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Mechanism of Action of ω-Amino Acids on Plasminogen Activation and Fibrinolysis Induced by Staphylokinase

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Abstract—Stimulation of Lys-plasminogen (Lys-Pg) and Glu-plasminogen (Glu-Pg) activation under the action of staphylokinase and Glu-Pg activation under the action of preformed plasmin–staphylokinase activator complex (Pm–STA) by low concentrations and inhibition by high concentrations of ω-amino acids (>90-140 mM) were found. Maximal stimulation of the activation was observed at concentrations of L-lysine, 6-aminohexanoic acid (6-AHA), and *trans*-(4-aminomethyl)cyclohexanecarboxylic acid 8.0, 2.0, and 0.8 mM, respectively. In contrast, the Lys-Pg activation rate by Pm–STA complex sharply decreased when concentrations of ω-amino acids exceeded the above-mentioned values. It was found that formation of Pm–STA complex from a mixture of equimolar concentrations of staphylokinase and Glu-Pg or Lys-Pg is stimulated by low concentrations (maximal at 10 mM) of 6-AHA. Negligible increase in the specific activities of plasmin and Pm–STA complex was detected at higher concentrations of 6-AHA (to maximal at 70 and 50 mM, respectively). Inhibitory effects of ω-amino acids on the rate of fibrinolysis induced by staphylokinase, Pm–STA complex, and plasmin were compared. It was found that inhibition of staphylokinase-induced fibrinolysis by ω-amino acids includes blocking of the reactions of Pm–STA complex formation, plasminogen activation by this complex, and lysis of fibrin by forming plasmin as a result of displacement of plasminogen and plasmin from the fibrin surface. Thus, the slow stage of Pm–STA complex formation plays an important role in the mechanism of action of ω-amino acids on Glu-Pg activation and fibrinolysis induced by staphylokinase. In addition to α→β change of Glu-Pg conformation, stimulation of Pm–STA complex formation leads to increase in Glu-Pg activation rate in the presence of low concentrations of ω-amino acids. Inhibition of Pm–STA complex formation on fibrin surface by ω-amino acids is responsible for appearance of long lag phases on curves of fibrinolysis induced by staphylokinase.

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Native human Glu-plasminogen (Glu-Pg) contains an N-terminal peptide (NTP, residues 1-77), five homologous kringle domains, and a protease domain (residues 562-791) [1]. A break of peptide bond Arg561–Val562 by activators of plasminogen converts the latter into plasmin, a two-chain enzyme that dissolves fibrin clots [2]. The light B-chain (protease domain) of plasmin is bound by two disulfide bonds to the heavy A-chain in which kringle domains are located. Lysine-binding sites (LBS) localized in kringles play a key role in specific binding of plasmin(ogen) to fibrin, α_2-antiplasmin, and cell surface [3-5]. Glu-plasminogen can have a compact closed α-conformation which is maintained by two intermolecular interactions (between LBS of kringle 5 and NTP and between LBS of kringle 4 and a ligand on kringle 3), semi-open β-conformation, when one of the two interactions is retained, and fully open γ-conformation, when both lysine-dependent intermolecular interactions are broken [1, 6, 7]. Lys-plasminogen (Lys-Pg) devoid of the N-terminal peptide adopts γ- or β-conformation required by

Abbreviations: AFK-pNA) *p*-nitroanilide CHO-Ala-Phe-Lys; 6-AHA) 6-aminohexanoic acid; *t*-AMCHA) *trans*-(4-aminomethyl)cyclohexanecarboxylic acid; Glu-Pg and Lys-Pg) Glu- and Lys-forms of plasminogen; LBS) lysine-binding sites; NTP) N-terminal peptide; Pm) plasmin; SK) streptokinase; STA) staphylokinase; Pg–SK and Pm–SK) equimolar plasminogen–streptokinase and plasmin–streptokinase complexes, respectively; Pg–STA and Pm–STA) equimolar plasminogen–staphylokinase and plasmin–staphylokinase complexes, respectively; tcu-PA) two-chain urokinase-type plasminogen activator; t-PA) tissue activator of plasminogen.

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the conditions and is activated more rapidly than Glu-Pg [8]. It was found that the rate of Glu-Pg activation by urokinase (tcu-PA) and tissue plasminogen activator (t-PA) markedly increased in the presence of inhibitors of fibrinolysis at low concentrations – L-lysine (L-Lys) and structurally similar ω-amino acids such as 6-aminohexanoic acid (6-AHA) and *trans*-(4-aminomethyl)cyclohexanecarboxylic acid (*t*-AMCHA) [1, 6, 9-11]. This effect is caused by the fact that binding of ω-amino acids to LBS of kringle 5 of Glu-Pg breaks its intermolecular bond between LBS of kringle 5 and NTP, which results in change in closed conformation to semi-open. At high concentrations, ω-amino acids inhibit activation of Glu-Pg as well as Lys-Pg [11, 12]. ω-Amino acids cause dose-dependent retardation of fibrinolysis induced by tcu-PA, t-PA, streptokinase (SK), and recombinant staphylokinase (STA); this is rationalized by saturation of high affinity LBS of plasminogen with ω-amino acids and its displacement from the surface of fibrin [12-14].

In contrast to direct activators of plasminogen (tcu-PA and t-PA), staphylokinase, like streptokinase, is not an enzyme. Both proteins form an equimolar complex with plasminogen. Whereas in Pg–SK complex an active site in the plasminogen molecule is exposed without proteolysis [15], Pg–STA complex is inactive and becomes active only after conversion into plasmin (Pm)–STA complex [16]. Mechanisms of action of ω-amino acids on activation of plasminogen by direct and indirect activators may differ, because their binding to LBS of plasminogen can influence not only activation of plasminogen substrate, but also the rate of formation and/or activity of Pg–SK and Pm–STA activator complexes. This is indicated by the fact that low 6-AHA concentrations inhibit activation of Glu-Pg by streptokinase as well as formation of Pg–SK activator complex [17-20]. Pg–SK complex (K_d = 0.05 nM [21]) is formed due to high-affinity interactions of streptokinase with the protease domain and kringle 5 of plasminogen [8, 17], whereas in Pg–STA complex (K_d = 10.75 nM [22]) the three times smaller staphylokinase molecule is bound only to the protease domain of plasminogen [16]. Taking into account the absence of kringle-dependent interaction in Pg–STA complex and a low rate of its conversion into active Pm–STA complex ($k_1 = 4 \cdot 10^{-7}$ sec^{-1} [23]), one can expect that the effect of ω-amino acids on plasminogen activation and fibrinolysis induced by staphylokinase may differ from that in case of streptokinase.

The goal of this work was to study the mechanism of action of ω-amino acids on plasminogen activation and fibrinolysis induced by staphylokinase.

MATERIALS AND METHODS

In this study we used a solution of highly purified recombinant staphylokinase (SAKSTAR, 15.5 kD, 0.98 mg/ml) from the Center for Molecular and Vascular Biology, University of Leuven (Belgium); streptokinase (47 kD, 6300 IU per mg of dry mass) from Reyon Pharmaceutical Co. Ltd (Korea); human fibrinogen, human thrombin, and *p*-nitroanilide HCO-Ala-Phe-Lys from Tekhnologiya-Standart (Russia); aprotinin (Gordox) from Gedeon Richter (Hungary); L-lysine and phenylmethylsulfonyl fluoride (PMSF) from Sigma (USA); 6-aminohexanoic acid from Merck (Germany); *trans*-(4-aminomethyl)cyclohexanecarboxylic acid from Acros Organics (USA); Lys-Sepharose 4B from Amersham Biosciences (Sweden); pool of citrated human blood plasma from the Hematological Center of the Health Ministry of Russia (Moscow).

Main buffer used was 0.1 M Tris-HCl, pH 7.4, containing 0.15 M NaCl (buffer A); in certain experiments it also contained other additional components.

Preparation of Lys-Pg and Glu-Pg. A pool of human blood plasma was the starting material for Lys-Pg preparation. To separate Glu-Pg, 6000 IU aprotinin was immediately added to 300 ml of fresh human plasma. Lys- and Glu-forms of plasminogen were isolated from plasma by affinity chromatography on Lys-Sepharose 4B at 4°C according to Castellino and Powell [24] but with some modifications. Protein peaks of Lys-Pg and Glu-Pg eluted from a column with 0.2 M 6-AHA were precipitated with ammonium sulfate (0.31 g/ml). After purification on Sephadex G-75 at 4°C, the plasminogens were treated with 1 mM PMSF for 3 h and dialyzed against 0.05 M Tris-HCl, pH 7.4, at 4°C. Protein concentration in the plasminogen preparations was determined according to Lowry [25]. Purity of preparations was tested by SDS-PAGE in 7.5% gel under non-reducing conditions according to Laemmli [26]. According to aprotinin titration of plasmin obtained by activation of Lys-Pg or Glu-Pg by streptokinase, zymogen content in the preparations was 90 and 95%, respectively.

Plasmin was prepared by activation of 3 μM Lys-Pg by streptokinase at catalytic concentration ([Pg]/[SK] = 100 : 1 M/M) in buffer A containing 20% glycerol (v/v) and 0.01% Tween-80 at 25°C. The degree of conversion of zymogen into enzyme was evaluated via the maximal amidase activity of samples. Plasmin solution was stored in aliquots at –20°C.

Pm–STA activator complex was prepared just before use by incubation of 2 μM Lys-Pg with equimolar concentration of streptokinase in buffer A containing 20% glycerol (v/v) for 20-25 min at 25°C.

Kinetics of formation of Pm–STA complex was studied as follows. A 0.4-μM solution of Glu-Pg (or Lys-Pg) in buffer A containing 6-AHA at various concentrations was incubated for 2-3 min at 37°C; then staphylokinase at equimolar concentration was added. Kinetics of formation of Pm–STA complex was monitored via increase in amidase activity of samples taken during incubation. To eliminate the effect of inhibitor on the estimated activity

of Pm–STA complex, the volume of samples added to a spectrophotometer cuvette was varied so that 6-AHA concentration did not exceed 8 mM. The rate of formation of Pm–STA complex in the presence of 6-AHA at various concentrations was determined as the slope of the initial portions of kinetic curves ($\Delta A_{405}/t$). Each experiment was performed four times.

Amidase activity of plasmin and Pm–STA complex was monitored spectrophotometrically at 405 nm via the initial rate of hydrolysis of 0.6 mM AFK-pNA solution in buffer A at 25°C, taking ε_M of *p*-nitroaniline as 10,000 $M^{-1}\cdot cm^{-1}$. Enzyme concentration in solution was 10-100 nM. When studying the effect of ω-amino acid on amidase activities of plasmin and Pm–STA complex, the reaction mixture contained 0-350 mM 6-AHA (each experiment was performed four times).

Kinetics of activation of Glu-Pg and Lys-Pg by the native STA and preformed Pm–STA complex was studied by a method developed by us. Buffer A (150 μl) containing 0.8 mM AFK-pNA, 0.67 μM Glu-Pg or Lys-Pg, and L-Lys, 6-AHA, or *t*-AMCHA at various concentrations was added to the wells of a microplate. After incubation of a closed microplate in a thermostatted Elmi ST-3 shaker–incubator for 15 min at 37°C, 50 μl of 0.52 nM solution of staphylokinase or Pm–STA complex in the same buffer were added to working wells. To control substrate hydrolysis by a possible residual plasmin (A^0_{405}), 50 μl of buffer was added to control wells instead of activator. Concentrations of L-Lys, 6-AHA, and *t*-AMCHA in the reaction mixture were varied from 0 to 500 mM. Immediately after addition of activator, closed plates were incubated in the shaker–incubator at 37°C. Kinetics of plasminogen activation was monitored via release of a product of AFK-pNA hydrolysis by the forming plasmin after 3-5 min intervals using an Anthos 2020 plate photometer (Austria) connected with a computer and set for measuring optical absorption at 405 nm (A_{405}). A_{405} values of working solutions were corrected for changes in controls (A^0_{405}) if the latter were detected. Each experiment was performed four times. Parabolic curves of *p*-nitroaniline formation in a conjugated reaction (A_{405} versus t) were linearized in $A_{405} - t^2$ coordinates, and the slopes of the lines gave the rates of plasminogen activation ($\Delta A_{405}/t^2$) [27].

For study of fibrinolysis kinetics, columns of fibrin gel were formed as follows in standard Salie test-tubes (d = 9.5 mm): 20 μl of thrombin solution (40 U/ml) was added to 0.78 ml of 0.3% (determined via coagulated protein) solution of human fibrinogen in buffer A, and the mixture was shaken and left in vertical position for 1.5 h at 25°C. To the obtained gels, 0.45 ml of buffer A containing various concentrations of corresponding ω-amino acid were added, and the test-tubes were thermostatted for 15 min at 37°C. Fibrinolysis was initiated by addition of 50 μl of staphylokinase or Pm–STA complex (0.52 nM) solutions. Concentrations of L-Lys, 6-AHA, or *t*-AMCHA in the reaction mixture were varied in the intervals 0-500, 0-5.0, and 0-0.8 mM, respectively. Each experiment was performed four times. Kinetics of fibrinolysis was monitored at 37°C via decrease in the gel column height (l) with time using a KM-6 cathetometer (Russia) [28]. The rate of fibrinolysis was determined as the slope of the initial portions of kinetic curves ($\Delta l/t$) in mm/min.

RESULTS

Figure 1a illustrates changes in the activation rates of Glu-Pg and Lys-Pg by the native staphylokinase in the presence of increased L-Lys, 6-AHA, and *t*-AMCHA concentrations. As shown, all ω-amino acids within certain concentration ranges stimulate activation of both plasminogen forms by staphylokinase. The maximal increase in the rates of staphylokinase-induced activation was 2.9, 3.9, and 4.6 times for Glu-Pg and 1.2, 1.4, and 1.5 times for Lys-Pg and it was observed at 8.0, 2.0, and 0.8 mM concentrations of L-Lys, 6-AHA, and *t*-AMCHA, respectively. Stimulating effect of ω-amino acids increased in the series: L-Lys < 6-AHA < *t*-AMCHA.

Activation of plasminogen by staphylokinase at catalytic concentration includes the following stages: 1) fast formation of staphylokinase–plasminogen complex; 2) slow conversion of inactive Pg–STA complex into the active Pm–STA complex; 3) activation of excess plasminogen to plasmin by Pm–STA complex.

$$\mathrm{Pg + STA} \underset{}{\overset{K_d}{\rightleftarrows}} \mathrm{Pg{-}STA}, \quad (1)$$

$$\mathrm{Pg{-}STA} \overset{k_1}{\rightleftarrows} \mathrm{Pm{-}STA}, \quad (2)$$

$$\mathrm{Pm{-}STA + Pg} \overset{K_m}{\rightleftarrows} \mathrm{Pm{-}STA \cdot Pg} \overset{k_{cat}}{\longrightarrow} \mathrm{Pm{-}STA + Pm}. \quad (3)$$

ω-Amino acids which bind to LBS of kringles of plasmin(ogen) A-chain are not suggested to influence formation of stable inactive Pg–STA complex (stage 1), because the small staphylokinase molecule is bound to the protease domain of plasminogen and does not interact with kringle domains [16]; however, ω-amino acids may modulate stages 2 and 3.

Figure 1b illustrates how the rates of activation of Glu-Pg and Lys-Pg by preformed active Pm–STA complex depend on L-Lys, 6-AHA, and *t*-AMCHA concentrations. These dependences demonstrate the effect of ω-amino acids only on stage 3. As shown, when preformed Pm–STA complex is used as an activator, the effect of stimulation of Lys-Pg activation by ω-amino acids is absent (curves *1*), whereas Glu-Pg activation (curves *2*) is stimulated approximately within the same ω-amino acid

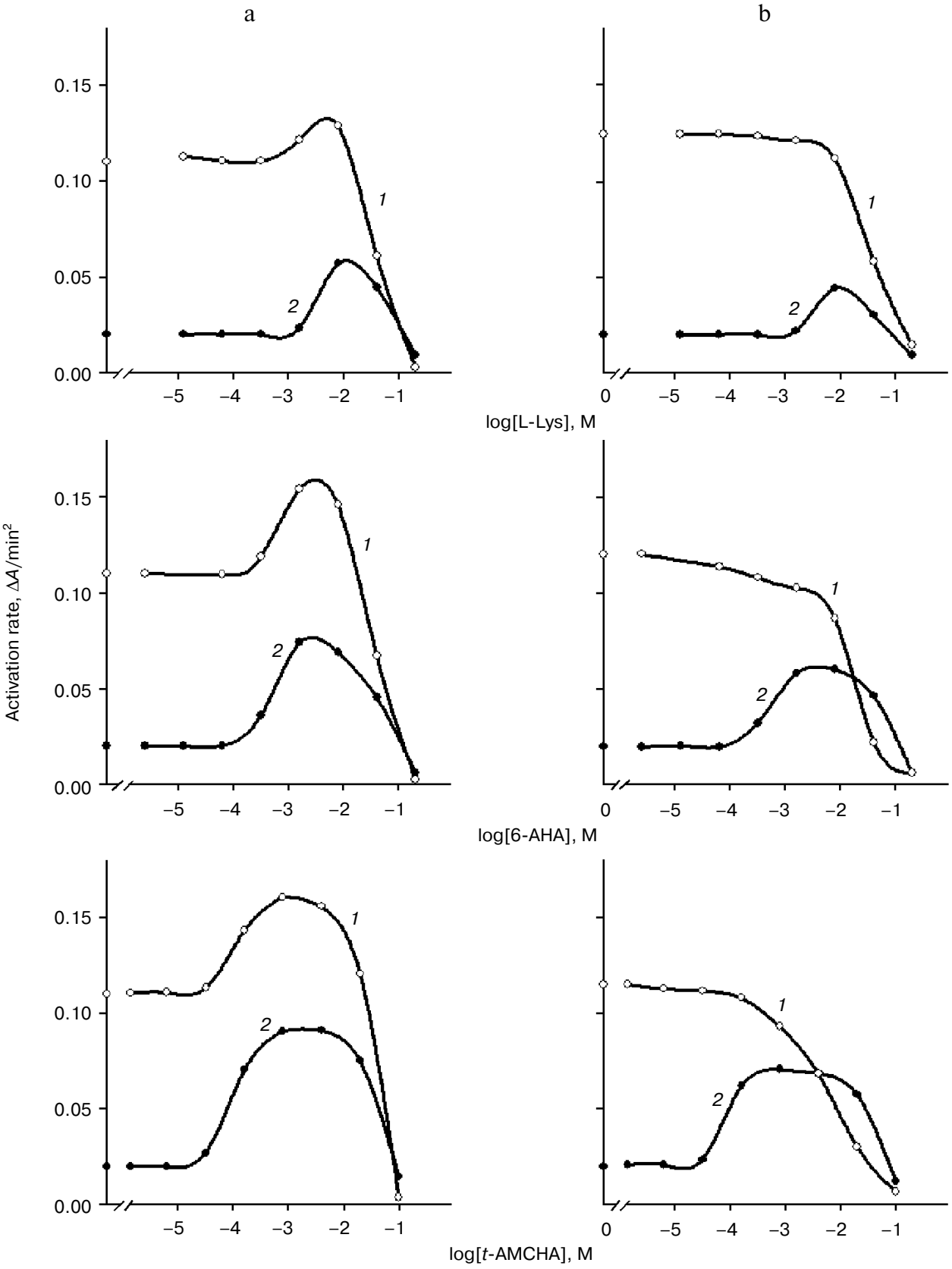

Fig. 1. Rates of activation of 0.5 μM Lys-Pg (*1*) and Glu-Pg (*2*) by 0.13 nM staphylokinase (a) and by 0.13 nM preformed Pm–STA complex (b) versus L-Lys, 6-AHA, and *t*-AMCHA concentrations ($p < 0.001$).

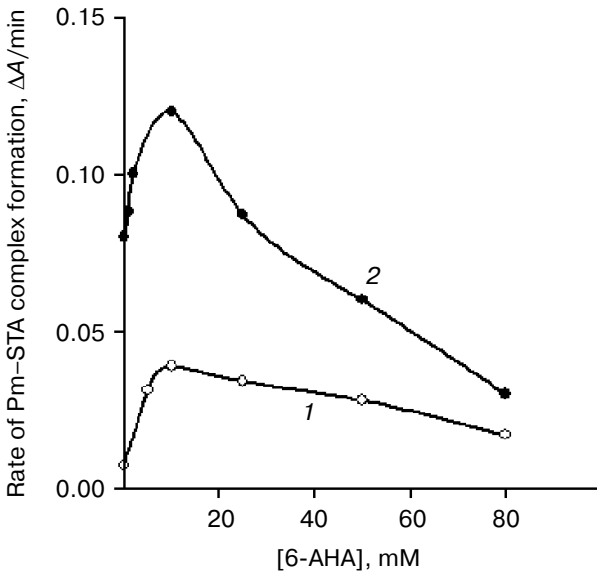

Fig. 2. Rate of formation of Pm–STA activator complex from a mixture of 0.4 μM staphylokinase with equimolar Lys-Pg (*1*) and Glu-Pg (*2*) concentrations versus 6-AHA concentration ($p < 0.001$).

concentration range in which stimulation of its activation by native staphylokinase (Fig. 1a, curves *2*) was observed. On increase in ω-amino acid concentration the rate of Lys-Pg activation by Pm–STA complex (curves *1*) initially decreased slowly and then sharply at L-Lys, 6-AHA, and *t*-AMCHA concentrations exceeding 8.0, 2.0, and 0.2 mM, respectively. However, inhibition of Glu-Pg and Lys-Pg activation by staphylokinase and also of Glu-Pg activation by Pm–STA complex was observed at ω-amino acid concentrations exceeding 90-140 mM. The maximal increase in the rate of Glu-Pg activation by Pm–STA complex was 2.2, 3.0, and 3.5 times and was observed at 8.0, 2.0, and 0.8 mM concentrations of L-Lys, 6-AHA, and *t*-AMCHA, respectively. Stimulation of Glu-Pg activation by ω-amino acids can be elucidated as follows: their binding to LBS of kringle 5 breaks the intermolecular bond between kringle 5 and the NTP in the Glu-Pg molecule; this leads to transfer from its closed α-conformation to a semi-open and more rapidly activated β-conformation. However, ω-amino acids do not influence conformation of Lys-Pg, which does not contain the NTP.

Observed stimulation by ω-amino acids of Lys-Pg activation by staphylokinase (Fig. 1a) but not by Pm–STA complex (Fig. 1b) indicates that they potentiate stage 2. It is also proved by the fact that the degree of ω-amino acids stimulation of Glu-Pg activation by staphylokinase is higher than its activation by Pm–STA complex. Figure 2 illustrates the effect of 6-AHA (as the most studied ω-amino acid) at various concentrations on the rate of formation of Pm–STA complex from an equimolar mixture of staphylokinase with Lys-Pg or Glu-Pg. The data indicate that the reaction of Pm–STA complex formation (stage 2) is stimulated by ω-amino acid, 10 mM 6-AHA causing the maximal increase in the reaction rate.

Figure 3 illustrates the effect of 6-AHA at various concentrations on amidase activity of plasmin (curve *1*) and preformed Pm–STA complex (curve *2*). As shown, ω-amino acid negligibly stimulates activity of the two enzymes. However, the maximal increase in activity of plasmin and Pm–STA complex (1.2-1.3 times) was observed in the presence of high 6-AHA concentrations (70 and 50 mM, respectively). Comparing Figs. 3 and 2, it can be seen that stimulation of amidase activity of plasmin and Pm–STA complex in the presence of 10 mM 6-AHA is negligibly low compared with the effect of 6-AHA on increase in the rate of formation of Pm–STA complex.

The effect of two low 6-AHA concentrations on kinetics of fibrinolysis initiated by staphylokinase and Pm–STA complex is presented in Fig. 4. In the absence of 6-AHA, the fibrinolytic action of both activators is identical (curves *1* and *2*): the kinetics is linear and the rate of fibrinolysis is equal. Addition of inhibitor decreases the rate of fibrinolysis by both activators. However, contrary to the action of Pm–STA complex (curves *4* and *6*), lysis of fibrin by the action of staphylokinase begins after a long lag phase, which increases with increase in 6-AHA concentration (curves *3* and *5*). Analogous lag phases for lysis by staphylokinase were also observed in the presence of L-Lys and *t*-AMCHA. Fibrinolysis by the

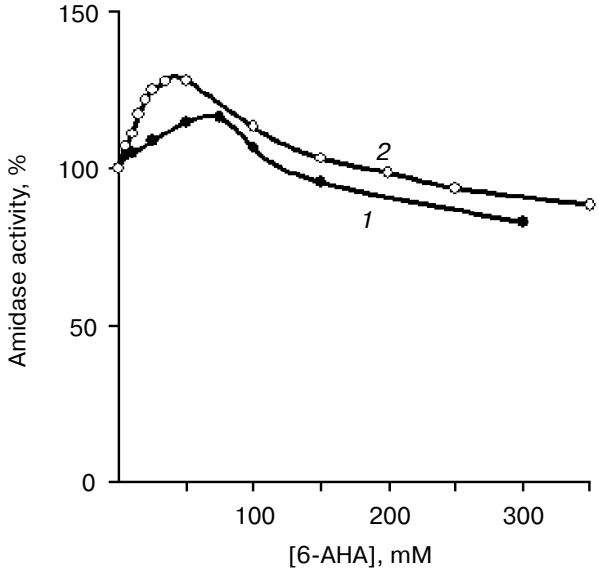

Fig. 3. Effect of various concentrations of 6-AHA on amidase activity of 0.02 μM plasmin (*1*) and Pm–STA complex (*2*) ($p < 0.001$).

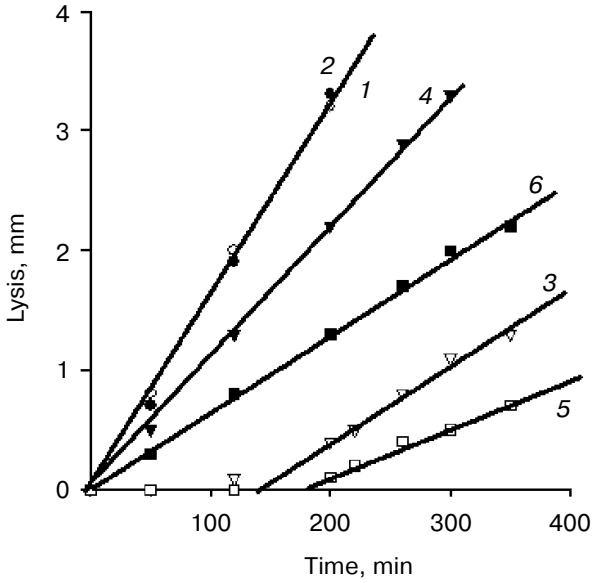

Fig. 4. Kinetics of lysis of fibrin clots by the action of 40 nM staphylokinase (open symbols) and Pm–STA complex (closed symbols) in the absence (*1* and *2*) and presence of 5 mM (*3* and *4*) and 10 mM 6-AHA (*5* and *6*) ($p < 0.001$).

action of staphylokinase and Pm–STA complex is based on activation of plasminogen, which is included in fibrin clot, and lysis of fibrin (Fn) by the forming plasmin to soluble products of its degradation (FDP):

$$\mathrm{Fn} \xrightarrow{\mathrm{Pm}} \mathrm{FDP}. \qquad (4)$$

Fibrinolytic action of staphylokinase includes stages 1-4, whereas the action of Pm–STA complex involves only stages 3 and 4. Consequently, appearance and increase in lag phase on kinetic curves of fibrinolysis by staphylokinase in the presence of increasing ω-amino acid concentrations is related with inhibition of Pm–STA complex formation (stage 2). The long lag phase of fibrinolysis by staphylokinase even at low ω-amino acid concentrations stimulated us to study the effect of various concentrations of the latter on kinetics of fibrinolysis by plasmin (stage 4) and Pm–STA complex (stages 3 and 4). The initial rates of fibrinolysis by the action of plasmin (curves *1*) and Pm–STA complex (curves *2*) versus L-Lys, 6-AHA, and *t*-AMCHA concentrations are presented in Fig. 5. Values of $[I]_{50}$ for fibrinolysis by plasmin were 270, 4.3, and 0.7 mM, while those for fibrinolysis by Pm–STA complex were 100, 1.3, and 0.3 mM for L-Lys, 6-AHA, and *t*-AMCHA, respectively. The data indicate that ω-amino acids inhibit fibrinolysis by Pm–STA complex mediated by a stage of plasminogen activation on fibrin to a greater extent than direct fibrinolysis by plasmin.

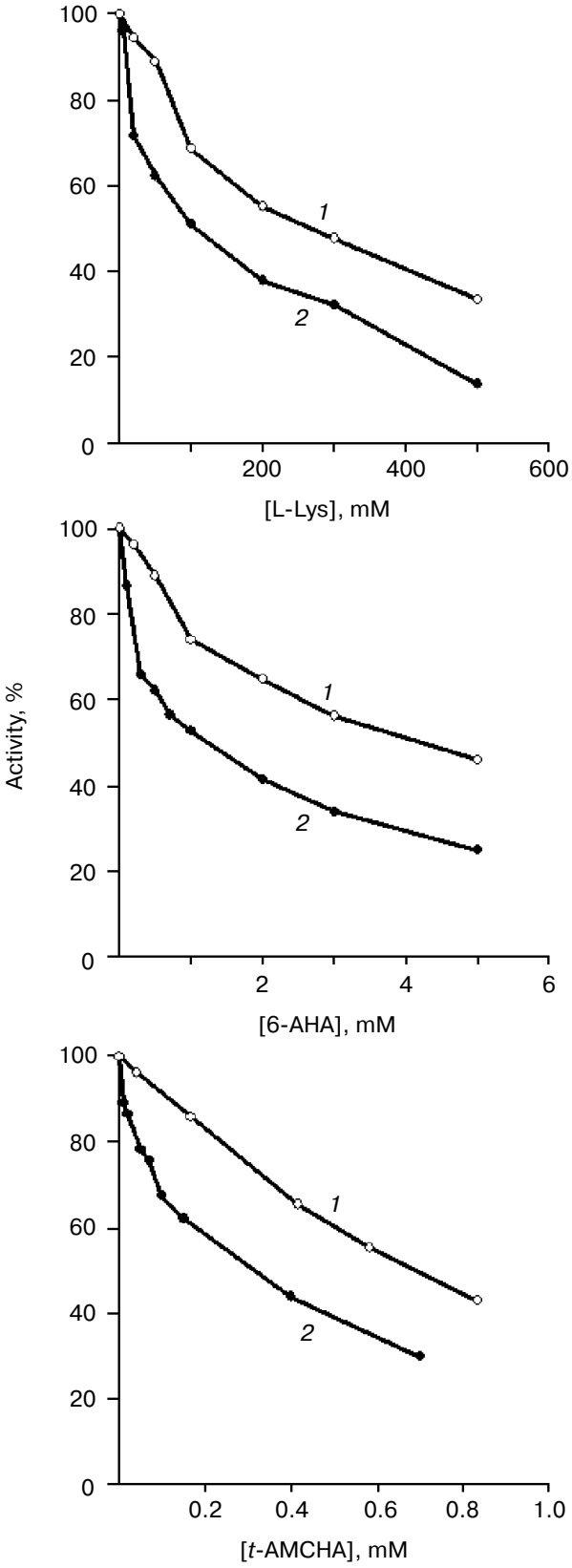

Fig. 5. Rates of fibrinolysis by the action of 0.7 μM plasmin (*1*) and 40 nM Pm–STA complex (*2*) versus L-Lys, 6-AHA, and *t*-AMCHA concentrations ($p < 0.001$).

DISCUSSION

ω-Amino acids are used in medical practice as inhibitors of fibrinolysis. Lysis of fibrin is caused by plasmin, which is formed from plasminogen by the action of plasminogen activators. High concentrations of ω-amino acids inhibit activation of plasminogen by its activators, and also plasmin activity and fibrinolysis. At low concentrations, they increase the rate of Glu-Pg activation by t-PA and tcu-PA due to conformational changes in the zymogen molecule [1, 6, 9], but inhibit its activation by streptokinase [18-20]. Recently it was shown that low 6-AHA concentrations inhibit Glu-Pg activation by streptokinase, blocking formation of Pg–SK activator complex and enzyme–substrate complex Pg–SK–Pg via a kringle-dependent mechanism [17].

There are certain distinctions in mechanisms of action of two indirect plasminogen activators – streptokinase and staphylokinase. These distinctions arise from different structures of staphylokinase and streptokinase as well as their complexes with plasmin(ogen). Hypothetically, each domain of streptokinase (SK_α, SK_β, and SK_γ) has different function (a proper pre-orientation, providing an anchor site and site of interaction with kringle-domain of plasminogen substrate and its activation) [29]. Based on structural similarity of staphylokinase and SK_α domain, it is suggested that their functions in plasminogen activation are similar. Both staphylokinase and SK_α bind to the protease domain of plasmin(ogen) in the neighborhood of the active site. The large streptokinase molecule mainly surrounds the protease domain of plasminogen, whereas the small one-domain staphylokinase molecule contacts with only one side of the protease domain. Additionally, SK_β domain interacts with the kringle domain of plasmin(ogen), whereas the kringle domains do not participate in staphylokinase binding [16, 29]. Inhibition of Glu-Pg activation by streptokinase in the presence of low 6-AHA concentrations seems to be caused by an ω-amino acid-induced break of a bond between SK_β domain and kringle domain of plasmin(ogen). To elucidate the mechanism of action of ω-amino acids on staphylokinase properties, it was necessary to study thoroughly how activator and fibrinolytic activities of staphylokinase depend on ω-amino acid concentrations, because it has not been previously studied.

It was found (Fig. 1a) that low ω-amino acid concentrations increase the rates of both Glu-Pg and Lys-Pg activation by staphylokinase. The effect of stimulation by ω-amino acids of Glu-Pg activation by the action of staphylokinase (curves *2*) as well as t-PA and tcu-PA can be rationalized by change in Glu-Pg conformation from α- to β-type as a result of a break of the intramolecular bond between kringle 5 and the NTP in the native zymogen molecule caused by ω-amino acids. However, ω-amino acids also increased the rate of activation of Lys-Pg by staphylokinase (curves *1*), and this was not observed while its activation by direct activators t-PA and tcu-PA [1, 6, 9, 10]. Since Lys-Pg does not contain an intramolecular bond between kringle 5 and NTP, it was suggested that the observed stimulation of its activation by staphylokinase can be related with the effect of ω-amino acids on formation of Pm–STA activator complex. To test this suggestion, we studied how the rates of Glu-Pg and Lys-Pg activation by preformed Pm–STA complex depend on concentrations of ω-amino acids (Fig. 1b). If stage 2 was excluded, low concentrations of ω-amino acids did not increase the rate of Lys-Pg activation (curves *1*), but stimulated Glu-Pg activation, although to a somewhat lesser extent (curves *2*). Higher rate of Lys-Pg activation (5-6 times) as compared with that of Glu-Pg activation by both types of activators in the absence of ω-amino acids and under the conditions of maximal stimulation by corresponding concentrations of ω-amino acids (Fig. 1) indicates that Lys-Pg adopts an open conformation which is not changed on binding to ω-amino acids. The data show that the above-mentioned suggestion is probable.

To test this suggestion, we studied kinetics of formation of Pm–STA complex from an equimolar mixture of Lys-Pg or Glu-Pg and staphylokinase in the presence of increasing 6-AHA concentration. Since the staphylokinase molecule binds only to the protease domain of plasminogen, ω-amino acid must not influence formation of inactive Pg–STA complex (stage 1). Results of this experiment characterizing stage 2 prove that low 6-AHA concentrations stimulate the rate of conversion of inactive Pg–STA complex into the active Pm–STA complex (Fig. 2). However, in the presence of 10 mM 6-AHA causing the maximal stimulation, the rate of formation of Pm–STA complex from Glu-Pg–STA complex increased 5.4-fold (curve *2*), whereas that from Lys-Pg–STA complex increased only 1.5-fold (curve *1*). This is probably related with the fact that in the case of Lys-Pg–STA complex, 6-AHA stimulates only the rate of its conversion into Pm–STA complex, whereas in case of Glu-Pg–STA complex 6-AHA also caused α→β conformational transfer of Glu-Pg molecule in the complex, and this contributes to increase in the total rate of Pm–STA complex formation. Comparison of our data and that in the literature demonstrates that the effects of low 6-AHA concentrations on formation of Pm–STA and Pg–SK activator complexes as well as on Glu-Pg activation by the native staphylokinase or streptokinase are opposite. This is caused by fine distinctions in mechanisms of action of the two indirect activators, activities of their complexes with plasminogen and the role of lysine-dependent interactions in formation of these complexes.

In some early studies of the effect of 6-AHA on plasmin activity [19, 20], it was shown that at concentrations lower than 100 mM it stimulates esterase activity of plasmin by 20-30%, but at higher concentrations it inhibits (K_i = 0.32 M). The mechanism of direct stimulation of

plasmin activity by 6-AHA is still unknown. We also observed the stimulating effect of 6-AHA on amidase activity of plasmin and Pm–STA complex (Fig. 3), which correlates with literature data. However, in the presence of 10 mM 6-AHA amidase activities of plasmin and Pm–STA complex increased negligibly (only by 4-5%). Consequently, the observed increase in the rate of formation of Pm–STA complex from Glu-Pg–STA and Lys-Pg–STA complexes in the presence of 6-AHA with a maximum at 10 mM (Fig. 3) is not caused by stimulation of intrinsic activity of Pm–STA complex. 6-AHA concentrations (50-70 mM) causing maximal (1.2-1.3-fold) increase in amidase activity of plasmin and Pm–STA complex correspond to descending portions of kinetic dependences: the rate of formation of Pm–STA complex (Fig. 2) and the rates of activation of the two forms of plasminogen by staphylokinase and Pm–STA complex (Fig. 1). Probably at high 6-AHA concentrations the effect of stimulation of activities of the two enzymes, negligibly compensating the effect of inhibition of reactions of Pm–STA complex formation and plasminogen activation, results in decreased slopes of descending portions of the above-mentioned dependences.

The necessity of plasmin and plasminogen (rarely of activator itself, e.g. fibrin-specific t-PA) sorption on fibrin surface is a basis for the mechanism of clot lysis by the action of plasmin or activators. Staphylokinase is a highly fibrin-specific activator of plasminogen, although in contrast to t-PA it has no affinity for fibrin but binds to plasmin(ogen) sorbed on fibrin [30]. Fibrin specificity of staphylokinase is caused by the fact that Pm–STA complex bound to fibrin is inhibited by α_2-antiplasmin 100 times slower than in solution. Anti-fibrinolytic action of ω-amino acids is based on their competition with lysine sites of fibrin for binding to high-affinity LBS on plasmin(ogen) localized in its kringles 1, 4, and 5 [3, 17, 31]; this results in displacement of plasmin(ogen) from fibrin surface. Binding of Glu-Pg to intact fibrin results in its conformational transfer from α- to β-type, and partial fibrin degradation by forming plasmin results in β→γ conformational transfer of the zymogen [1, 6, 7]. That is why low concentrations of ω-amino acids do not stimulate activation of Glu-Pg by activators on fibrin surface. We have found that kinetic curves of fibrinolysis by the action of staphylokinase and preformed Pm–STA complex in the presence of ω-amino acids have different shapes; this was demonstrated in the case of 6-AHA (Fig. 4), although L-Lys and *t*-AMCHA caused similar effects. During fibrinolysis by staphylokinase even low concentrations of ω-amino acids caused not only decrease in the rate of fibrinolysis, as during fibrinolysis by Pm–STA complex, but also an appearance of long lag periods, and this did not allow studying how the rates of fibrinolysis by staphylokinase depend on concentration of inhibitors. Existence and elongation of lag period with increase in ω-amino acid concentration on the curves of fibrinolysis by staphylokinase including stages 1-4 (in contrast to the curves of fibrinolysis by Pm–STA complex including stages 3 and 4) seems to be related with displacement of plasminogen from fibrin surface. As a result, concentrations of plasminogen substrate as well as that of forming Pg–STA complex on fibrin surface decrease, and consequently the rate of conversion of Pg–STA complex into Pm–STA (stage 2) also decreases ($k_1 = 4 \cdot 10^{-7}$ sec^{-1} [23]). This suggestion was supported by the fact that the presence of ω-amino acids does not result in lag periods on the curves of fibrinolysis by streptokinase [14], which immediately forms an active complex with plasminogen (Pg–SK) in contrast to staphylokinase.

Comparing dependences of the rates of fibrinolysis by the action of plasmin and preformed Pm–STA complex on L-Lys, 6-AHA, and *t*-AMCHA concentrations (Fig. 5), it is evident that ω-amino acids cause a stronger inhibitory effect on fibrinolysis induced by Pm–STA complex than on that induced by plasmin. This may be rationalized as follows: in contrast to direct lysis of fibrin by plasmin (stage 4), the action of Pm–STA complex on fibrin is mediated via activation of plasminogen adsorbed on fibrin surface (stages 3 and 4). Displacing plasminogen from fibrin surface, ω-amino acids decrease the rate of plasmin formation on the surface of its solid-phase substrate. Since plasmin has a higher affinity for fibrin (K_d = 0.1 μM) than plasminogen (K_d = 0.5 μM) [32], ω-amino acids inhibit fibrinolysis by added plasmin to a lesser extent than by added Pm–STA complex. Inhibitory effect of ω-amino acids on fibrinolysis increased in the series: L-Lys < 6-AHA < *t*-AMCHA ($[I]_{50}$ = 270, 4.3, and 0.7 mM and 100, 1.3, and 0.3 mM for plasmin and Pm–STA complex, respectively). The data indicate that the effect of ω-amino acids on the complex process of fibrinolysis induced by native staphylokinase includes inhibition of stages 2-4 as a result of desorption of plasminogen and plasmin from fibrin surface, sensitivity of three stages to inhibition by ω-amino acids being increased in the series: 4 < 3 < 2.

So, the mechanism of action of ω-amino acids on the activator and fibrinolytic activities of staphylokinase differ from the mechanism of their action on corresponding activities of streptokinase, t-PA and tcu-PA. Contrary to direct activators and rapidly forming Pg–SK activator complex, Pm–STA activator complex is formed slowly. Formation of inactive Pg–STA complex in which lysine-dependent interactions are absent is not inhibited by ω-amino acids, in contrast to Pg–SK complex. Conversion of Glu-Pg–STA and Lys-Pg–STA complexes into active Pm–STA complex is stimulated by low concentrations of ω-amino acids, although the stimulation mechanism of conversion of Lys-Pg–STA complex into Pm–STA complex is unclear. Increase in the rate of Lys-Pg activation by staphylokinase observed at low concentrations of ω-amino acids is related with stimulation by them of Pm–STA complex formation, whereas increase

in the rate of Glu-Pg activation by staphylokinase is caused by α→β conformational change of Glu-Pg (in the substrate molecule and consisting of Glu-Pg–STA complex) and by stimulation of the rate of Pm–STA complex formation. Inhibition of staphylokinase-induced fibrinolysis by ω-amino acids includes retardation of three stages: formation of Pm–STA complex, activation of plasminogen by the latter, and lysis of fibrin by forming plasmin as a result of displacement of plasminogen and plasmin from fibrin surface. Long lag periods on kinetic curves of fibrinolysis by staphylokinase in the presence of ω-amino acids are mainly related with their blocking of Pm–STA complex formation.

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