FIBRINOLYTIC ACTION OF AN ENZYME PREPARATION COVALENTLY BOUND WITH MODIFIED THROMBIN

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The effectiveness of treatment can be enhanced by the creation of systems of targeted drug transport [11]. Antibodies against the corresponding organ or tissue have proved themselves to be promising vector molecules for use in such systems [5, 12]. Other substances whose molecules might perform similar functions also have been suggested. For instance, to increase the affinity of urokinase for the fibrin clot, modification of the enzyme by fibrinogen has been used [9]. Since the rate of saturation with thrombin is increased in zones of platelet concentration [3], this enzyme may be regarded as a vector for the recognition of such zones. We know that the active center of a biocatalyst does not take part in binding of the enzyme with platelets, but it is necessary for the subsequent development of their aggregation [1]. With the above facts in mind it was decided to undertake chemical modification of thrombin so that, while losing its catalytic activity, it preserved its affinity for platelet receptors. It was pointed out previously that modification of thrombin at the tryptophan, tyrosine, arginine, and lysine residues impairs its affinity for fibrinogen [7]. Since a long binding site of macromolecular substrate in the thrombin molecule is responsible for binding of the enzyme with the platelet membrane also [1, 4], it is probably preferable to carry out the modification at other amino-acid residues.

The aim of this investigation was to confirm experimentally the validity of this approach to the targeted modification of α-thrombin in order to obtain a protein polymer matrix with affinity for centers of thrombus formation.

EXPERIMENTAL METHOD

Modified derivatives of α-thrombin (from Sigma, USA) were obtained as follows. After activation of the carboxyl group of thrombin by 1-ethyl-3(3'-dimethylaminopropyl)carbodiimide (from Serva, West Germany) by the method in [10], a 10^{-3}-10^{-2} M solution of the corresponding aliphatic diamine (1,4-tetramethylenediamine, 1,6-hexamethylenediamine, 1,12-dodecamethylenediamine from Fluka, Switzerland, was used) in 0.1 M phosphate buffer, pH 8.2, was added to a solution of the enzyme in distilled water, and the mixture was incubated with stirring overnight at room temperature. To prevent the formation of intermolecular aggregates the concentration of the enzyme in the incubation mixture must not exceed $5 \cdot 10^{-6}$ M. Low-molecular-weight substances were separated by ultrafiltration on an Amicon apparatus (USA) with VM-10 filter, using distilled water for washing (elution).

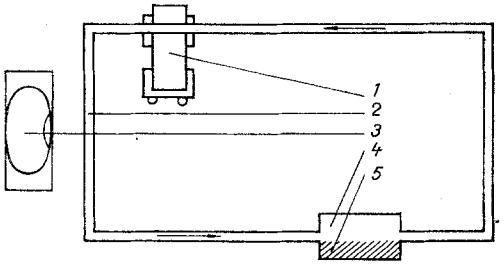

Fig. 1. Diagram of circulation system *in vitro* for estimating velocity of fibrinolysis: 1) peristaltic pump; 2) channel of circulation of solution; 3) γ-counter; 4) continuous flow cuvette; 5) fibrin clot.

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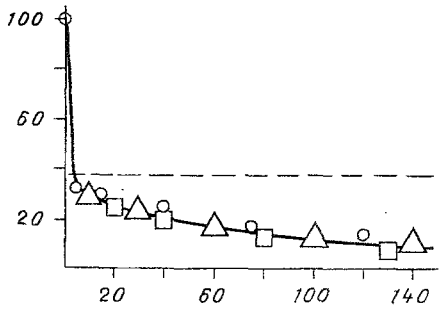

Fig. 2. Changes in esterase (BAEE) activity of thrombin on its modification by carbodiimide (level of enzyme activity indicated by broken line) and subsequent treatment with aliphatic diamines. Abscissa, inducation time (in min); ordinate, esterase activity preserved (in %). Modification of carboxyl-activated thrombin by: 1,4-tetramethylenediamine (circles); 1, 6-hexamethylenediamine (triangles); and 1,12-dodecamethylenediamine (squares).

Covalent binding of α-chymotrypsin (Koch-Light, England) to the diamine-modified thrombin was carried out after activation of the α-chymotrypsin with carbodiimide [10] by addition of the enzyme solution at pH 8.2 (0.1 M phosphate buffer) and incubation of the mixture overnight at room temperature. The resulting chymotrypsin–thrombin derivatives were isolated in an Amicon apparatus with VM-30 filter (using distilled water as the eluent).

At all stages of obtaining the preparations the level of esterase activity of the enzymes was monitored by means of a pH-stat (Radiometer, Denmark) by the method of initial reaction velocities at room temperature. Thrombin activity was monitored relative to hydrolysis of a 0.01 M solution of the ethyl ester of N-benzoyl-L-arginine (BAEE, from Sigma, in 0.01 M KCl solution at pH 7.5 and of a 10^{-4} M solution of the ethyl ester of p-tosyl-L-arginine (TAME, from Sigma) in 0.01 M KCl at pH 8.5, and chymotrypsin activity was monitored relative to hydrolysis of a 0.01 M solution of the ethyl ester of N-acetyl-L-tyrosine (ATEE, from Sigma) in 0.01 M KCl, pH 7.5. Incidentally, thrombin catalyzes hydrolysis of BAEE and TAME, but not of ATEE, whereas chymotrypsin catalyzes hydrolysis of ATEE and BAEE, but not TAME.

The fibrinogen-clotting activity of the derivatives was determined as described previously [2], by estimating the mean time of formation of a fibrin clot in a solution of fibrinogen (10 mg/ml, from Sigma) in 0.1 M phosphate buffer, pH 7.4, in the presence of various thrombin preparations (their concentration as protein was 10^{-7} M). The esterase and fibrinogen-clotting activity of the enzymes was determined in percentages of initial activity of the corresponding native biocatalyst (± 2%).

The fibrinolytic activity of the enzymes was determined *in vitro*. A fibrin clot was obtained in the cuvette of the circulation system (Fig. 1) [2] by interaction of fibrinogen (from Sigma), 5% of the quantity of which was added was fibrinogen TCK-19 (from Sorin Biomedica S.p.A., Italy), labeled with ^{99m}Tc [8], and thrombin. The cuvette was perfused with 0.1 M phosphate buffer, pH 7.4, containing preparations of native chymotrypsin and of chymotrypsin bound with premodified thrombin, with identical esterase (ATEE) activity ($4 \cdot 10^{-8}$ M), by means of a "rabbit" peristaltic pump (Rainin, France). In the control experiments preparations of native and premodified thrombin were used in the circulation system in concentrations identical with respect to protein. Radioactivity (γ-activity) in the channel of the system, evidence of destruction of the fibrin clot in the cuvette, was recorded on the RD-1600 analyzer with portable detector and teletype (Wallac, Finland). Three circulation systems, in the channels of which γ-activity was measured in turn, functioned simultaneously and in parallel. After lysis under dynamic conditions (about 6 h, rate of flow 0.08 ml/sec) the pump was disconnected and γ-activity determined in the system after about 13 h. The total volume of circulating solution was 3.5 ml. Since the duration of the experiment was commensurate with the half-decay time of ^{99m}Tc, during calculation of the changes in radioactivity a correction 2 t/T was introduced (where t stands for the time of determination of γ-activity and T for the half-decay period, or 6.04 h), obtained from the law of radioactive decay [6].

Platelet aggregation (concentration of cells $5.6 \cdot 10^5/\mu l$) induced by native or diamine-modified thrombin, in concentrations equal with respect to protein (about $4 \cdot 10^{-8}$ M) was monitored [2] by means of an aggregometer (All-Union Cardiologic Scientific Center, Academy of Medical Sciences of the USSR).

EXPERIMENTAL RESULTS

During modification of the carboxyl group of thrombin with carbodiimide the esterase (BAEE) activity of the biocatalyst was reduced by 2.5-3 times. Subsequent treatment of the

TABLE 1. Catalytic Activity (in %) of Thrombin Derivatives

Derivative	Native thrombin	Thrombin treated with carbodiimide	Thrombin modified by carbodiimide and diamines $H_2N-(CH_2)n-NH_2$			Thrombin—diamine—chymotrypsin conjugate			Native chymotrypsin
			$n=4$	$n=6$	$n=12$	$n=4$	$n=6$	$n=12$	
BAEE	100	38	16	12	10	—	—	—	—
TAME	100	—	15	13	11	0	0	0	—
ATEE	—	—	—	—	—	4	5	6	100
Fibrinogen	100	—	20	15	12	0	0	0	—

enzyme with diamines led to a further decrease of its catalytic activity (Fig. 2). The fibrinogen-clotting activity of the modified thrombin derivatives also was reduced (Table 1).

Native thrombin and its diamine-modified derivatives behaved similarly in the model system *in vitro* (Fig. 1). In the aggregometer, however, modified throbmin, unlike native, did not induce platelet aggregation. Chymotrypsin in the native form, or covalently bound with premodified thrombin, possessed varied fibrinolytic activity *in vitro* (Fig. 3). The modified derivatives were more active. When circulation of the solution in the system stopped (when the pump was disconnected) lysis was supported only by the enzyme derivative present on the fibrin clot. The difference in the fibrinolytic action of the preparations, based on the value of the γ-activity in the system, was proportional to the sorptive capacity of the derivatives tested. Adsorption of the modified derivative on the fibrin clot is probably maintained by the fibrinogen, or structures similar to it, in them, for fibrinogen can interact with fibrin monomer [13], and polyclonal antibodies to fibrinogen attach themselves to the fibrin clot [5]. The experimental results show (Fig. 3) that affinity of the chymotrypsin—diamine—thrombin preparations to the location of the fibrin clot is six to nine times greater than in the case of native chymotrypsin.

Modification of thrombin thus enables a polymer matrix, with affinity for the site of the fibrin clot, to be obtained. On its basis both fibrinolytic preparation with targeted action and also, probably, thrombin derivatives with anticoagulant activity can be created [1].

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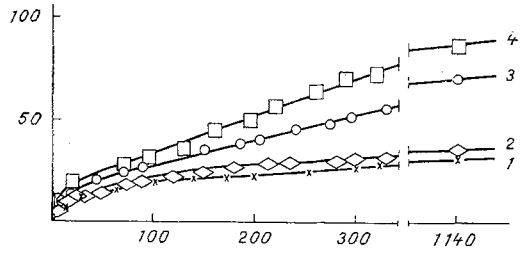

Fig. 3. Changes in velocity of fibrinolysis under the influence of native and modified chymotrypsin. Abscissa, time of measurement (in min); ordinate, - activity in system (in %, γ-activity in system on total lysis of fibrin clot taken as 100%). 1) Control (0.1 M phosphate buffer, pH 7.4); 2) native chymotrypsin; 3, 4) chymotrypsin covalently bound with thrombin, premodified by 1,4-tetramethylenediamine and 1,12-dodecamethylenediamine, respectively.

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INDUCTION OF LIPID PEROXIDATION BY THE LENS

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The lens tissue is avascular and its metabolism is entirely dependent on the aqueous humor. The possibility therefore cannot be ruled out that the causes of opacity of the lens during cataract development may be connected with disturbance of the normal composition of the aqueous humor [2]. Because of the role of lipid peroxidation (LPO) in the genesis of opacity of the lens [1], it was decided to study whether LPO products may not appear in the aqueous humor. The presence of high concentrations of hydrogen peroxide (H_2O_2) in the aqueous humor of some cataract patients was demonstrated previously [10]. Meanwhile, we know that the aqueous contains about 4 μg/ml of high-density lipoproteins, which evidently take part in the renewal of the lipid composition of the lens [4], and, in principle, they may undergo oxidation.

In the investigation described below two problems were studied: 1) Does the concentration of LPO products in the aqueous of the lens change in cataracts; 2) Can the isolated lens affect the concentration of LPO products in the surrounding medium.

EXPERIMENTAL METHOD

The aqueous extracted from the anterior chamber of 27 eyes from cataract patients during operations for intracapsular cryoextraction was used as the test material. The aqueous from 10 eyes from donors, supplied from a corneal transplant bank, was used as the control. The average age of the patients was 65 ± 10 years. Immediately after the material had been obtained, lipids were extracted by the method described previously [1]. The relative concentration of lipids in the extract was determined by their absorption at 206 nm (D_{206}). The concentration of primary (diene conjugates) and secondary (ketodienes) LPO products was estimated spectrophotometrically from their characteristic absorption in the region of the maxima at 232 nm (D_{232}) and 280 nm (D_{280}) on a Hitachi-557 spectrophotometer, and expressed

TABLE 1. Concentrations of LPO Products in Aqueous Humor of Cataract Patients (M ± m)

Parameter tested	Control (10)	Cataract (27)
D_{232}/D_{206}	0,331±0,030	0,448±0,027*
D_{280}/D_{206}	0,122±0,006	0,170±0,1*
Fluorescent products, relative units	47,4±18,3	231,5±61,9*

Legend. Number of experiments in parentheses. *P < 0.01 compared with control.

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