Porcine tissue plasminogen activator

Immunoaffinity purification, structural properties and glycosylation pattern

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Tissue plasminogen activator was purified in high yield from pig heart by immunoaffinity chromatography and characterized by analysis of the glycosylation pattern and the N-terminal amino acid sequence. Comparisons with the human enzyme reveals residue exchanges in the A-chain at positions 3 (porcine Arg/human Gln) and 5 (Thr/Ile), and in the B-chain at positions 6 (Tyr/Phe), 10 (Thr/Ala) and 20 (Val/Ala). The glycosylation pattern for the porcine activator was determined by endoglycosidase treatment followed by gel electrophoresis. The A-chain contains a single high-mannose type of *N*-linked glycan structure and the B-chain contains a complex type of oligosaccharide. A similar but not identical pattern has been observed for the human activator, purified from melanoma cells.

Amino acid sequence analysis *Endoglycosidase treatment* *Electrophoresis*

1. INTRODUCTION

Blood clots formed in the vascular system can be dissolved by plasmin. This proteolytic enzyme which is present in the blood as an inactive proenzyme, plasminogen, has to be activated by plasminogen activators. Two activators of human origin, urokinase (u-PA) and tissue plasminogen activator (t-PA) have been structurally characterized [1–9]. They are both serine proteases and activate plasminogen by a single proteolytic cleavage [10]. However, one difference is that t-PA requires fibrin in order to be a potent activator [11,12], whereas u-PA activates plasminogen equally well in the absence of fibrin. It has been shown that t-PA is the most important plasminogen activator in the blood [13].

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The human t-PA molecule has been characterized at both protein [1–5] and DNA levels [6,7]. It is synthesized as a single-chain protein which is readily cleaved by plasmin or trypsin into a two-chain form [14]. The disulfide bonded chains of the cleaved molecule are designated A-chain (N-terminal, heavy chain) and B-chain (C-terminal, light chain). The B-chain harbours the active site of the enzyme [2], whereas the non-catalytic A-chain mediates fibrin binding and possibly other protein-protein interactions. This part of the activator contains amino acid sequences which are homologous to a number of plasma proteins (review [15]).

However, in many studies the porcine activator has been utilized, and for this protein no amino acid or DNA sequence data have been presented. In the present work we have purified tissue plasminogen activator from pig hearts by the use of immobilized antibodies directed against the human enzyme. The porcine activator has been characterized by N-terminal amino acid sequence analysis

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and the glycosylation pattern has been studied by the use of endoglycosidases.

2. MATERIALS AND METHODS

Tissue plasminogen activator was extracted from homogenized and acetone-delipidated pig hearts using 0.45 M potassium acetate buffer, pH 4.2, and was further purified by fractionated ammonium sulphate precipitation. A detailed presentation of these steps in relation to the adsorption of t-PA to fibrin is given in [14]. To about 4 l of the dissolved precipitate, representing 15 kg heart tissue, an aliquot of 30 g of Sepharose-coupled antibodies against human melanoma t-PA was added. After adsorption and washing, the active material was eluted from the gel and was further purified by chromatography on arginine-Sepharose and on Sephadex G-150 columns. Immunization of goats, preparation of chromatographic media and purification by chromatography were performed as in [2].

Fibrinolytic activity was determined using a clot lysis assay [14] with urokinase as reference standard. Protein concentrations were determined with a modified Beckman 120C analyzer after hydrolysis in evacuated tubes with 5.7 M HCl for 24 h at 110°C. The final product had a specific activity of about 220000 IU/mg (international urokinase units), and the purification yield was 60%. Preparations usually contained a mixture of the single-chain and the two-chain forms of the enzyme.

S-Carboxymethylation was performed with ^{14}C-labeled iodoacetate [2], and the single-chain molecules were separated from the two-chain molecules on a Sephadex G-150 column (135×1.6 cm) in 50 mM ammonium bicarbonate, 0.1% SDS. Radioactive fractions were analyzed by SDS-polyacrylamide gel electrophoresis [16]. Fractions containing alkylated single-chain and two-chain forms of the activator were concentrated under a stream of nitrogen. Liquid-phase sequencer degradations were performed in a Beckman 890D instrument, and phenylthiohydantoin derivatives were analyzed by reverse-phase high-performance liquid chromatography [17] using a Hewlett-Packard 1090 instrument.

Different samples of about 100 μg *S*-carboxymethylated material (mixture of single and two-chain forms) were treated with endo-β-*N*-acetylglucosaminidases F (400 mU) and H (1.5 mU), respectively. The enzymes were from New England Nuclear and reactions were performed in buffers recommended by the supplier. Deglycosylation was monitored by SDS-polyacrylamide gel electrophoresis [16].

3. RESULTS AND DISCUSSION

3.1. *Purification*

The four-step purification procedure used in this work employed: 1, ammonium sulfate precipitation; 2, immunoadsorption; 3, arginine-Sepharose chromatography; 4, size exclusion chromatography. It compares favourably with the five-step fibrin adsorption procedure used before [14]. Utilizing the same assay method, specific activity is slightly lower in the four-step immunoadsorption method, 220000 IU/mg compared with 260000 IU/mg, but purification yield is significantly better. We now obtain a yield of 60% over the four steps, whereas the five-step procedure gives a yield of about 25%. Thus, by taking advantage of the immunological cross-reactivity of t-PA from different species (man and pig), immunoadsorption media can be prepared, with high enough affinity to specifically adsorb the activator, but with low enough affinity to allow elution in high yield.

3.2. *N-terminal sequence analysis*

For the single-chain material, one major sequence was obtained and the N-terminal 20 residues were identified (table 1). Also seen was a clearly minor amino acid sequence derived from the B-chain, indicating that the material contains about 15% of the two-chain form. When the two-chain material was analyzed, the same two sequences were found, but now in equal yield. By combining these results we could identify the first 20 residues of both the A- and the B-chain and compare the sequences with the corresponding parts of the human enzyme.

3.3. *Comparison with the human tissue plasminogen activator*

As expected, the amino acid sequences obtained for the porcine activator are highly homologous to corresponding parts of the human protein. The two-chain porcine t-PA has apparently been

Table 1

Amino acid sequence analyses of single-chain and two-chain forms of porcine tissue plasminogen activator

Chain	Sequence																			
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
	Ser	Tyr	Arg	Val	Thr	Cys	Arg	Asp	Glu	Lys	Thr	Gln	Met	Ile	Tyr	Gln	Gln	His	Gln	Ser
Single-chain form		(27)		(38)									(22)	(26)	(18)					
A-chain of two-chain form		(17)		(23)									(19)	(10)	(9)					
	Ile	Lys	Gly	Gly	Leu	Tyr	Ala	Asp	Ile	Thr	Ser	His	Pro	Trp	Gln	Ala	Ala	Ile	Phe	Val
B-chain of two-chain form	(13)				(16)	(15)	(19)		(20)							(8)	(13)	(5)	(7)	(7)

Residues were identified by high-performance liquid chromatography, and recoveries in nmol of stable derivatives are shown within parentheses. The two-chain preparation showed two sequences in equal yield, and assignments of residues to A- and B-chains were done by comparison with the single-chain form

The two residues which differ among the first 20 residues in the A-chain were found at positions 3 and 5 (table 2). The exchanges observed in the B-chain (i.e. positions 6, 10 and 20) between human and porcine activator apparently occur in a domain where residue exchanges are common between species (table 3). However, the residues at positions 3, 4, 13 and 14 are identical in all of the eight enzymes shown in table 3, relating them to each other and to serine proteases in general. The residues at these positions are highly conserved in the serine protease family.

For the 10 positions where species differences occur, only one position (pos. 20) shows variations in all three enzymes, whereas 7 positions have species variations unique for t-PA, u-PA or plasmin. The fact that species variations preferably occur at unique and different positions for related proteins may reflect their functional differences.

Interestingly, the lysine residue at position 2 in the B-chain of human t-PA was also found in the porcine enzyme. As suggested before [2], a positively charged residue at this position may force the single-chain zymogen-like enzyme into a conformation similar to the active two-chain form. This may explain the unusually high reactivity of the single-chain form of both porcine and human t-PA [23,24] with low-M_r substrates and inhibitors as compared with other serine protease zymogens. Indeed, t-PA is unique in this group of enzymes in having a positively charged residue at position 2 of the B-chain.

Table 2

Residue differences found between porcine and human tissue plasminogen activator for segments 1-20 of both the A- and B-chain

Chain	Position	Porcine activator	Human activator
A	3	Arg	Gln
A	5	Thr	Ile
B	6	Tyr	Phe
B	10	Thr	Ala
B	20	Val	Ala

3.4. *Glycosylation pattern*

When the *S*-carboxymethylated two-chain human melanoma t-PA was analyzed by SDS-polyacrylamide gel electrophoresis the A-chain migrated as a doublet, whereas the B-chain appeared as a single band on the gel [2]. Endoglycosidase treatment of this material [25] has shown that the A-chain size heterogeneity is caused by a complex type of *N*-linked glycan, present only in the variant I [26] of the melanoma activator. In the A-chain of both variants I and II a high-mannose glycan structure is located, and in the B-chain a complex type of oligosaccharide.

Table 3

Comparison of N-terminal sequences from the B-chains of tissue plasminogen activator (t-PA), urokinase (u-PA) and plasmin (pli)

Enzyme	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	References
t-PA (porcine)	I	K	G	G	L	Ⓨ	A	D	I	Ⓣ	S	H	P	W	Q	A	A	I	F	Ⓥ	this work
t-PA (human)	I	K	G	G	L	Ⓕ	A	D	I	Ⓐ	S	H	P	W	Q	A	A	I	F	Ⓐ	2
u-PA (porcine)	I	Ⓥ	G	G	Ⓚ	Ⓢ	T	Ⓣ	Ⓘ	E	N	Q	P	W	F	A	A	I	Y	Ⓡ	18
u-PA (human)	I	Ⓘ	G	G	Ⓔ	Ⓕ	T	Ⓣ	Ⓘ	E	N	Q	P	W	F	A	A	I	Y	Ⓡ	9
u-PA (murine)	I	Ⓥ	G	G	Ⓔ	Ⓕ	T	Ⓔ	Ⓥ	E	N	Q	P	W	F	A	A	I	Y	Ⓠ	17
pli (porcine)	Ⓥ	V	G	G	C	V	Ⓢ	Ⓘ	P	H	S	W	P	W	Q	Ⓘ	S	L	R	Ⓨ	22
pli (human)	Ⓥ	V	G	G	C	V	Ⓐ	Ⓗ	P	H	S	W	P	W	Q	Ⓥ	S	L	R	Ⓣ	21
pli (bovine)	Ⓘ	V	G	G	C	V	Ⓢ	Ⓚ	P	H	S	W	P	W	Q	Ⓥ	S	L	R	Ⓡ	20

One-letter codes are used and residues which differ between species for the same enzyme are encircled

cleaved (before or during purification) at the same site as the human enzyme when treated with plasmin or trypsin. The N-terminal three residue extension seen in the single-chain human melanoma t-PA [2,3] was not observed for the porcine activator, neither the truncated form present in t-PA from human uterus [5].

No A-chain size heterogeneity was observed for the alkylated porcine activator (fig.1, lanes 1 and 5). Treatment of this porcine material (mixture of single-chain and two-chain forms) with endoglycosidase F resulted in a size reduction for both the A-chain and the B-chain (fig.1, lanes 5–8), indicating that each of the two chains carries an *N*-linked oligosaccharide. Endoglycosidase F has the ability to cleave off all types of N-linked glycans [27], whereas endoglycosidase H is more specific, cleaving only high-mannose types of structures [28]. Treatment of the porcine activator with endoglycosidase H resulted in a size reduction only for the A-chain (fig.1, lanes 1–4), showing that the A-chain contains a high-mannose type of glycan structure.

Some deglycosylation of the B-chain was observed for the endoglycosidase H treated samples (fig.1, lanes 1–4). It could be due to heterogeneity in the B-chain glycans, or to the presence of less specific glycosidases in the endoglycosidase H preparation. After treatment with endoglycosidase F for 16 h (fig.1, lane 8) some weakly stained bands were observed in the gel at sizes about M_r 13 000, 30 000 and 60 000. These protein bands are probably the results of contaminating protease ac-

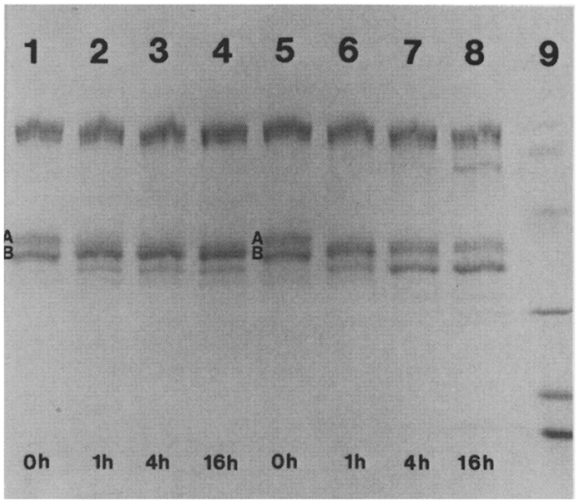

Fig.1. SDS-polyacrylamide gel electrophoresis of *S*-carboxymethylated porcine tissue plasminogen activator after treatment with endoglycosidases H and F. Samples of 100 μg of the alkylated protein (mixture of single- and two-chain forms) were treated with endoglycosidase H and F (1.5 and 400 mU, respectively) at 37°C. Aliquots were taken at the times indicated and boiled for 5 min in 2% SDS to stop the reaction. Electrophoresis was performed on a 10% slab gel [16] which was stained with Coomassie brilliant blue. The positions of the untreated A- and B-chains are indicated. The material which was treated with endoglycosidase H is shown in lanes 2–4 and the corresponding sample treated with endoglycosidase F is shown in lanes 6–8. Molecular mass markers (Da) in lane 9 are ovotransferrin (77 000), albumin (66 200), ovalbumin (45 000), carbonic anhydrase (30 000), myoglobin (17 200) and cytochrome *c* (12 300).

tivity in the endoglycosidase F preparation.

The results show that porcine t-PA contains at least two *N*-linked glycan structures. The A-chain has a high-mannose type, and the B-chain a complex type of oligosaccharide. This is very similar to the glycosylation pattern observed for the human melanoma t-PA. However, in the melanoma t-PA a third glycan is present on about 50% of the molecules [25] causing the variant I and II heterogeneity [26]. This heterogeneity is not observed in the porcine activator studied in this work.

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REFERENCES

[1] Rijken, D.C. and Collen, D. (1981) J. Biol. Chem. 256, 7035-7041.
[2] Wallén, P., Pohl, G., Bergsdorf, N., Rånby, M., Ny, T. and Jörnvall, H. (1983) Eur. J. Biochem. 132, 681-686.
[3] Jörnvall, H., Pohl, G., Bergsdorf, N. and Wallén, P. (1983) FEBS Lett. 156, 47-50.
[4] Pohl, G., Källström, M., Bergsdorf, N., Wallén, P. and Jörnvall, H. (1984) Biochemistry 23, 3701-3707.
[5] Pohl, G., Kaplan, L., Einarsson, M., Wallén, P. and Jörnvall, H. (1984) FEBS Lett. 168, 29-32.
[6] Pennica, D., Holmes, W.E., Kohr, W.J., Harkins, R.N., Vehar, G.A., Ward, C.A., Bennett, W.F., Ylverton, E., Seeburg, P.H., Heyneker, H.L., Goeddel, D.V. and Collen, D. (1983) Nature 301, 214-221.
[7] Ny, T., Elgh, F. and Lund, B. (1984) Proc. Natl. Acad. Sci. USA 81, 5355-5359.
[8] Günzler, W.A., Steffens, G.J., Ötting, F., Kim, S.-M.A., Frankus, E. and Flohé, L. (1982) Hoppe-Seyler's Z. Physiol. Chem. 363, 1155-1165.
[9] Steffens, G.J., Günzler, W.A., Ötting, F., Frankus, E. and Flohé, L. (1982) Hoppe-Seyler's Z. Physiol. Chem. 362, 1043-1058.
[10] Robbins, K.C., Summaria, L., Hsieh, B. and Shah, R.J. (1967) J. Biol. Chem. 242, 2333-2342.
[11] Rijken, D.C., Hoylaerts, M. and Collen, D. (1982) J. Biol. Chem. 257, 2920-2925.
[12] Rånby, M. (1982) Biochim. Biophys. Acta 704, 461-469.
[13] Wun, T.-C. and Capuano, A. (1985) J. Biol. Chem. 260, 5061-5066.
[14] Wallén, P., Bergsdorf, N. and Rånby, M. (1982) Biochim. Biophys. Acta 719, 318-328.
[15] Patthy, L. (1985) Cell 41, 657-663.
[16] Laemmli, U.K. (1970) Nature 227, 680-685.
[17] Zimmerman, C.L., Appella, E. and Pisano, J.J. (1977) Anal. Biochem. 77, 569-573.
[18] Nagamine, Y., Pearson, D., Altus, M.S. and Reich, E. (1984) Nucleic Acids Res. 12, 9525-9541.
[19] Belin, D., Vassalli, J.-D., Combepine, C., Godeau, F. and Nagamine, Y. (1985) Eur. J. Biochem. 148, 225-232.
[20] Schaller, J., Moser, P.W., Dannegger-Müller, G.A.K., Rosselet, S.J., Kampfer, U. and Rickli, E.E. (1985) Eur. J. Biochem. 149, 267-278.
[21] Dayhoff, M.O. (1972) Atlas of Protein Sequence and Structure, vol.5, National Biomedical Research Foundation, Washington D.C.
[22] Marti, T., Schaller, J. and Rickli, E.E. (1985) Eur. J. Biochem. 149, 279-285.
[23] Rånby, M., Bergsdorf, N. and Nilsson, T. (1982) Thromb. Res. 27, 175-183.
[24] Heussen, C., Joubert, P. and Dowdle, E.B. (1984) J. Biol. Chem. 259, 11635-11638.
[25] Pohl, G., Einarsson, M., Nilsson, B. and Svensson, S. (1985) Thrombos. Haemostas. 54, Abstr. 965.
[26] Rånby, M., Bergsdorf, N., Pohl, G. and Wallén, P. (1982) FEBS Lett. 146, 289-292.
[27] Elder, J.H. and Alexander, S. (1982) Proc. Natl. Acad. Sci. USA 79, 4540-4544.
[28] Tarentino, A.L. and Maley, F. (1974) J. Biol. Chem. 249, 811-817.