Antifibrinolytic Activities of $\alpha$-N-Acetyl-L-Lysine Methyl Ester, $\varepsilon$-Aminocaproic Acid, and Tranexamic Acid

Importance of Kringle Interactions and Active Site Inhibition

Patrick K. Anonick, Jayanand Vasudevan, and Steven L. Gonias

$\alpha$-N-acetyl-L-lysine methyl ester (NALME) is a lysine analogue that reportedly binds to low-affinity lysine binding sites in plasmin(ogen) and miniplasmin(ogen). In the studies presented here, we show that NALME has antifibrinolytic activity; however, unlike the therapeutic agents $\varepsilon$-amino-n-caproic acid ($\varepsilon$ACA) and tranexamic acid (TEA), the activity of NALME is based on inhibition of the plasmin active site. NALME (0.1-10 mM) significantly inhibited the amidase activity of plasmin, miniplasmin, and streptokinase–plasmin complex without affecting $\alpha$-thrombin or tissue plasminogen activator. $\varepsilon$ACA and TEA (0.1-10 mM) did not affect the amidase activity of plasmin or miniplasmin. A kinetic analysis showed that NALME is a competitive inhibitor of $\text{D-Val-L-Leu-L-Lys-p-nitroanilide \ HCl (S-2251)}$ hydrolysis by plasmin; NALME binding to plasmin completely prevented $\text{S-2251}$ binding. The $K_i$ for the plasmin–NALME interaction was 0.4 mM. $\varepsilon$ACA and TEA inhibited fibrin monomer digestion by plasmin and miniplasmin without binding to the active site of either enzyme. This result suggests that $\varepsilon$ACA and TEA function as antifibrinolitics by disrupting the noncovalent association of fibrin monomer with a domain common to both plasmin and miniplasmin (probably kringle 5). NALME inhibited fibrin monomer digestion principally by decreasing amidase activity. NALME was the only lysine analogue that prevented fragment X formation; TEA and $\varepsilon$ACA primarily inhibited the formation of fragments Y and D. When plasmin was incubated simultaneously with $\alpha$-antiplasmin and $\alpha$-macroglobulin, $\varepsilon$ACA increased the fraction of plasmin reacting with $\alpha$-macroglobulin; NALME had no effect on the plasmin distribution. $\varepsilon$ACA, TEA, and NALME increased the euglobulin clot lysis time of normal plasma. NALME did not prolong the prothrombin time or activated partial thromboplastin time. These studies demonstrate that the antifibrinolytic activity of NALME is based on inhibition of the plasmin active site, whereas $\varepsilon$ACA and TEA are active due to kringle domain interactions. (Arteriosclerosis and Thrombosis 1992;12:708–716)

KEYWORDS • plasmin • fibrin • $\alpha$-antiplasmin • $\alpha$-macroglobulin • lysine • $\varepsilon$-aminocaproic acid • tranexamic acid • kringle domains

In the circulation, fibrinolysis and fibrinogenolysis are mediated primarily by plasmin, the active serine proteinase counterpart of the zymogen plasminogen.1,2 The intact structure of plasminogen includes 791 amino acids with an N-terminal glutamic acid2–5 and either one or two oligosaccharide chains.6–8 Plasminogen activation results from the cleavage of the Arg$^{990}$-Val$^{991}$ peptide bond.9 Low concentrations of plasmin cleave [Glu$^1$]-plasminogen to form [Lys$^{78}$]-plasminogen, a second more readily activatable form of the zymogen.10 Plasminogen activation and the subsequent function of plasmin are modulated by noncovalent binding interactions with a variety of macromolecules, including fibrin$,11–19$ extracellular matrix proteins,20 and cellular receptors.21–23 These noncovalent interactions are mediated by a series of five homologous triple-disulfide-bonded plasmin(ogen) domains termed kringles.2–3 Each of the kringles apparently expresses a single binding site for lysine analogues such as $\varepsilon$-amino-n-caproic acid ($\varepsilon$ACA) and trans-(4-aminomethyl)-cyclohexancarboxylic acid (tranexamic acid, or TEA) (Figure 1).24–34 Kringle 1 (K1), near the N-terminus of plasmin(ogen), has the highest affinity for $\varepsilon$ACA (9.0 $\mu$M) and TEA (1.1 $\mu$M),$^{24,33}$; K4 contains a binding site with intermediate affinity, which is probably expressed only in [Lys$^{78}$]-plasmin(ogen).22 The other kringles contain binding sites with lower affinities (0.1–10 mM)$^{24,27,28}$ A sixth binding site for lysine analogues may be the plasmin active site.$^{29,36}$ The role of the various kringle domains and the interrelation between these structures in promoting plasmin activity remain incompletely understood. Considerable attention has focused on the fibrin–plasmin(ogen) interaction.$^{12–19}$ Christensen and colleagues$^{30,37,38}$ suggested that there are two classes of functional fibrin
binding sites in plasminogen. The lysine binding site in K1 provides a high-affinity interaction; this interaction requires C-terminal lysine residues that are present in fibrin only after partial digestion. By contrast, a low-affinity binding site in K5 (termed the aminohexyl or AH site) may mediate the initial association of plasminogen with fibrin. The role of K5 in fibrin binding is supported by studies demonstrating fibrin's association with miniplasminogen (an elastase digestion fragment of plasminogen that includes the active site and the K5 domain). Urano et al proposed that the antifibrinolytic activity of eACA is based on disruption of plasminogen-fibrin interactions and is not due to inhibition of the tissue plasminogen activator (t-PA)-fibrin interaction. The same investigators also showed that eACA comparably inhibits fibrin clot lysis by plasmin or miniplasmin.

A second potentially important role for the lysine binding sites involves the conformation of plasminogen. In the presence of physiological concentrations of chloride ion, [Glu']-plasminogen exists in a "closed," poorly activated conformation while the conformation of [Lys']-plasminogen is "open" and more readily activated. Concentrations of eACA near 2.0 mM convert the conformation of [Glu']-plasminogen into the open form. Based on the affinity of the eACA-K5 interaction, Christensen and Mølgaard suggested that the binding of eACA to K5 may be critical in promoting plasminogen conformational change.

In studies with AH-Sepharose, Christensen identified α-N-acetyl-l-lysine methyl ester (NALME) (Figure 1) as a high-affinity ligand (K 30 μM) for the AH site in miniplasminogen and [Lys']-plasminogen. By comparison, the K 300 μM for the binding of eACA in the same system was 300 μM. Because both eACA and TEA are used as intravascular antifibrinolytic agents in patients, the affinity of NALME for the AH site (K 30 μM) suggested a possible therapeutic role for this lysine analogue. Whewel et al studied purified K5 by nuclear magnetic resonance spectroscopy. These investigators demonstrated that the K 30 for the binding of NALME to purified K5 is 1.7 mM, significantly higher than the constant determined with eACA (94 μM).

The antifibrinolytic activity of lysine analogues may involve multiple mechanisms, including 1) inhibition of plasminogen binding to sites of rapid activation, 2) direct inhibition of the plasmin active site. In this investigation, the antifibrinolytic activity of NALME was studied in comparison with eACA and TEA. Plasmin amidase activity, fibrinolytic activity, and reaction with α-antiplasmin (α2AP) and α2-macroglobulin (α2M) were studied in the presence of different concentrations of each agent. Our results demonstrate that the antifibrinolytic activity of NALME is unique because it is based on inhibition of the plasmin active site, unlike eACA and TEA, which function primarily by interacting with the plasminogen kringle domains. NALME was the only lysine analogue that inhibited the initial steps of fibrin digestion, which may be important in priming the clot for enhanced plasminogen activation. Therefore, NALME could potentially be used to regulate fibrinolysis in a manner that complements the activity of eACA or TEA.

Methods

Materials

p-Nitrophenyl-p'-guanidinobenzoate hydrochloride (PNPGB), N-benzoyl-D,L-arginine-p-nitroanilide hydrochloride (BAPNA), eACA, TEA, and NALME were purchased from Sigma Chemical Co., St. Louis, Mo. D-Val-I-Leu-i-Lys-p-nitroanilide hydrochloride (S-2251) and D-Ile-i-Pro-i-Arg-p-nitroanilide hydrochloride (S-2288) were from Kabi Vitrum, Stockholm, Sweden. Na125I was from Amersham, Amersham Corp., Arlington Heights, Ill., and iodobeads were from Pierce Chemical Co., Rockford, Ill.

Proteins

[Glu']-plasminogen was prepared from human plasma by the method of Deutsch and Mertz. Miniplasminogen was prepared by digesting human plasminogen with porcine pancreatic elastase and was purified by chromatography on lysine-Sepharose. Miniplasminogen and plasminogen were activated with low-molecular-weight urokinase (Calbiochem Corp., La Jolla, Calif.) as previously described. The concentration of active proteinase was determined by the rate of S-2251 substrate hydrolysis at 22°C using a Michaelis constant (K) of 0.18 mM and a of 11.8 second 1 for plasmin and a K of 0.13 mM and a of 10.8 second 1 for miniplasmin.

α2M and α2AP were purified from human plasma by the methods of Imber and Pizzo and Wiman, respectively. Bowes melanoma one-chain t-PA was purchased from American Diagnostica (Greenwich, Conn.), and bovine thrombin was purchased from Sigma. Human α-thrombin was a gift from Dr. John Fenton II (Albany, N.Y.). Streptokinase was purified from Kabikinase by the method of Castellino et al. Streptokinase–plasmin complex was prepared by incubating plasminogen with a twofold molar excess of streptokinase for 20 minutes at 37°C as previously described. Human fibrinogen, grade I, was obtained from Kabi Vitrum; this fibrinogen was used to prepare plasminogen-free fibrin monomer (Fn-M) as previously described.

Radioiodination

Plasminogen was radioiodinated with Iodobeads as described by the manufacturer (Pierce). Desalting was performed on Sephadex G-25 (Pharmacia Fine Chemi-
Amidase Activity

Plasmin (18 nM) and different concentrations of eACA, TEA, or NALME (0–10 mM) were incubated in the sample cuvette of a Hewlett-Packard 8450 diode-array spectrophotometer for 5 minutes at 22°C. The incubation buffer was 20 mM sodium phosphate, 150 mM NaCl, and 0.05% Tween-80, pH 7.4 (PBS-T). S-2251 (0.09–0.72 mM) was then added, and the absorbance at 406 nm was measured every 2 seconds for 100 seconds. The velocity of substrate hydrolysis was determined by transforming absorbance measurements with the first-derivative function (dA_406nm/dt). Equivalent studies were performed using miniplasmin and streptokinase-plasmin complex. All experiments were performed at least in triplicate.

The hydrolysis of S-2288 by human α-thrombin (2.8 nM) and t-PA (30 nM) was studied in the presence of NALME (0–10 mM) as described above. The substrate concentration was 0.2 mM for α-thrombin and 0.8 mM for t-PA.

Fibrinolysis by Plasmin and Miniplasmin

Plasmin or miniplasmin (18 nM) was incubated with eACA, TEA, or NALME (0–10 mM) in PBS-T for 5 minutes. Fn-M was then added, and incubation was continued for 30 minutes at 22°C. Reactions were stopped with the addition of PNPG (0.4 mM). Samples were denatured under nonreducing conditions and subjected to sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) on 8% slabs using the McLellan 55 as modified by Gonias and Figler. The molecular weights of fragments Y and D were 140,000 and 90,000, respectively, in good agreement with published values.57,58

Reaction of Plasmin With α2-Antiplasmin

The reaction of plasmin with α2AP was studied in the presence of S-2251 and different concentrations of eACA, TEA, or NALME (0–10 mM). Plasmin (18 nM) was incubated with the lysine analogues in PBS-T for 5 minutes at 22°C. The absorbance at 406 nm was measured every 2 seconds for 100 seconds. The velocity of substrate hydrolysis was determined by transforming absorbance measurements with the first-derivative function (dA_406nm/dt). Equivalent studies were performed using miniplasmin and streptokinase-plasmin complex. All experiments were performed at least in triplicate.

The reaction of plasmin with α2M was then added. The final concentrations of plasmin, α2AP, and α2M were 3.33 nM, 33.3 nM, and 1.96 μM, respectively. These concentrations were selected so that the plasmin would distribute nearly equally between the two inhibitors in the absence of lysine analogues. After incubation for 15 minutes at 22°C, reactions were terminated with addition of 0.4 mM PNPG and analyzed by SDS-PAGE, autoradiography, and gel slicing.

Prothrombin Time and Activated Partial Thromboplastin Time Determinations

Normal pooled plasma (3.8% wt/vol sodium citrate) was incubated with eACA, TEA, or NALME (0.2–10 mM) for 5 minutes at 4°C. Prothrombin time (PT) and activated partial thromboplastin time (APTT) values were determined by standard methods using a Coagamate X-2 automated analyzer. The PT reagent was Simplastin Excel (prepared from rabbit brain by Organon Teknika, Rockville, Md.). The APTT reagent consisted of micronized silica and rabbit brain phospholipid (Organon Teknika). Each value was determined in duplicate.

Euglobulin Clot Lysis Times

The euglobulin clot lysis time (ECLT) provides an index of plasminogen activation by intrinsic t-PA in the absence of plasmin inhibitors and in the presence of a fibrin clot. The effects of eACA, TEA, and NALME on the ECLT were determined by the method of Triplet and Harms. Briefly, plasma from normal human donors was anticoagulated with 3.8% sodium citrate, and aliquots (0.5 ml) were placed into several
TABLE 1. Effect of α-N-Acetyl-L-Lysine Methyl Ester on Amidase Activity of Proteinases*

<table>
<thead>
<tr>
<th>NALME concentration (mM)</th>
<th>Proteinase</th>
<th>Activity (% ± SEM)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Plasmin</td>
<td>Miniplasmin</td>
</tr>
<tr>
<td>0</td>
<td>100.0±1.7</td>
<td>100.0±1.0</td>
</tr>
<tr>
<td>0.1</td>
<td>88.3±1.7</td>
<td>92.7±2.0</td>
</tr>
<tr>
<td>1.0</td>
<td>63.9±0.8</td>
<td>65.5±3.8</td>
</tr>
<tr>
<td>10.0</td>
<td>14.3±1.8</td>
<td>15.4±1.2</td>
</tr>
</tbody>
</table>

NALME, α-N-acetyl-L-lysine methyl ester; t-PA, tissue plasminogen activator; SkPm, streptokinase-plasmin complex.

*Proteinase was incubated with NALME for 5 minutes in the reaction cuvette before addition of substrate as described in the text. Absorbance at 406 nm was measured every 2 seconds for 100 seconds. The velocity of substrate hydrolysis was determined from \( \frac{dA}{dt} \) tubes containing 6.0 ml deionized water. Acetic acid (0.1 ml of a 1% [vol/vol] solution) was added, and the samples were incubated at 4°C for 10 minutes before centrifugation at 650g in a Beckman model TJ-6 centrifuge (Beckman Instruments, Fullerton, Calif.). The precipitate was redissolved in 0.35 ml PBS-T. The lysine analogues (0-10 mM) were added to the samples, followed by 1.25 National Institutes of Health units of bovine thrombin. Each sample clotted within 30 seconds. The time required for complete lysis was determined by visual inspection. Each concentration of lysine analogue was studied in quadruplicate.

Results

Plasmin Amidase Activity

The amidase activity of plasmin was studied at 22°C in the presence of TEA and eACA (0.1-10 mM) using a single concentration of S-2251 (0.54 mM). Neither agent affected the rate of substrate hydrolysis by more than 7% in the specified concentration range (data not shown), consistent with the results of previous investigations.29,40-62-63 Equivalent results were obtained with miniplasmin and streptokinase-plasmin complex.

NALME markedly inhibited the hydrolysis of S-2251 by plasmin, miniplasmin, and streptokinase-plasmin complex (Table 1). The hydrolysis of S-2288 by human α-thrombin and t-PA was unaffected by NALME, eACA, or TEA (0.1-10 mM).

Figure 2A shows a reciprocal plot of the kinetics of S-2251 hydrolysis by plasmin in the presence of NALME. The apparent \( K_M \) was increased without a significant change in apparent \( k_{cat} \). A replot of the slopes from panel A versus NALME concentration was linear (correlation coefficient, 0.99) (Figure 2B), indicating that NALME is a simple competitive inhibitor of S-2251 hydrolysis by plasmin. The \( K_I \) for the binding of NALME to plasmin, based on the replot, was 0.4 mM.

Fibrinolysis by Plasmin in the Presence of Lysine Analogues

The lysis of Fn-M by preactivated plasmin was studied after incubating the plasmin with eACA, TEA, or NALME (Figure 3). eACA and TEA at concentrations of 1.0 mM almost completely inhibited the formation of fibrin fragments Y and D. Marked inhibition was observed with 0.1 mM TEA, and a slight effect (mostly in the digestion of fragment Y) was seen with 0.1 mM eACA. Importantly, the concentrations of eACA and TEA that inhibited Fn-M digestion had no effect on plasmin amidase activity. The formation of fragment X was not significantly inhibited by eACA or TEA even at the highest concentration studied (10.0 mM).

Fn-M digestion by plasmin was not affected by 0.1 mM NALME (Figure 3). Inhibition of Fn-M digestion was observed with 1.0 mM NALME; however, the extent of inhibition was less than that demonstrated with 1.0 mM eACA or TEA. NALME at 10 mM almost completely inhibited the formation of fragments Y and D. Fragment X formation was also decreased; this activity was unique to NALME compared with eACA and TEA.

Fibrinolysis by Miniplasmin in the Presence of Lysine Analogues

Fn-M digestion by preactivated miniplasmin was studied after incubating the miniplasmin with eACA,
FIGURE 3. Fibrin monomer (Fn-M) digestion by plasmin in the presence of lysine analogues. Lane 1 shows the undigested Fn-M preparation. Lane 2 shows digestion products after incubation with plasmin (18 nM) at 22°C for 30 minutes. Lanes 3–5 show digestion in the presence of 0.1, 1.0, and 10.0 mM eACA, respectively. Lanes 6–8 show digestion in the presence of 0.1, 1.0, and 10.0 mM TEA, respectively. Lanes 9–11 show digestion in the presence of 0.1, 1.0, and 10.0 mM NALME, respectively. Frag, fragment; eACA, e-amino-n-caproic acid; TEA, trans-(4-aminomethyl)-cyclohexanecarboxylic acid; NALME, a-N-acetyl-L-lysine methyl ester. Coomassie blue-stained gel.

TEA, or NALME (Figure 4). As demonstrated previously,63,64 the Fn-M fragments generated by miniplasmin were equivalent to those observed with plasmin. The extent of Fn-M digestion by miniplasmin after 30 minutes was somewhat decreased compared with the equivalent concentration of active plasmin, confirming the work of Ney and Pizzo.64 eACA and TEA inhibited Fn-M digestion by miniplasmin, and the concentration dependency of this activity was almost identical to that observed with plasmin. Miniplasmin lacks four of the five kringle domains found in plasmin.3 Because the miniplasmin active site was unaffected by eACA and TEA (as determined by amidase activity), interaction of these lysine analogues with the K5 domain was probably responsible for the inhibition of Fn-M digestion. eACA and TEA (even at 10 mM) had little effect on the formation of fragment X by miniplasmin.

FIGURE 4. Fibrin monomer (Fn-M) digestion by miniplasmin in the presence of lysine analogues. Lane 1 shows the undigested Fn-M preparation. Lane 2 shows digestion products after incubation with miniplasmin (18 nM) at 22°C for 30 minutes. Lanes 3–5 show digestion in the presence of 0.1, 1.0, and 10.0 mM eACA, respectively. Lanes 6–8 show digestion in the presence of 0.1, 1.0, and 10.0 mM TEA, respectively. Lanes 9–11 show digestion in the presence of 0.1, 1.0, and 10.0 mM NALME, respectively. eACA, e-amino-n-caproic acid; TEA, trans-(4-aminomethyl)-cyclohexanecarboxylic acid; NALME, a-N-acetyl-L-lysine methyl ester. Coomassie blue-stained gel.

TABLE 2. Plasmin Inhibition by α,-Antiplasmin in the Presence of Lysine Analogues

<table>
<thead>
<tr>
<th>Lysine analogue</th>
<th>eACA k&quot;app</th>
<th>TEA k&quot;app</th>
<th>NALME k&quot;app</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.2±0.2×10²</td>
<td>1.2±0.2×10²</td>
<td>1.2±0.2×10²</td>
</tr>
<tr>
<td>0.1</td>
<td>7.3±0.3×10⁴</td>
<td>3.5±0.7×10⁴</td>
<td>1.1±0.1×10⁵</td>
</tr>
<tr>
<td>1.0</td>
<td>2.6±0.1×10⁶</td>
<td>7.2±0.5×10⁶</td>
<td>8.9±1.1×10⁶</td>
</tr>
<tr>
<td>10.0</td>
<td>3.9±0.4×10⁸</td>
<td>2.9±0.6×10⁹</td>
<td>ND</td>
</tr>
</tbody>
</table>

k"app values (in M⁻¹·sec⁻¹) were determined as described in text and represent mean±SEM of at least three experiments. eACA, e- amino-n-caproic acid; TEA, trans-(4-aminomethyl)-cyclohexanecarboxylic acid; NALME, α-N-acetyl-L-lysine methyl ester; ND, not determined.

NALME inhibited Fn-M digestion by miniplasmin when present at 1.0 mM or greater. NALME (10 mM) also inhibited fragment X formation. These results were comparable to those obtained with plasmin.

Reaction of Plasmin With α,-Antiplasmin in the Presence of Lysine Analogues

αAP is the primary antiplasmin in human plasma.60,66 The second-order rate constant (k"app) for the inhibition of plasmin by αAP is ~2.0×10⁷ M⁻¹·sec⁻¹; this rapid reaction depends on noncovalent association of αAP with the plasmin K1–3 domain.66 Table 2 shows k"app values determined for the inhibition of plasmin by αAP in the presence of lysine analogues. eACA or TEA (0.1 or 1.0 mM) significantly decreased the k"app; the 30-fold decrease caused by 10 mM eACA is consistent with the results of previous studies.62-67 As much as a 40-fold decrease in k"app was observed with TEA. These results probably reflect binding of eACA or TEA to the K1–3 domain of plasmin. By contrast, 0.1 mM NALME did not significantly affect the reaction of plasmin with αAP. With 1.0 mM NALME, the k"app was decreased by <25%. This minor change in reaction rate was close to the 40% decrease that would be predicted for 1.0 mM NALME functioning strictly as a competitive inhibitor in the presence of a second competitive inhibitor (0.54 mM S-2251). Experiments with the highest concentration of NALME (10 mM) were precluded by the decrease in S-2251 hydrolysis rate (Table 1).

Reaction of Plasmin With α-Macroglobulin in the Presence of Lysine Analogues

αM is the second major antiplasmin; the rate constant for the reaction of plasmin with αM is 5×10⁴ M⁻¹·sec⁻¹.19,68-70 Unlike αAP, the kringle domains probably do not play an important role in the plasmin/αM reaction; eACA does not significantly affect the rate of reaction.69 Although plasmin binding to αM is almost always covalent,54,59 nucleophilic amines can inhibit covalent bond formation without affecting irreversible αM–proteinase complex formation. To address this possibility, 125I-plasmin (3.3 nM) was preincubated with eACA (0.01–10.0 mM) and then reacted with 33 nM for 1 hour. The extent of covalent bond formation between 125I-plasmin and αM was not changed, as determined by SDS-PAGE (data not shown). This preliminary study demonstrated that the SDS-PAGE method could be used to study the reaction
of 125I-plasmin with α2M in the presence of lysine analogues.

As shown in Table 3, εACA and TEA slightly decreased the amount of α2M-plasmin complex formed in 15 minutes. A slight decrease in $k_{app}$ was confirmed when the reaction of plasmin with α2M in the presence of εACA or TEA was studied as a function of time (data not shown). By contrast, NALME significantly inhibited the reaction of α2M with plasmin. The extent of inhibition was consistent with the demonstrated effect of NALME on plasmin amidase activity.

**Inhibition of Plasmin by Mixtures of α2-Macroglobulin and α2-Antiplasmin**

125I-plasmin (3.33 nM) was incubated with either εACA or NALME (0–10 mM) for 5 minutes. Premixed solutions of α2AP (33.3 nM) and α2M (1.96 μM) were added, and reactions were conducted for 15 minutes. Because of the extremely high concentrations of proteinase inhibitors used in these experiments, the plasmin completely reacted within the allotted incubation time (with or without εACA and NALME). In the absence of εACA or NALME, approximately 50% of the plasmin bound to each inhibitor (Table 4). εACA altered the distribution of 125I-plasmin so that more α2M-plasmin was formed; with 10 mM εACA, ~80% of the plasmin bound to α2M. This result is consistent with the known effect of εACA on the noncovalent interaction of α2AP with plasmin.2,6,7

NALME had no effect on the distribution of 125I-plasmin between α2AP and α2M, supporting the hypothesis that NALME principally affects the plasmin active site. In a reversible equilibrium system when NALME is not bound to the plasmin active site, the plasmin reacts with α2AP or α2M with unchanged specificity.

**Effect of α-N-Acetyl-L-Lysine Methyl Ester on Coagulation**

NALME, εACA, and TEA at concentrations up to 10 mM were incubated with normal pooled plasma. The PT and APTT were not affected by any of the lysine analogues (data not shown).

**Effect of Lysine Analogues on the Euglobulin Clot Lysis Time**

The ECLT may be affected by inactivation of t-PA, disruption of the fibrin clot–dependent plasminogen activation mechanism, dissociation of plasmin from the clot, or direct inhibition of plasmin. Table 5 compares the effects of εACA, TEA, and NALME on the ECLT of normal plasma. εACA and TEA, even at 0.1 mM, prolonged the ECLT, probably reflecting disruption of the fibrin clot–dependent plasminogen activation mechanism. NALME also prolonged the ECLT but to a lesser extent than εACA or TEA. With 10 mM NALME, the ECLT was 2.2 times the normal value (9.6 hours). This prolongation is probably caused by the interaction of NALME with the plasmin active site; however, we cannot exclude other mechanisms in this experimental system.

**Discussion**

While it is clear that noncovalent binding of plasminogen to fibrin is critical in fibrinolysis, the relative importance of the various kringle domains in mediating this interaction remains unresolved. One theory proposes that the K5 domain mediates the initial interaction of plasminogen with fibrin.20 Binding of K5 to fibrin is thermodynamically weak but necessary for initiating the cleavage of fibrin, a process that generates new C-terminal lysine residues. These C-terminal lysine residues then provide new plasminogen binding sites that...
interact preferentially with the K1 plasmin(ogen) domain.37

Christensen30 identified NALME as a reagent that can
displace plasminogen from AH-Sepharose by inter-
acting with a low-affinity lysine binding site termed the
AH site. Subsequent studies suggested that the AH site
is equivalent to the K5 lysine binding site.27,38 Because
the K5 domain of plasmin(ogen) may play a critical role
in fibrinolysis, we hypothesized that NALME might
demonstrate unique antifibrinolytic properties com-
pared with the established therapeutic agents εACA
and TEA.

Any lysine analogue may affect fibrinolysis by inter-
acting with the plasmin(ogen) kringle domains or the
plasmin active site. In the present investigation, we
compared the antifibrinolytic activities of NALME,
εACA, and TEA using chromogenic substrate hydroly-
sis experiments, fibrin cleavage studies, proteinase in-
hibitor experiments, and ECLT assays. The results of
our studies and the work of others strongly suggest that
each lysine analogue is capable of interacting with
multiple sites in the plasmin(ogen) structure. Neverthe-
less, for each agent, a specific interaction or class of
interactions is primarily responsible for the antifibrin-
olytic activity. For εACA and TEA, our studies confirm
that antifibrinolytic activity is due primarily to interac-
tion with the kringle domains.39 As shown here and
elsewhere,29 εACA and TEA interact minimally with the
plasmin active site when present at concentrations
that are sufficient to significantly alter fibrinolytic activity
(10 mM or less). By contrast, the activity of NALME
is based primarily on inhibition of the plasmin active
site. While indeed NALME may interact with K5 when
present in sufficient concentration, this interaction
probably contributes minimally to the antifibrinolytic
activity of this agent.

The plasmin amidase activity studies demonstrated
that NALME is a simple competitive inhibitor of S-2251
hydrolysis. Accordingly, the plasmin–NALME complex
is completely incapable of binding S-2251. These data
are most simply explained by direct association of
NALME with the plasmin active site. Similar competi-
tive inhibition may be observed if NALME binds to a
separate area of plasmin, allosterically altering the
structure of the active site so that binding of S-2251 is
completely prevented. This second explanation is con-
sidered less likely, as NALME binding sites other than
the active site are probably located in distinct plasmin
domains that have minimal potential for altering active
site structure.43 In addition, in our studies TEA and
εACA probably saturated the kringle domain lysine
binding sites without affecting plasmin amidase activity.

The $K_i$ for the plasmin–NALME interaction, based
on the analysis of S-2251 hydrolysis, was 0.4 mM. In the
presence of other plasmin substrates, such as S-2251 or
fibrin, concentrations of NALME somewhat higher than
the $K_i$ should be necessary to occupy 50% of the
plasmin active sites. Therefore, the $K_i$ (0.4 mM) is
consistent with the concentrations of NALME (1–2
mM) that eliminated 50% of the activity of plasmin in
the different experimental systems studied here.

In Fn-M digestion experiments, the lowest concen-
tration of TEA (0.1 mM) significantly but incompletely
inhibited the formation of fragments Y and D. εACA at
the same concentration had only a slight effect. These
results indicate that TEA and εACA are functioning at
concentrations significantly above the reported $K_i$
values for the respective K1 interactions. In addition, the
efficacies of TEA and εACA in preventing Fn-M diges-
tion were essentially identical with plasmin and
miniplasmin. These data strongly suggest that cleavage of
Fn-M to form fragments Y and D requires noncoval-
ent binding of the Fn-M to a domain present in both
plasmin and miniplasmin. Furthermore, because this
particular domain behaves as a low-affinity lysine bind-
ing site, it is most likely that K5 mediates the critical
plasmin–Fn-M interaction, resulting in soluble fibrin
digestion. These studies are consistent with the work of
Uran et al.39

The initial cleavage of fibrin to form early digestion
products (fragment X) may be extremely important
because this digestion results in the formation of new
high-affinity plasminogen binding sites and enhances
the activation of plasminogen by t-PA.18,47,48 NALME
was unique among the lysine analogues in its ability to
inhibit fragment X formation. In this capacity, εACA
and TEA were relatively ineffective. These data suggest
that the initial steps of fibrin digestion may occur
independent of noncovalent plasmin–fibrin interactions,
unlike the later steps that result in the formation of
fragments Y, D, and E.

In a previous investigation,19 we presented evidence
for a high-affinity interaction of Fn-M with plasmin but
not with miniplasmin. The apparent $K_i$ for the plas-
m–Fn-M interaction was 20 nM, as determined by an
analysis of S-2251 hydrolysis (the actual $K_i$ may have
been somewhat higher if each fibrin monomer provided
more than one plasmin binding site). As a result of
Fn-M binding, the reaction of plasmin with α2AP was
inhibited; however, as suggested in the previous study,19
the Fn-M preparation was partially degraded by plas-
m. This modification was probably necessary for the
high-affinity interaction. Figures 3 and 4 show that
Fn-M binding sites specific for plasmin and not
miniplasmin were not important in determining the rate
of soluble fibrin cleavage. Of course, a more synergistic
relation may exist between binding sites in the lysis of
polymerized fibrin as opposed to soluble fibrin. In the
intact polymer, different kringles may function together
to permit plasmin movement across the degrading clot.

Overall, this study demonstrates that the antifibrin-
olytic activities of lysine analogues vary in a qualitative
(mechanism of activity) as well as a quantitative (con-
centration dependence) manner. The complementary
modes of action of NALME and εACA or TEA suggest
that these agents might be efficacious as therapeutics
when used in combination.

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References

1. Lijnen HR, Collen D: Interaction of plasminogen activators and
inhibitors with plasminogen and fibrin. *Semin Thromb Hemost*
1982;8:2–10
Hemost* 1984;10:18–23
3. Sotrup-Jensen L, Claes H, Zajeil M, Petersen TE, Magnusson S:
The primary structure of human plasminogen: Isolation of two
lysine-binding fragments and one "mini"-plasminogen (MW,
by guest on October 30, 2017 http://atvb.ahajournals.org/ Downloaded from

26. Lerch PG, Rickli EE, Lergier W, Gillessen D: Localization of


49.  

24. Markus G, DePasquale JL, Wissler FC: Quantitative determina-

20. Knudsen BS, Silverstein RL, Leung LLK, Harpel PC, Nachman 

15. Lucas MA, Fretto LJ, McKee PA: The binding of human plasmin-

14. Bok R, Mangel W: Quantitative characterization of the binding of

41.  


12. Thorsen S, Clemmensen I, Sottrup-Jensen L, Magnusson S: 


8. Hayes M, Castellino FJ: Carbohydrate of the human plasminogen

33.  

Biochem  

43.  

19. Hajjar KA, Harpel PC, Jaffe EA, Nachman RL: Binding of

21. Hajjar KA, Harpel PC, Jaffe EA, Nachman RL: Binding of

17. Hayes M, Castellino FJ: Carbohydrate of the human plasminogen

21. Hajjar KA, Harpel PC, Jaffe EA, Nachman RL: Binding of

35.  

20. Knudsen BS, Silverstein RL, Leung LLK, Harpel PC, Nachman 

10. Wallen P, Wiman B: Characterization of human plasminogen: II. 


38,000) by elastase-catalyzed-specific limited proteolysis. Prog 

30. Christensen U: The AH-site of plasminogen and two C-terminal 

fragments. Biochem J 1984;223:413–421

31. Novokhatny VV, Matsuka YW, Kudinov SA: Analysis of ligand 

binding to kringles 4 and 5 fragments from human plasminogen. 


32. Sehl LC, Castellino FJ: Thermodynamic properties of the binding of 

α-α-amino-carboxylic acid-binding sites in human plasminogen. 

J Biol Chem 1982;257:2104–2110

33. Menhart N, Sehl LC, Kelley RF, Castellino FJ: Construction, 

expression, and purification of recombinant kringle 1 of human 

plasminogen and analysis of its interaction with α-α-amino acids. 


34. Markus G, Priore RL, Wissler FC: The binding of tranexamic acid 

to native (Glu) and modified (Lys) human plasminogen and its 

35. Christensen U, Ipsen HH: Steady-state kinetics of plasmin- 

and trypsin-catalyzed hydrolysis of a number of tripeptide-

nitroanilides, Biochim Biophys Acta 1979;569:177–183

36. Christensen U: C-terminal lysine residues of fibrinogen fragments 

essential for binding to plasmin. FEBS Lett 1985;182:43–46

37. Christensen U, Malgaard L: Stopped-flow fluorescence kinetic 

studies of Glu-plasminogen: Conformation changes triggered by 


38. Urano S, Metzger AR, Castellino FJ: Plasmin-mediated fibrinoly-

sis by variant recombinant tissue plasminogen activators. Proc 

Natl Acad Sci U S A 1989;86:2588–2571

39. Brockway WJ, Castellino FJ: The mechanism of the inhibition of 

plasmin activity by e-aminocaproic acid. J Biol Chem 1971;246: 

4641–4647

40. Urano T, Chibber BAK, Castellino FJ: The reciprocal effects of 

e-aminohexanoic acid and chloride ion on the activation of human 

Glu-plasminogen by human urokinase. Proc Natl Acad Sci U S A  

1987;84:4031–4034

41. Castellino FJ, Urano T, de Serrano V, Morris JP, Chibber BAK: 

Control of human plasminogen activation. Haemostasis 1988; 

18(suppl 1):15–23

42. Mangel WF, Lin B, Ramakrishnan V: Characterization of an 

extremely large, ligand-induced conformational change in plasmi-


43. Griffin JD, Elman L: Epsilon-aminocaproic acid (EACA). Semin 

Thromb Hemost 1978;5:27–40

44. Nilsson EM: Clinical pharmacology of aminocaproic and tranex-


45. Verstraete M: Clinical application of inhibitors of fibrinolysis. 

Drugs 1985;29:236–261

46. Harpel PC, Chang TS, Verderber E: Tissue plasminogen activator 

and urokinase mediate the binding of Glu-plasminogen to plasma 


47. Tran-Thang C, Kruithof EKO, Atkinson J, Bachmann F: High- 

affinity binding sites for human Glu-plasminogen unsealed by 

limited plasmin degradation of human fibrin. Eur J Biochem 

1986;160:599–604

48. Deutsch DG, Mertz ET: Plasminogen: Purification from human 


49. Christensen U, Sottrup-Jensen L, Magnusson S, Petersen TE, 

Clemmensen I: Enzymic properties of the neo-plasmin-human a2-


50. Christensen U, Ipsen HH: Steady-state kinetics of plasmin- 

and trypsin-catalyzed hydrolysis of a number of tripeptide-p-

nitroanilides, Biochim Biophys Acta 1979;569:177–183

51. Verstraete M: Clinical application of inhibitors of fibrinolysis. 

Drugs 1985;29:236–261

52. Harpel PC, Chang TS, Verderber E: Tissue plasminogen activator 

and urokinase mediate the binding of Glu-plasminogen to plasma 


53. Tran-Thang C, Kruithof EKO, Atkinson J, Bachmann F: High-

affinity binding sites for human Glu-plasminogen unsealed by 

limited plasmin degradation of human fibrin. Eur J Biochem 

1986;160:599–604

54. Deutsch DG, Mertz ET: Plasminogen: Purification from human 


55. Christensen U, Sottrup-Jensen L, Magnusson S, Petersen TE, 

Clemmensen I: Enzymic properties of the neo-plasmin-human a2-


56. Christensen U, Ipsen HH: Steady-state kinetics of plasmin- 

and trypsin-catalyzed hydrolysis of a number of tripeptide-p-

nitroanilides, Biochim Biophys Acta 1979;569:177–183

57. Verstraete M: Clinical application of inhibitors of fibrinolysis. 

Drugs 1985;29:236–261

58. Harpel PC, Chang TS, Verderber E: Tissue plasminogen activator 

and urokinase mediate the binding of Glu-plasminogen to plasma 


59. Tran-Thang C, Kruithof EKO, Atkinson J, Bachmann F: High-

affinity binding sites for human Glu-plasminogen unsealed by 

limited plasmin degradation of human fibrin. Eur J Biochem 

1986;160:599–604

60. Deutsch DG, Mertz ET: Plasminogen: Purification from human 


61. Imber MJ, Pizzo SV: Clearance and binding of two electrophoretic 

"fast" forms of human α2-macroglobulin. J Biol Chem 1981;256: 

8134–8139

62. Wiman B: Affinity-chromatographic purification of human α2-


63. Castellino FJ, Sodetz JM, Brockway WJ, Siefring GE, Castellino 

FJ: Construction, expression, and purification of recombinant 

kringle 1 of human plasminogen and analysis of its interaction with 


64. Gonias SL, Figler NL: α-Macroglobulin is the primary inhibitor 

of miniplasmin in vitro and in vivo in the mouse. J Biol Chem 

1988;263:725–730


Sci 1988;26:1–41
59. Pochon F: Some consequences of the covalent and non-covalent
binding modes of plasmin with α2-macroglobulin. Biochim Biophys
Acta 1987;915:37–45
60. Chakrabarti R, Bielawiec M, Evans JF, Fearnley GR: Methodolog-
ical study and a recommended technique for determining the
61. Triplett DA, Harms CS: Euglobulin clot lysis time, in Procedures
for the Clinical Laboratory. Chicago, American Society of Clinical
Pathologists, 1981, pp 139–143
62. Wiman B, Collen D: On the kinetics of the reaction between
63. Morris JP, Blatt S, Powell JR, Strickland DK, Castellino FJ: Role
of lysine binding regions in the kinetic properties of human
64. Ney KA, Pizzo SV: Fibrinolysis and fibrinogenolysis by Val-Leu-
plasmin. Biochim Biophys Acta 1982;708:218–224
65. Aoki N, Harpel PC: Inhibitors of the fibrinolytic enzyme system.
Semin Thromb Hemost 1984;10:24–41
67. Wiman B, Boman L, Collen D: On the kinetics of the reaction
between human antiplasmin and a low-molecular-weight form of
plasmin. Eur J Biochem 1978;87:143–146
68. Christensen U, Sottrup-Jensen L: Mechanism of α2-macroglobulin–
proteinase interactions: Studies with trypsin and plasmin. Bio-
chemistry 1984;23:6619–6626
69. Steiner JP, Migliorini M, Strickland DK: Characterization of the
reaction of plasmin with α2-macroglobulin: Effect of antifibrinolytic
agents. Biochemistry 1987;26:8487–8495
70. Anonick PK, Wolf BB, Gonias SL: Regulation of plasmin,
imiplatin, and streptokinase–plasmin complex by α2-antiplas-
min, α2-macroglobulin, and antithrombin III in the presence of
Antifibrinolytic activities of alpha-N-acetyl-L-lysine methyl ester, epsilon-aminocaproic acid, and tranexamic acid. Importance of kringle interactions and active site inhibition.

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