Synthetic Analog of the Thymosin$_{\alpha 1}$ Fragment 24-28 Alters the Coagulating and Aggregating Activities of α-Thrombin

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A pentapeptide (EEAEN), which is the 24-28 fragment of the COOH-terminal sequence in the thymosin$_{\alpha 1}$ molecule highly homologous with the 54-58 region of hirudin (with the complementary anion-binding exosite in the thrombin molecule), was synthesized by a solid-phase method. Preincubation of α-thrombin with EEAEN in concentrations of 0.1 pM to 1 nM reduced its clotting activity while preincubation of this enzyme with EEAEN in concentrations of 0.01 to 1 nM reduced its platelet-aggregating activity. The reaction of EEAEN with thrombin is shown to be similar to the reaction of the entire thymosin$_{\alpha 1}$ molecule. It is concluded that the COOH-terminal thymosin$_{\alpha 1}$ peptide EEAEN may be the reactive site responsible for the antithrombin activity of thymosin$_{\alpha 1}$.

Key Words: *thrombin; thymosin; platelets; blood coagulation*

The broad range of physiological and pathophysiological functions performed by thrombin including, in particular, the regulation of hemostasis, vascular tonus, mitogenesis, inflammation, atherogenesis, and some other processes, are mediated through its interaction with receptors of blood cells, vessel walls, and connective tissue [7]. The high specificity of this enzyme for membrane receptors and physiological substrates is due to the presence in its molecule of an additional recognition site consisting of several subsites [5,7]. This additional site, which is located outside the active site, is held to be an allosteric center that alters the properties of thrombin following its binding to substrates or receptors [5]. The presence in the thrombin molecule of a recognition site with subsites differing from each other in nature allows for a targeted regulation of thrombin activity by natural and synthetic modulators. A number of regulatory peptides possessing clusters complementary to the anion-binding and other subsites of the thrombin's recognition site have been identified [1,3,4]. One such peptide is thymosin$_{\alpha 1}$ - an immunologically active thymic factor whose COOH terminus contains a negatively charged 24-28 cluster having a high degree of homology with the 54-58 fragment of the COOH terminus in hirudin, a highly specific exogenous thrombin inhibitor interacting with the active and recognition sites of the thrombin molecule [8,10]. Thymosin$_{\alpha 1}$ has been shown to inhibit the fibrinogen-clotting and aggregating activities of thrombin as well as its ability to activate Na-H exchange in mast cells [1,4]. The high degree of homology between the COOH-terminal fragments of thymosin$_{\alpha 1}$ and hirudin suggests that the 24-28 peptide of thymosin$_{\alpha 1}$ will also interact with anion-binding subsite 1 in the α-thrombin molecule and modulate the activity of this subsite. An EEAEN pentapeptide (Glu-Glu-Ala-Glu-Asn), analogous to the 24-28 fragment of the thymosin$_{\alpha 1}$ terminus was therefore synthesized. As indicated by preliminary data [9], this pentapeptide is capable of blocking the clotting activity of thrombin.

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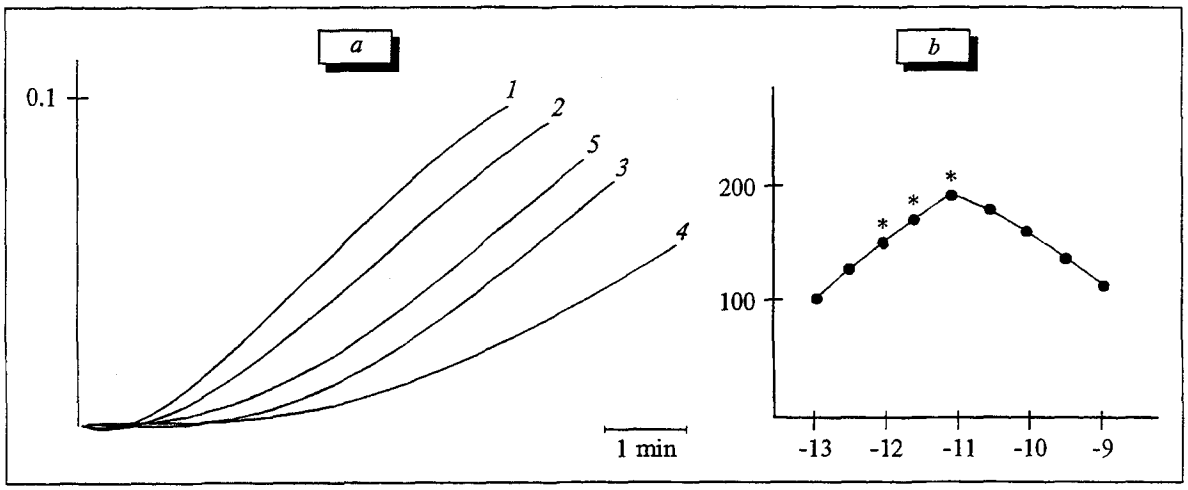

Fig. 1. Variation of α-thrombin clotting activity in the presence of the EEAEN peptide. *a*) curves describing the variation of light scattering by fibrinogen (τ=350 nm) in the course of fibrin formation under the action of 10 nM α-thrombin (*1*) and in the presence of EEAEN in concentrations of 0.1 (*2*), 1 (*3*), 10 (*4*), and 100 (*5*) pM. *b*) changes in the duration of the induction period (as expressed in percent of the control value) for the reaction of fibrinogen clotting under the action of α-thrombin in the presence of EEAEN in various concentrations. Abscissa: log molar concentrations of the peptide; *$p<0.05$.

The present study was undertaken to test EEAEN for its effects on the clotting activity of thrombin and on the ability of this enzyme to induce aggregation of human platelets.

MATERIALS AND METHIODS

In this study we used α-thrombin obtained as described by Dugina *et al.* [1] and NIH thrombin (100 units/ml) in concentrations of 25-62 nM (0.014-0.035 units/ml); thymosin$_{\alpha 1}$ (0.01 nM-10 μM) synthesized at the Research Institute of Extra Pure Preparations (St. Petersburg); hirudin from leeches (0.014-1 units/ml) (Sigma); and an EEAEN pentapeptide synthesized by the solid-phase method on PAM polymer [6]. A BOC group was used to protect the NH_2 function and cyclohexyl ester to protect the lateral (Glu) function. Condensations were performed sequentially, using a method of symmetrical anhydrides, until the reaction was >99% completed. After its separation from the polymer carrier and its unblocking by means of liquid hydrogen fluoride, the EEAEN peptide was freed from salt on a G-10 Sephadex column and purified with high performance liquid chromatography (HPLC) on a 25×250 mm Diasorb-130 C16T column (at a flow rate of 10 ml/min and an acetonitrile gradient of 0 to 70% over 45 min in 0.05% aqueous TFA). The peptide was characterized by mass spectrometry (using the FAB method and a Kratos MS50 TC instrument) and reverse-phase HPLC.

Platelets were isolated from stabilized human donor blood plasma by centrifugation two times at 1500 rpm for 15 min (platelet-enriched plasma was obtained by centrifuging blood samples at 1000 rpm for 10 min). For washing off and resuspension of the platelets, a buffer of the following composition was used: 120 mM NaCl, 15.4 mM KCl, 13.3 mM Tris-HCl, 6 mM glucose, and 1.54 mM EDTA, pH 6.5. The final platelet concentration in the suspension was 10^8 cells/ml. Platelet aggregation was tested by a light-transmission technique at 37°C using a device described by Samal' *et al.* [2]; for the tests, 0.3 ml of platelet suspension was added to a cuvette containing 1 ml of phosphate-buffered saline with $CaCl_2$ (pH 7.35), followed by the addition 2 min later of thrombin preincubated with one of the compounds under study for 1 min at room temperature.

RESULTS

The EEAEN peptide inhibited the clotting activity of thrombin in concentrations of 0.01 pM to 0.1 nM (Fig. 1). Preincubation of 10 nM thrombin with EEAEN in concentrations of 0.1 pM to 1 nM led: 1) to a prolongation of the period required for induction of a polymerizatiuon curve, indicating that the peptide prolonged the time during which fibril monomers accumulated and protofibrils were formed; 2) to a more shallow slope of the curve - an indication of reduced polymerization rate (reduced lateral association of protofibrils). The equilibrium constant for inhibition of thrombin clotting activity (K_i), calculated in Dixon's coordinates, equaled 0.01 nM and was close to the K_i estimated in tests with thymosin$_{\alpha 1}$. The incomplete inhibition of thrombin clotting activity by EEAEN suggests that this thymosin$_{\alpha 1}$ fragment, like

thymosin$_{\alpha 1}$ itself, binds to a subsite of the additional recognition site in the thrombin molecule rather than to the active site of the enzyme. After 10 nM thrombin was incubated with thymosin$_{\alpha 1}$ in concentrations above 1 nM, no significant change in its activity was observed.

In the next series of tests, we evaluated EEAEN for its impact on the platelet-aggregating activity of thrombin. Figure 2 shows curves describing how thrombin preincubated with this peptide in various concentrations altered light transmission by the platelet suspension. When EEAEN was present in the incubation medium at a low concentration (10 pM), the aggregating activity of thrombin remained unchanged. In higher concentrations (0.01 to 1.0 nM), the peptide inhibited thrombin activity: after the incubation, thrombin initiated alterations in the shape of platelets and their aggregation, but these processes proceeded at slow rates. In still higher concentrations, EEAEN inhibited thrombin activity to a lesser extent and in the concentration of 1 μM failed to inhibit it at all. The effects of different EEAEN concentrations on thrombin-induced platelet aggregation are shown in Fig. 2, *a*. Thus, as can be seen, the greatest reduction in the aggregating activity of α-thrombin was recorded after its incubation with EEAEN in the concentration of 1.0 nM. Incubation of α-thrombin with thymosin$_{\alpha 1}$ under the same conditions as its incubation with EEAEN produced results (details not shown) similar to those we obtained earlier [3], confirming that thymosin$_{\alpha 1}$ partially inhibits α-thrombin's aggregating activity. For example, 1-minute incubation of α-thrombin with thymosin$_{\alpha 1}$ in concentrations of 0.1 to 1 nM lowered the activity of the enzyme by approximately 50-70%, whereas incubation with thymosin$_{\alpha 1}$ in higher concentrations (0.1-1 μM) did not affect its activity. These tests thus showed that the interaction of thymosin$_{\alpha 1}$ with α-thrombin is similar to that of EEAEN with this enzyme.

In further tests, the EEAEN peptide was added to a platelet suspension and the results are summarized in Fig. 3. As shown in this figure, the reaction of platelets to thrombin was altered in the presence of EEAEN, although the inhibitory effect of this pentapeptide was much weaker than in the case of its preincubation with thrombin.

Figure 4 shows platelet aggregatograms obtained when platelets were exposed to intact thrombin and to thrombin preincubated with EEAEN or with hirudin. It can be seen that the time taken to reach minimal light transmission was longer and the platelet aggregation rate was slower when the lower thrombin concentration was used (cf. curves

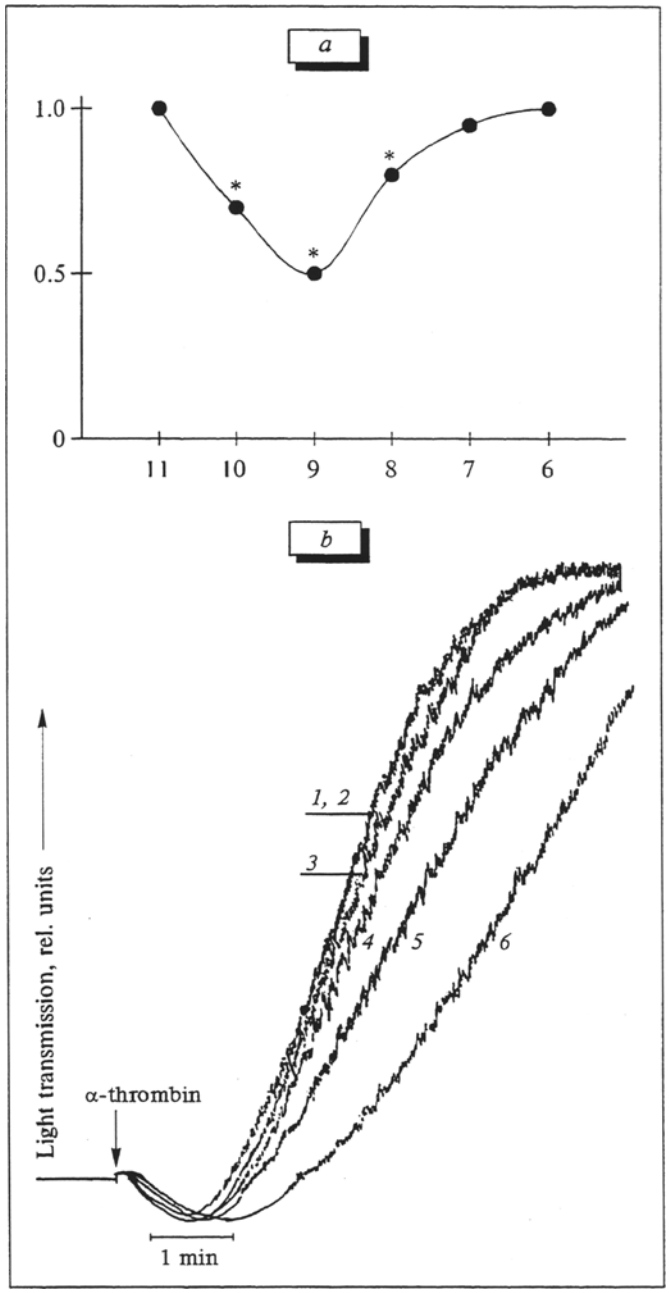

Fig. 2. Aggregating activity of thrombin as a function of EEAEN concentration (*a*) and variation in the light transmission by platelet suspension (*b*) induced with 6 nM thrombin (*1*) preincubated with EEAEN in concentrations of 0 (control), 1.0 μM (*2*), 0.1 μM (*3*), 10 nM (*4*), 0.1 nM (*5*), and 1.0 nM (*6*). *a*) ordinate: the v/v_o ratio, where v and v_o are the rates of thrombin–induced platelet aggregation in the presence and absence of EEAEN, respectively; abscissa: negative log molar concentrations of the peptide; $^*p<0.05$.

1 and *2* in Fig. 4); that the platelet responses to α-thrombin (4.5 nM) preincubated with hirudin at 0.02 units/ml (curve *3*) and with EEAEN at 1 nM (curve *4*) were virtually the same; and that the latter two curves are similar to curve *2* describing the process of platelet aggregation under the action of thrombin in the lower (2.5 nM) concentration.

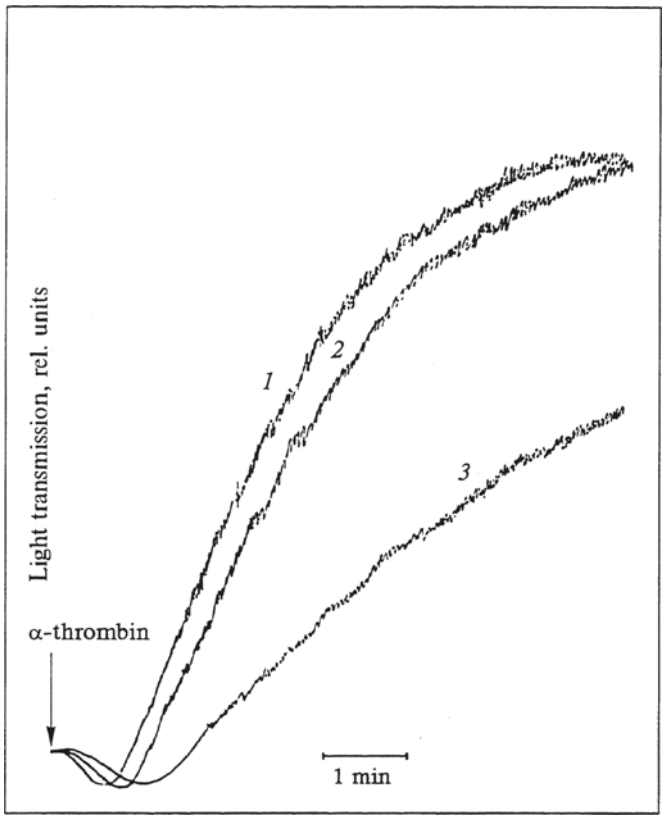

Fig. 3. Effect of EEAEN incubated with platelets (*2*) and thrombin (*3*) on thrombin–induced platelet aggregation. *1*) control. a – Thrombin concentration = 6 nM, EEAEN concentration = 1.0 nM.

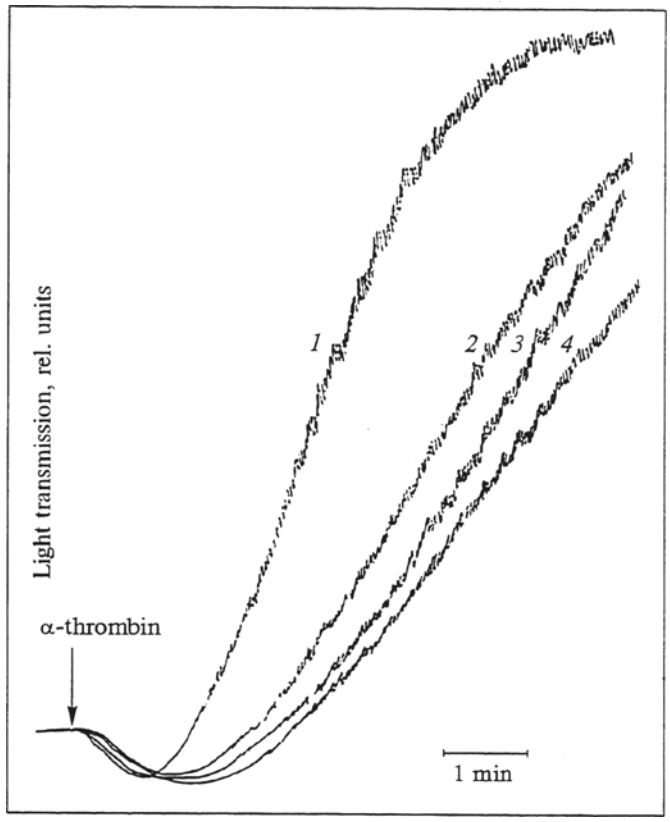

Fig. 4. Curves of thrombin–induced platelet aggregation in the presence of hirudin (0.02 units/ml) (*3*) and 1 nM EEAEN (*4*); thrombin concentrations: 4.5 nM in *1*, *3*, and *4* and 2.5 nM in *2*.

It should be noted that, unlike hirudin, EEAEN and thymosin$_{\alpha 1}$ in high concentrations failed to inhibit the platelet-aggregating activity of thrombin (data not shown). This difference probably occurred because hirudin interacts with both the recognition and reactive sites in thrombin [8,10], whereas EEAEN and thymosin$_{\alpha 1}$ apparently bind only to the recognition site. Since the physiological concentrations of thymosin$_{\alpha 1}$ do not exceed 1 nM, this thymic factor may be classed among the weak endogenous inhibitors of thrombin activity. The COOH-terminal peptide EEAEN of thymosin$_{\alpha 1}$ may be thought to be the reactive site responsible for its antithrombin activity.

Thus, as this study indicates, a peptide fragment capable of controlling thrombin activity is contained in the thymosin$_{\alpha 1}$ molecule in addition to the previously identified site (positions 20-24) responsible for the immunoregulatory properties of this thymic factor.

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