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Studies on Plasminogen Activators

III. Assay of Fibrinolytic Activator Enzymes from Plasma und Urine*

ULLA HAMBERG and MAIJA-LIISA SAVOLAINEN

Department of Biochemistry, University of Helsinki, Helsinki, Finland

An esterase assay with acetyl-L-lysine methyl ester (ALMe) was found to be useful for the determination of the specific activity in terms of μmoles hydrolyzed per hour per mg of protein with a plasminogen activator enzyme complex which is formed in normal human plasma after incubation with streptokinase (SK). The method may be applied for the study of SK-induced fibrinolytic activation in human plasma, and is also applicable for an evaluation of the urokinase content in small samples of human and rabbit urine, first submitted to a gel filtration procedure. The identity with urokinase of this crude fibrinolytically active urinary enzyme was further sustained by its action on ALMe.

In the course of studies concerning the fibrinolytically active protein complex formed with streptokinase (SK) in human plasma [1,2] the need of a sensitive method measuring the specific activity of the SK-activator enzyme became evident. This enzyme is formed specifically in human plasma by an interaction between plasminogen or plasmin and SK, which is equimolar in the purified *in vitro* systems.[3] In total human plasma, however, the interaction with other plasma proteins leads to the formation of a larger molecular complex having the enzymatic activity of an SK-activator.[2] Casein,[4] fibrin,[5–7] and the synthetic esters of arginine or lysine [8,9] are commonly used substrates for the measurement of fibrinolytic enzyme activity. The often applied two-step reaction measures the proteolytic activity on fibrin after the primary activation of plasminogen. The relatively high plasminogen content in purified bovine fibrinogen makes this protein preparation a suitable substrate for the two-step method, applied either in the fibrin plate methods,[5,6] the clot lysis test,[7] or measuring the activator-induced plasmin on casein.[4] Variations in the plasminogen content of commercially available purified fibrinogen

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preparations, as well as species differences between various types of plasma, make these assays purely comparative. The SK-activated human plasminogen contains an enzyme that hydrolyses esters of lysine and arginine.[9,10] A recent study by Sherry *et al.*[11] shows that the highest degree of substrate sensitivity with urokinase, the plasminogen activator enzyme from urine, occurred with the acetylated lysine methyl ester (ALMe). In view of the possibility to use this substrate also for other plasminogen activators[11] we have applied the esterase action on ALMe for studies of the enzymatic activity of the SK activator enzyme complex isolated from normal human plasma.[2] The experimental details presented in this paper deal with the determination of SK activator activity in terms of μmoles of methanol released per hour per mg of protein with ALMe as substrate.

In view of the possible relationship between the physiological plasminogen activator enzyme in blood and urokinase, we recently designed a method for the study of this problem with small urine samples.[12] The results showed that a plasminogen activating enzyme with action on lysine methyl ester could be separated from fibrinolytically inactive material with high absorbancy at 260 and 280 mμ. As demonstrated below this purified enzyme fraction from human and rabbit urine also has action on ALMe. This esterase assay on ALMe therefore proved to be useful also for the determination of urokinase with small samples of gelfiltrated urine.

EXPERIMENTAL

The SK activator enzyme complex was prepared and isolated as described[1-2] by incubation of normal human plasma with 1000 units per ml of a highly purified streptokinase (Kabikinas 250 000 units, AB Kabi, Stockholm, Sweden) for 15 min at 37°C and pH 7.8. The enzyme complex was collected in the first eluates following V_0 from Sephadex G-200 by measurement of protein elution at 280 mμ and tracing of the plasminogen activator activity with the fibrin plate methods.[2]

Specific activity determinations were performed with the pooled eluates after concentration by ultrafiltration[2] and dialysis at +5°C for 12 h against 0.15 M NaCl.

The enzyme assays were performed using the methanol procedures developed by Siegelman *et al.*[13] for serum "trypsin", as adapted for urokinase assay by Sherry *et al.*[11] and by Harpel[14] for the C′ 1 esterase using the synthetic *N*-α-acetyl-L-lysine methyl ester monohydrochloride (ALMe) as substrate (Cyclo Chemical Corporation, Los Angeles, California, lot M 3170-).*

ε-Aminocaproic acid (ε-ACA)[16] (a commercial preparation from AB Kabi, Stockholm, Sweden) and soybean trypsin inhibitor (SBI) (crystallized, Worthington, New Jersey, USA) were applied in experiments investigating the inhibitory effects on the esterase activity on ALMe with the SK activator enzyme complex. Both inhibitors were dissolved in Tris-HCl buffer 0.06 M, pH 8.0 containing 0.09 M NaCl, to various concentrations.

Urine was gel filtrated by the previously described method,[12] however, the Sephadex G-200 column was equilibrated with 0.1 M ammonium acetate buffer pH 6.0, containing 1 M NaCl. After tracing the urokinase activity with bovine fibrin plates,[12] the pooled eluates were concentrated by ultrafiltration to 10–20 ml, and dialyzed against 0.15 M NaCl at + 5°C. All procedures were kept within the same time limits (total time for procedures 76 h).

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The urokinase standard preparations contained 2400 or 25 000 units (Leo, Denmark) and 5000 units (The Green Gross, Osaka, Japan) per vial (Ploug or Leo units).[13] The results are expressed in CTA units (Committee on Thrombolytic Agents of the United States Public Health Service, National Heart Institute) and may be converted according to the data given by Johnson and Newman[17] by which one CTA unit equals 0.75 Leo units approximately.

The urokinase standard curve was linear between 2.5 and 60.0 CTA units, corresponding to the absorbancy range 0.010 to 0.295 at 580 mμ. This measuring range was found to be comparable to that obtained by Harpel[14] with pure urokinase[15] (see footnote p. 1453).

Standard urokinase mixed with urine was submitted to the same gel filtration and preparation procedures. The recovery measured with ALMe was 75 % with human, and 74 % with rabbit urine.

Reagents

(1) Tris-HCl buffer 0.06 M, pH 8.0 containing 0.09 M NaCl (with urine incubations at pH 7.5[11]).

(2) ALMe solution in buffer, 3.6 mg per ml (0.015 M).

(3) Enzyme preparation diluted with buffer to contain about 0.33—1.00 mg protein per ml.

(4) Perchloric acid, 0.75 M solution.

(5) Sodium sulfite 10 % w/v, freshly prepared solution.

(6) Potassium permanganate, 2 % w/v solution.

(7) Sodium salt of chromotropic acid, 2 % w/v solution.

(8) Sulphuric acid, 67 % v/v solution (by dilution of concentrated acid with 1/2 the volume of distilled water).

(9) Chromotropic acid reagent: prepared freshly by mixing in the proportion 1:10 with sulphuric acid (8), and cooled.

(10) Methanol standard: prepared by refluxing with calcium oxide and redistillation at 65°C; diluted with buffer to 0.1—1.5 μmoles per ml.

Procedures

The *enzyme preparations* were diluted with buffer to correspond, in terms of mg of protein per 0.65 ml, to the amount of methanol released as determined by the straight line part of the curve shown in Fig. 1. Fig. 2 presents the typical dilution curves, showing the measuring range in relation to protein content with a SK activator preparation. The enzyme sample was mixed with 0.5 ml ALMe solution and incubated in a closed test tube for 1 h at 37°C. An aliquot of 1 ml of the incubation mixture was pipetted into 0.5 ml of 0.75 M perchloric acid in a centrifuge tube. After 30 min the precipitate was centrifuged off with a Servall RC-2-P centrifuge, 15 min at 0°C and 3000 *g*. For the colour reaction 1 ml of clear supernatant was used. With scarce material the proportions were reduced to 1/2 these amounts.

The *spontaneous hydrolysis* of ALMe[11] was determined with a control incubation using 0.5 ml ALMe solution (2); after 1 hour at 37°C the enzyme solution, 0.65 ml, was added and 1 ml of the mixture immediately added to 0.5 ml of perchloric acid.

Determination of methanol. To 1 ml supernatant was added 0.1 ml potassium permanganate solution (6). After 1 min 0.1 ml sodium sulfite solution (5) was added for the removal of the colour, and 4.0 ml chromotropic acid reagent (9). The tubes were closed with a cork stopper with a glass tubing for reflux. Heating was performed at 100°C for 30 min in a dry heating block (Type TB 4; Wissenschaftlich-technische Werkstätten G.m.b.H., West Germany). Cooling was performed for 3 min at+15°C in a water bath. The absorbancy was measured in a Beckman DU spectrometer at 580 mμ using a water blank.

Inhibition with ε-ACA and SBI of the SK activator enzyme was performed with the esterase assay system. The total volume of the incubation mixture was increased to 2.3 ml; 0.66 mg of enzyme protein (0.65 ml) was added to 1 ml of the ALMe solution (2) together with 0.65 ml of the inhibitor solution. A control incubation was performed in parallel

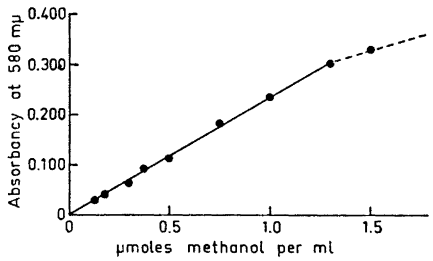

Fig. 1. Methanol standard curve measuring the absorbancy at 580 mμ obtained with the formaldehyde-chromotropic acid complex formed in reaction with the methanol. The linear range was applied in enzyme activity determinations.

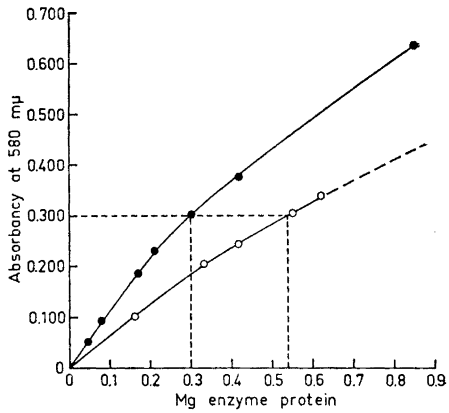

Fig. 2. Measurement of methanol release with increasing concentrations of enzyme protein per 0.65 ml sample used in the reaction. The area indicated by the squares shows the suitable ranges of protein concentration possible to use to match the methanol standard curve (Fig. 1). The preparations contained 6.5 (●) and 5.1 (○) mg of protein per ml, respectively, using 0.0065 M ALMe.

for each one of the different inhibitor concentrations used. In the controls the enzyme and inhibitor solutions were added after the incubation period and before precipitation with perchloric acid.

Calculation of results. Considering the control value for spontaneous hydrolysis of the ester the amount of methanol released by the enzyme was obtained from the standard methanol curve (Fig. 1), and corrected for volume (1.15 ml incubation mixture). Dimensions: μmoles/hour/mg protein.

The reproducibility of the method was tested with an SK activator enzyme preparation corresponding to 50 ml of normal human plasma and isolated by the gel filtration procedure.[2] The standard deviation (12 assays) was 3.3 %; standard error of the mean 1 %.

RESULTS AND DISCUSSION

The method used for the enzyme assay is based upon the reaction between formaldehyde and chromotropic acid by a mechanism suggested by Eegrive [18] and Feigl,[19] originally applied for the determination of methanol after oxidation with permanganate. A critical experimental evaluation of the method was given by Reynolds and Irwin.[20] Siegelman *et al.*[13] first applied the reaction for determination of serum "trypsin" to demonstrate the leakage of this enzyme in pancreatic disease. Synthetic substrates have been widely used for determination of fibrinolytic enzymes such as plasmin applying different techniques for the assays.[8,21] An evaluation of esterase assays for urokinase was given by Sherry *et al.*[11] who also found the methanol procedure convenient to use for multiple determinations in comparing the reaction rates

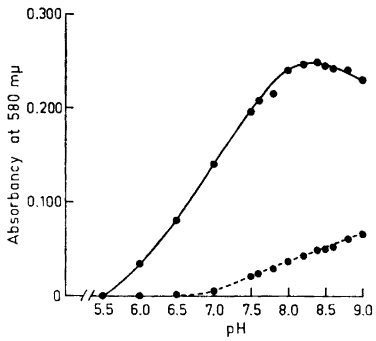

Fig. 3. Influence of pH upon the enzyme catalyzed (———) and spontaneous hydrolysis (- - - - -) of ALMe measured by absorbancy (○). The pH curve for enzyme activity is drawn without correction for spontaneous hydrolysis (*cf.* Fig. 4).

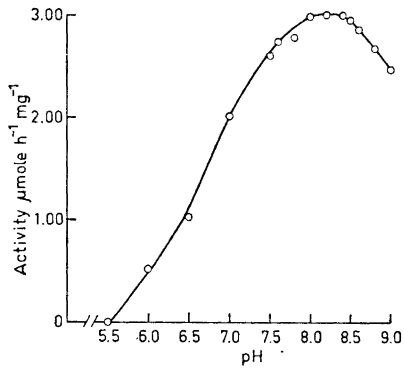

Fig. 4. The pH optimum for the SK activator enzyme activity on ALMe corrected for spontaneous hydrolysis at the corresponding pH values.

with methyl esters of L-arginine and L-lysine. With ALMe a considerably higher rate of hydrolysis (approximately 4 to 20 times) was obtained compared to other esters. Lorand and Mozen[22] and Lorand and Condit[23] used *N*-α-carbobenzoxy-L-tyrosine-*p*-nitrophenyl ester which is as sensitive a substrate for urokinase as ALMe. Recently Walton[24] introduced the crystalline *N*-α-acetyl-glycyl-L-lysine methyl ester, more suitable for kinetic studies with pure urokinase, and obtained K_m values comparable to the rate of hydrolysis with ALMe by titration of carboxyl groups. A high sensitivity range was obtained in the present studies by applying the measuring range as indicated in Fig. 1—2 using the determination of methanol for the assay of substrate hydrolysis.[11,14]

The optimum for the SK activator enzyme activity on ALMe was obtained at pH 8 in the present experimental conditions. As demonstrated in Fig. 3 a different pH dependence appeared in the spontaneous hydrolysis which increased toward higher pH values. Variation of pH did not change the two reactions in parallel. When corrected for spontaneous hydrolysis the true pH-optimum for the reaction appeared to be around pH 8 (Fig. 4). At this pH the spontaneous reaction was comparatively low, and suitable for the assay.

The enzyme assays were performed at a substrate concentration of 0.0065 M ALMe. The reaction reached maximum velocity at approximately 0.0055—0.0065 M concentration of substrate under the present experimental conditions and remained unchanged at 0.0086 M concentration. Using these values the K_m with an impure SK activator enzyme preparation was 3.2×10^{-3} M.

Activation of human plasminogen may occur with proteolytic enzymes such as trypsin or plasmin or autocatalytically after the removal of fibrinolytic inhibitors. A proteolytic step is involved in the final stage of conversion of

plasminogen [25] to plasmin. The reactions leading to the formation of a plasminogen activator enzyme other than plasmin in human plasma are less known. The use of SK in thrombolytic therapy necessitates a determination of the so called "titrated initial dose" (T.I.D.) [26] of SK, *i.e.* an estimation of the dose necessary to induce free fibrinolytic activity in circulating blood. The amount of SK used in the present investigation to produce the plasminogen activator enzyme in plasma is higher than generally applied for clinical thrombolytic effects. It may therefore be suspected that eventually small amounts of a free protease are present in a preparation of the SK activator enzyme complex from total human plasma. With the plasma or urine enzyme preparations used in these studies no proteolytic effect on heated fibrin plates was generally obtained.[2,12] On the other hand strong activity on undenatured bovine fibrinogen was obtained in the fibrin plate test. This suggests that the effect measured on ALMe is due mainly to an esterase with no proteolytic activity on fibrin. The range of activity measured on ALMe is shown in Table 1 with the SK activator enzyme from human plasma. The results obtained with SBI further sustain that the enzyme complex lacks the characteristics of a proteolytic enzyme (Table 2). While none or very low inhibition was obtained with SBI, the ε-ACA inhibited the esterase action however, in concentrations resembling those necessary to inhibit plasmin.[25] It has long been established that ε-ACA is a potent competitive inhibitor of the activation of human and bovine plasminogen, while noncompetitive inhibition is obtained with plasmin and trypsin at higher ε-ACA concentrations.[25,26] Furthermore the results of Alkjærsig *et al.*[25] and Ablondi *et al.*[26] demonstrated that the main effect of ε-ACA is to inhibit the activation of plasminogen if added to the test system [27] before SK was added, or simultaneously. If activation of plasminogen was performed before the addition of ε-ACA [25] there was no

Table 1. Esterase activity on ALMe with the enzyme complex obtained from normal human plasma after activation with SK.

	ml plasma used	protein mg/ml	activity μmoles/hour/mg protein
1.	30	6.5	5.4
2.	40	12.5	3.8
3.	40	31.5	3.3
4.	40	35.0	3.3

The table presents preparations collected after a batch-wise gel filtration on Sephadex G-200 of 10 ml SK-incubated samples of normal human plasma.[1,2] Concentration was made by ultrafiltration at pH 6.0. Dialysis was thereafter performed against 0.15 M NaCl to reduce the ionic strength to $\mu = 0.15$. All operations were performed in a cold room at + 5°C. The decrease in specific activity observed with prolonged ultrafiltration periods accounts for the lower activities generally obtained with the more concentrated preparations.[29]

Protein determinations were performed by the Folin-Cu method according to Lowry *et al.*[30]

Table 2. Effects of ε-aminocaproic acid (ε-ACA) and soybean trypsin inhibitor (SBI) on the esterase activity on ALMe with the SK activator enzyme.

Inhibitor	Concentration	% activity of standard
ε-ACA	M 0.0006	106
	0.0012	105
	0.0020	100
	0.0040	95
	0.0100	92
	0.0200	90
	0.0400	87
	0.0600	84
	0.1000	76
	0.2000	46
	0.3000	13
	0.4000	0
	0.6000	0
SBI μg/ml	34	100
	68	99
	340	93
	680	100

The enzyme preparation corresponds to 40 ml of normal human plasma.[1,2]

significant inhibition of plasmin. Due to the very different test systems applied a strict comparison with the present results is difficult. Similarities may be found, however, to the recently presented results by Ganrot,[28] who showed that plasmin and trypsin can bind to α_2-macroglobulin in an enzyme-inhibitor complex resistant to SBI but retaining its esterase activity. A similar mechanism apparently accounts for the action on ALMe and withstanding the inhibitory effects of SBI and the lower concentrations of ε-ACA found in the present studies with human SK-activated plasma. This further suggests that the esterase assay provides an accurate measure in the study of the SK-induced fibrinolytic activation in human plasma. As shown in previous work [2] the SK activation in human plasma causes the formation of an enzymatically active protein complex larger than accounted for by the equimolar reaction between plasminogen and SK, however, capable of activating human and bovine plasminogen. Further studies concerning the SK-induced esterase complex in human plasma will be presented separately.[29]

The activities on ALMe measured with crude urokinase preparations from human and rabbit urine are shown in Table 3. A preparation of human urine was found to contain 67.5 μg protein per ml after gel filtration and concentration according to the previously described procedures.[12] The specific activity, when corrected for recovery, was 4.4 μmoles/hour/mg of protein, corresponding to 186 CTA units per mg. These values are not directly comparable with the data given by Sherry *et al.*[11] for the ALMe esterase equivalent of the CTA urokinase unit, obtained with different preparations. Comparison between

Table 3. Esterase activity on ALMe obtained with Sephadex G-200 gel filtrated human and rabbit urine.

	Urine samples tested	Activity in CTA units per ml corrected for recovery
Human	1.	5.3
	2.	6.1
	3. *	10.9
	4. *	5.4
Rabbit	1.	21.2
	2.	32.9
	3.	59.7
	4. *	58.1

All experiments were performed with 10 ml urine samples * (female). The human urine was collected in the morning. The rabbits were starved for about 16 h before collecting the urine directly from the bladder of the narcotized animals (with Nembutal, Abbot Laboratories, 50—60 mg per kg, i.p.). The urine samples were prepared according to the previously published method.[12] Urinary enzyme determinations are expressed in CTA units [11,17] and were performed with 0.65 ml samples of ultrafiltrated and dialyzed preparations.

the activities of this crude urokinase and the pure crystalline enzyme,[15,31,32] in terms of CTA units per mg of protein, however, allows an estimation of the degree of purity obtained by gel filtration of human urine.[12] Although a comparatively low degree of purity is obtained (approximately 0.2 %), these results establish that human and rabbit urine samples, that have been submitted to gel filtration, may be enzymatically assayed with ALMe, allowing a more accurate evaluation of the excretion of urokinase in terms of specific esterase activity.

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