Isolation of two variants of native one-chain tissue plasminogen activator

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The native one-chain tissue plasminogen activator from the human melanoma cell line (Bowes) occurs in two variants as demonstrated by sodium dodecylsulphate–polyacrylamide gel electrophoresis of reduced and carboxymethylated samples. The two variants were isolated by affinity chromatography on arginine–Sepharose using a guanidinium–HCl gradient. in the order of elution, the variants were designated tissue plasminogen activator I and II. Variant I was found to be 3000 M_r larger than variant II and the difference was found to reside in the N-terminal half of the molecule. No substantial difference in carbohydrate content nor in enzymatic activity was demonstrated.

Plasminogen activator *Tissue plasminogen activator* *Extrinsic plasminogen activator*
Arginine–Sepharose chromatography *Carbohydrate* *Enzymatic activity*

1. INTRODUCTION

Here, two variants of native one-chain tissue plasminogen activator (tissue PA) have been isolated; their difference is shown to reside in the N-terminus. The isolation procedure works equally well with reduced and carboxymethylated preparations and provides a convenient method for the isolation of electrophoretically homogenous preparations corresponding to the A and B chains of two-chain tissue PA.

Tissue PA can be extracted from most tissue homogenates [1]. It is similar or identical to the vascular activator and the activator of the extrinsic fibrinolytic pathway [2]. Native tissue PA (M_r ~ 70 000) is a one-chain polypeptide and has been shown by amino acid and cDNA sequence analysis to be homologous to serine protease zymogens [3,4]. However, native one-chain tissue PA is an active serine protease [5–7]. This irregularity is reflected in (or perhaps due to) a lysine residue at a position in the activation cleavage site region (–Arg↓Ile–Lys–Gly–Gly–) that typically harbours a hydrophobic residue [8].

When digested with plasmin either in purified systems [5] or in fibrin clots [6] the native one-chain activator is transformed into a two-chain form by cleavage at the zymogen activation site [3,4]. The two disulfide connected chains of M_r ~ 40 000 and 30 000 are designated A and B, respectively [3,5]. The B chain originates from the C-terminal of one-chain tissue PA and is homologous to trypsin [3]. In the presence of fibrin one-chain and two-chain tissue PA have similar enzymatic properties [6,7], but in the absence of fibrin the two-chain form is generally 2–10-fold more efficient [5,7,9,10].

Electrophoretic analysis of reduced and carboxymethylated preparations of two-chain tissue PA from a human melanoma cell line show that the A chain (the N-terminal part of one-chain molecule) exists in two variants with apparent M_r-values of 40 000 and 37 000 [3].

2. MATERIALS AND METHODS

Culture medium from confluent human melanoma cells [11,12] sustained [3] on modified Eagle's minimal essential medium containing 50 KIU/ml aprotinin was the source of tissue PA. Immunoglobulins against porcine tissue PA were isolated from goat antiserum and immobilized on Sepharose 4B as in [3,5]. Fibrinolytic activity was measured with a clot lysis method using a urokinase standard (code no. 66/46, WHO International Laboratory for Biological Standards, Holly Hill, Hampstead).

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Reduction with dithioerythritol and carboxymethylation with iodo-acetic acid was performed as in [3]. Sodium dodecylsulphate–polyacrylamide gel electrophoresis (SDS–PAGE) analysis was performed [13] on 7% polyacrylamide gels using an M_r calibration kit. Protein content was determined by amino acid analysis [14]. Carbohydrate content was determined with anthrone [15] using a multiple standard addition method on lyophilized G-150 purified samples. A 1:1 mixture of D-galactose/D-mannose was used as standard and non-protein containing G-150 eluate as blank. Neuraminidase (*Clostridium perfringens*, type VIII, Sigma, St Louis MO) treatment was in 0.5 M sodium acetate buffer (pH 4.5) [17]. Aminolytic activity was determined [5] on D-Val–Gly–Arg–pNA and D-Ile–Pro–Arg–pNA (S-2322 and S-2288, courtesy of Dr Petter Friberger, Kabi Peptide Research, Mölndal). Arginine–Sepharose was prepared in the same way as lysine–Sepharose [16]. Sepharose 4B, G-150 and the M_r calibration kit were purchased from Pharmacia (Uppsala), the non-ionic detergent Tween 80 from Kebo (Stockholm) and aprotinin (Trasylol R) from Bayer (Leverkusen).

3. RESULTS

3.1. *One-chain tissue PA I and II*

Tissue PA was purified from 50 l melanoma cell culture medium by affinity chromatography on 90 g anti-porcine tissue PA immunoglobulin–Sepharose [3,5]. The activator activity (in 3 M KSCN) was diluted 20-fold with 0.1 M NH_4HCO_3, 0.1 g/l Tween 80, applied to an arginine–Sepharose column and eluted with a linear guanidinium gradient. The activity eluted in 2 partially separated peaks (designated I and II in order of their appearance) as shown in fig.1. Pooled fractions (each containing ~4 mg tissue PA) were concentrated to ~15 ml and separately gel filtered on G-150 columns (5 cm^2 × 80 cm) equilibrated with 1 M NH_4HCO_3, 0.1 g/l Tween 80.

SDS–PAGE of reduced and carboxymethylated samples of one-chain tissue PA I and II mixtures resolved 2 closely-spaced bands (seen as a broad band in fig.2).

3.2. *Two-chain tissue PA I and II*

About 1 mg of one-chain tissue PA I or II dissolved in 2 ml 1 M ammonium bicarbonate, 0.1 g/l Tween 80 was digested with 15 mg plasmin–Sepharose (containing 0.1 mg plasmin) as in [5]. SDS–PAGE analysis of reduced and carboxymethylated samples (fig.2) showed that one-chain tissue PA I and II both were quantitatively converted to the two-chain form. The B chains of the activator variants I and II were apparently identical, but the A chains showed a size difference of ~3000 M_r. The apparent M_r-values of the A chains were 40 000 and 37 000 for two-chain tissue PA variants I and II, respectively. The difference did not disappear on neuraminidase treatment of the two-chain activator prior to reduction and carboxymethylation.

Two-chain tissue PA variants I and II chromatographed in the same way as the one-chain variants on arginine–Sepharose. When reduced and carboxymethylated samples of two-chain

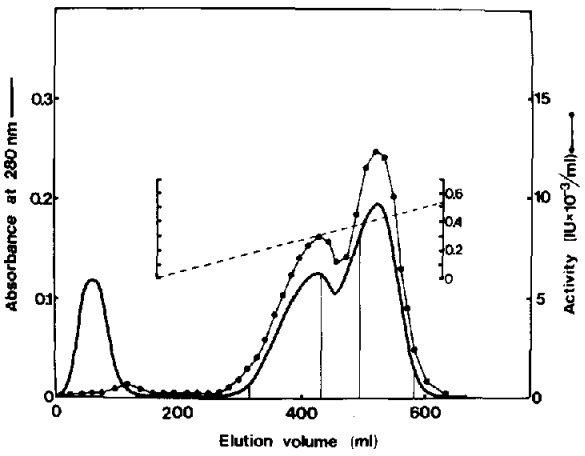

Fig.1. Arginine–Sepharose chromatography of 3 × 10^6 IU (~14 mg) immunosorbent purified one-chain tissue PA. The preparation was dissolved in 100 ml 0.1 M NH_4HCO_3, 0.15 M KSCN and applied to a column (5 cm^2 × 6 cm) of arginine–Sepharose. The column was washed with 50 ml 0.1 M NH_4HCO_3 and adsorbed material eluted with a linear guanidinium–HCl gradient (mixing vessel, 300 ml 0.1 M NH_4HCO_3; limiting vessel, 300 ml 0.1 M NH_4HCO_3, 0.7 M guanidinium–HCl). The chromatography was performed at 4°C and 30 ml/h. All solvents contained 25 KIU/ml aprotinin and 0.1 g/l Tween 80. The absorbance at 280 nm (——) was continuously monitored and the fibrinolytic activity (•——•) was pooled as indicated by the vertical lines (310 – 430 ml, variant I; 500 – 590 ml, variant II).

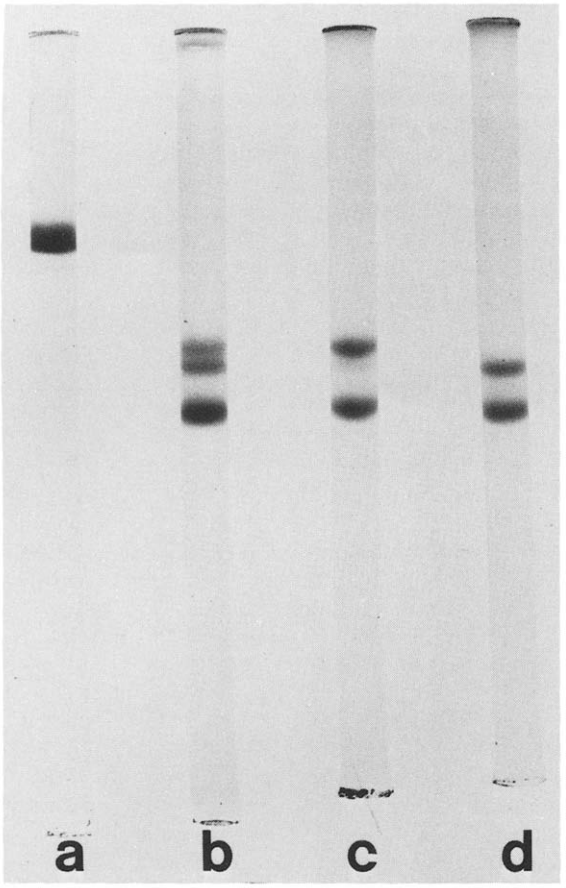

Fig.2. SDS–PAGE analysis of reduced and carboxymethylated samples of tissue PA purified by immunosorbent, arginine–Sepharose and G-150 chromatography. Two-chain tissue PA was prepared by in vitro digestion of one-chain tissue PA with plasmin–Sepharose: (a) mixture of one-chain tissue PA variants I and II; (b) mixture of two-chain tissue PA variants I and II; (c) two-chain tissue PA I; (d) two-chain tissue PA II.

tissue PA were chromatographed, the A chains were found to bind to the gel and to elute with the guanidinium gradient (not shown).

3.3. *Carbohydrate content and enzymatic properties*

Variants I and II of one-chain tissue PA contained 6.9 and 6.7% (w/w) carbohydrates, respectively. The difference is not significant since the standard deviation was 0.7% for both determinations.

The specific fibrinolytic activity was 240 000 and 260 000 IU/mg for one-chain tissue PA I and II and 210 000 and 240 000 IU/mg for two-chain tissue PA I and II. The amidolytic activity on 0.4 mM D-Val–Gly–Arg–pNA (S-2322) at 25°C in 0.1 M NaCl, 0.05 Tris (pH 7.5) expressed in ΔA_{405}/min for 1 μg enzyme/ml was 0.0089, 0.0069, 0.058 and 0.049 for one-chain tissue PA I and II and two-chain tissue PA I and II. The corresponding values on D-Ile–Pro–Arg–pNA (S-2288) were 0.0096, 0.012, 0.056 and 0.062. All determinations were performed in triplicate and the standard deviation never exceeded 5%.

4. DISCUSSION

Two variants of one-chain tissue PA were isolated by affinity chromatography on arginine–Sepharose using a guanidinium gradient. Variant I eluted first. The difference was found to reside in the N-terminal part of the molecule (A chain of the two-chain tissue PA). Two-chain tissue PA I and II, and to our surprise, reduced and carboxymethylated A chain variants behaved in the same way as one-chain tissue PA on arginine–Sepharose. When analysed on SDS–PAGE the A chain variants I and II had apparent M_r-values of 40 000 and 37 000, respectively. This does not indicate differences in sialic acid content since neuraminidase treatment did not change the SDS–PAGE results. No significant difference in carbohydrate content and no substantial difference in fibrinolytic nor amidolytic activity between the variants was observed.

Plasmin digestion of one-chain tissue PA I results in a two-chain activator with homogenous A chain. This rules out that the A chain heterogeneity observed in [3] is due to partial removal of a terminal 3000 M_r peptide.

It is tempting to speculate that the two variants of tissue PA are correlated physiologically to the plasminogen variants I and II (isolated in a similar manner) [18]. Plasminogen I and II occur in many mammalian species [19] indicating a physiological function yet to be unraveled.

The findings presented here may be of importance for cDNA work on tissue PA. Studies on the genetic origin (two alleles or two loci), physiological functions (thrombolysis and turn-over), in vitro kinetics and sequence analysis are in progress.

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