. I) Optical density at $\lambda$ = 280 nm, II) intensity of fluorescence at $\lambda_{exc}$ = 436 nm, $\lambda_{em}$ = 485 nm. Volume of each sample 200 $\mu$l. 1) FITC-labeled $\alpha$-thrombin; 2) heparin; 3) free FITC.

Interaction of thrombin with MC was studied as follows. To a sample of 20 $\mu$l ($2 \cdot 10^6$ cells) were added 20 $\mu$l of FITC-labeled $\alpha$-thrombin and 20 $\mu$l of balanced buffer solution or (to determine specific binding) 20 $\mu$l of 0.1 $\mu$M DIP-$\alpha$-thrombin. Samples were incubated for 5 min at 20°C. The excess of label was removed by repeated washing and centrifugation. The residue was suspended in 1 ml balanced buffer solution, transferred to a cuvette, in which fluorescence was measured on an "Automated Fluorescence Polarization Analyzer." The results were analyzed on an IBM PC/XT computer, by "Delta" program [5]. Specific binding was found as the difference between total and nonspecific binding. To study the effect of temperature on the kinetics of specific binding of FITC-labeled $\alpha$-thrombin with MC, the binding reaction was stopped after incubation for 30 sec, 1, and 1.5 min, and so on, by dilution with 20 volumes of cold (4°C) buffer. MC were incubated with 1 nM labeled $\alpha$-thrombin in the presence and absence of a 100-fold molar excess of DIP-$\alpha$-thrombin.

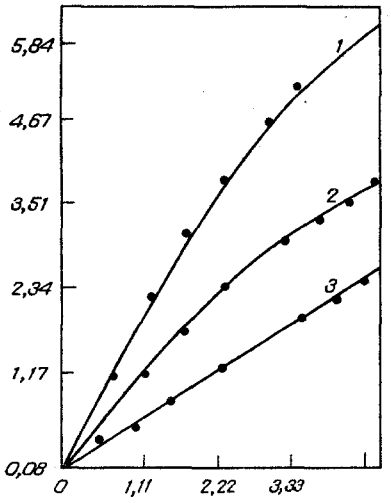
## EXPERIMENTAL RESULTS

To determine the mechanism of interaction of thrombin with MC a method of obtaining a conjugate of $\alpha$-thrombin with FITC on the basis of modification of the method in [6] was developed. Since the fluorophore reacts mainly with free protein amino groups, the anion-binding subcenter of the recognition center for high-molecular-weight compounds (containing amino acid residues arginine and lysine), responsible for binding with cells and specific substrates inhibitors [2], in the $\alpha$-thrombin molecule had to be protected. For this purpose, the heparin polyanion was used, for it blocks the anion-binding subcenter in the thrombin molecule reversibly and selectively. Elution profiles of FITC-labeled $\alpha$-thrombin, purified by gel-filtration under high pressure (HPLC) are given in Fig. 1.

The results indicate that the conjugate of FITC with $\alpha$-thrombin is eluted with the first peak, heparin with the second peak, and unbound FITC with the third peak.

Taking the coefficient of molar extinction for FITC (EM 492 nm) to be 61,000 $M^{-1} \cdot cm^{-1}$, and considering the concentration of FITC-labeled $\alpha$-thrombin, it was calculated that 1 M $\alpha$-thrombin binds 0.16 mole FITC, which is quite sufficient for recording labeled thrombin.

FITC-labeled $\alpha$-thrombin contains traces of heparin (0.02 USP unit/ml), i.e., 5 $\mu$M heparin to 1 M modified $\alpha$-thrombin (0.0001% of the quantity added). On gel filtration, approximately 15% of the added heparin (over 0.5 USP unit/ml) was found in the second peak. Most heparin was separated by dialysis. These data indicate virtually complete removal of the heparin from the FITC-labeled $\alpha$-thrombin. Measurement of the rate of accumulation of fibrin monomers during the action of FITC-labeled $\alpha$-thrombin on fibrinogen demonstrated the low level of clotting activity of the modified



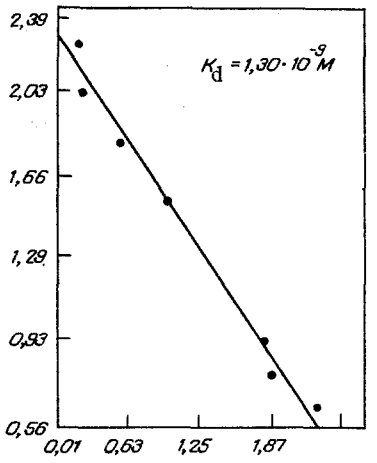




Fig. 2 Fig. 3

Fig. 2. Binding of FITC-labeled $\alpha$-thrombin to MC. Abscissa, concentration of free thrombin (in nM); ordinate, concentration of bound thrombin (in nM). 1) Total binding; 2) specific binding; 3) nonspecific binding.

Fig. 3. Scatchard plot for specific binding of FITC-labeled $\alpha$-thrombin with MC. Abscissa, concentration of specifically bound thrombin (in nM); ordinate, ratio of concentration of specifically bound thrombin and free thrombin.

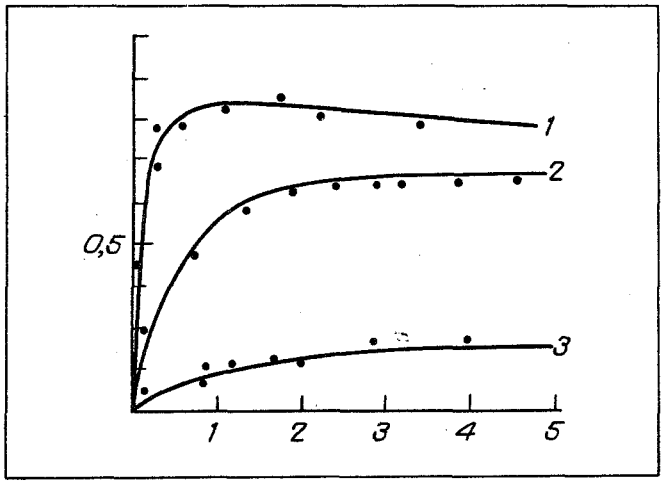



Fig. 4. Effect of temperature on kinetic curves of specific binding of 1 nM FITC-labeled $\alpha$-thrombin with receptors of MC in presence of a 100-fold excess of DIP-$\alpha$-thrombin. Abscissa, incubation time (in min); ordinate, concentration of specifically bound thrombin (in nM). 1) 37°C, 2) 20°C, 3) 4°C.

thrombin. Amidase activity of the FITC-labeled $\alpha$-thrombin and the residual clotting activity amounted to 0.01% of the original $\alpha$-thrombin.

Binding of FITC-labeled $\alpha$-thrombin with rat peritoneal MC was studied. Curves characterizing total, specific, and nonspecific binding are given in Fig. 2.

To assess the character of specific binding, the results were subjected to graphic analysis in Scatchard plots, and parameters of complex formation were determined: the dissociation constant ($K_d$) and the number of binding sites. The Scatchard plot for specific binding of FITC-labeled $\alpha$-thrombin with MC is a straight line, indicating the presence of one type of binding sites (Fig. 3). The value of $K_d$ calculated for specific binding was $1.3 \cdot 10^{-9}$ M and the number of binding sites was 54,200.

The effect of temperature (37, 20, and 4°C) on the kinetics of specific binding of FITC-labeled $\alpha$-thrombin with MC was studied. Figure 3 gives the results showing dependence of specific binding of FITC-labeled $\alpha$-thrombin with MC on time, and the effect of temperature is shown in Fig. 4. The best binding was observed at 37°C. It was shown to take place rapidly, and during the first minute of incubation saturation was reached. The efficiency of the process fell sharply at room temperature and it virtually ceased at 4°C.

The investigation thus showed that thrombin binds with high affinity to receptors of peritoneal MC: $K_d = 1.3 \cdot 10^{-9}$ M. Binding is characterized by specificity and saturability. The specific binding of $\alpha$-thrombin with MC thus revealed indicates that high-affinity thrombin receptors are present on the surface of MC, just as on other connective-tissue cells, and they mediate the reaction of the enzyme with the cells by a mechanism of ligand-receptor interaction.

The mechanism of signal realization during specific binding of thrombin with peritoneal MC (Na/H exchange, anion transport, intracellular $Ca^{2+}$, and other secondary messengers) is being investigated.

According to preliminary data obtained with the aid of the pH-sensitive probe BCECF, interaction of $\alpha$-thrombin with MC evokes a dose-dependent fall of intracellular pH, followed by a rise. The first phase, one of rapid initial fall of intracellular pH, may be due to the contribution of anionic transport, as has been shown in experiments with the anionic channel blocker SITS, the second phase, a stable rise, is due to activation of Na/H exchange, the existence of which in peritoneal MC was confirmed by our experiments with nigericin (a $K^+/H^+$-ionophore) and ammonium chloride. It can be tentatively suggested that specific binding of $\alpha$-thrombin with peritoneal MC leads to activation of ionic transport and secondary messengers, and ultimately to the secretion of heparin and other agents involved in the regulation of hemostasis and homeostasis.

## LITERATURE CITED


1. S. M. Strukova, Biochemistry of Animals and Man [in Russian], No. 6, Kiev (1982), pp. 27-38.
2. S. M. Strukova, A. A. Sereiskaya, and T. V. Osadchuk, Usp. Sov. Biol., **107**, 41 (1989).
3. B. A. Umarova, S. V. Khlgatyan, and S. M. Strukova, Byull. Éksp. Biol. Med., No. 2, 131 (1989).
4. J. W. Fenton, II, Thromb. Hemostas., **14**, 229 (1988).
5. E. M. Melikhova, I. M. Kurochkin, S. V. Zaitsev, and S. D. Varfolomeev, Analyt. Biochem., **175**, No. 1-2, 507 (1988).
6. A. Pappenhagen, J. L. Koppel, and J. H. Olwin, J. Lab. Clin. Med., **59**, No. 6, 1039 (1962).
7. E. Razin, D. Baranes, and G. Marx, Exp. Cell Res., **160**, No. 2, 380 (1985).
8. H. Sato and A. Nakajima, Thromb. Res., **33**, 645 (1984).
9. M. A. Shuman, Ann. New York Acad. Sci., **485**, 228 (1986).
10. I. L. Thon and B. Uvnas, Acta Physiol. Scand., **71**, 303 (1967).