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Involvement of residues 296–299 in the enzymatic activity of tissue-type plasminogen activator

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The tetra-alanine substitution variant KHRR 296–299 AAAA of tissue-type plasminogen activator (t-PA) was previously shown to have enhanced fibrin specificity and enhanced activity in the presence of fibrin compared with the wild-type form of the molecule. The structural requirements for these alterations in enzymatic activity were investigated by constructing several amino acid substitution variants at each of the positions from 296 to 299 and evaluating their activities under a variety of conditions. Effects on plasminogen activator activity were common among the point mutants at positions 296–299; nearly all had a phenotype similar to the KHRR 296–299 AAAA variant. The greatest effects on enzymatic function were found with multiple substitution variants, but some single charge reversals and proline substitutions had substantial effects. The enhanced fibrin specificity of KHRR 296–299 AAAA t-PA results in less fibrinogenolysis than seen with wild-type t-PA. Approximately four times greater concentration of KHRR 296–299 AAAA compared with wild-type t-PA was required to consume 50% of the fibrinogen in human plasma.

Key words: tissue-type plasminogen activator/mutagenesis/fibrinolysis

Introduction

Tissue-type plasminogen activator (t-PA) is a serine protease that functions in the fibrinolytic system by activating the proenzyme plasminogen in the active enzyme plasmin. Plasmin then degrades fibrin to soluble fibrin degradation products. t-PA is composed of five domains which have been identified by virtue of their amino acid sequence homologies to other proteins (Pennica *et al.*, 1983; Patthy, 1985). Starting from the N terminus, t-PA contains a finger and growth factor domain similar to those found in fibronectin and epidermal growth factor respectively, two kringle structures and a serine protease. The five t-PA domains have been variously implicated in several functions including (but not limited to) fibrin binding, lysine binding, reaction with inhibitors, rapid plasma clearance and fibrin stimulation and specificity (for a review see Higgins and Bennett, 1990).

We recently reported the results of a study where a technique we called a 'clustered charged-to-alanine scan' was used to associate structural determinants on t-PA with several of its inherent properties, thus producing a map of the functional determinants of the molecule (Bennett *et al.*, 1991). In this approach, small groups of charged residues throughout the primary sequence of the molecule were systematically changed to alanine. In total, 64 variants were constructed with from one to four alanine substitutions each. These variants were then tested using a battery of assays to evaluate their amidolytic, plasminogen activator and clot lysis activities; their fibrin and lysine binding; and their resistance to inhibition by plasminogen activator inhibitor-1 (PAI-1). One of the interesting observations in that study was that the tetra-alanine substituted variant KHRR 296–299 AAAA had significantly altered enzymatic properties compared with wild-type t-PA. This variant, whose parent residues lie in an insertion loop absent from most serine proteases (Madison *et al.*, 1989; Bennett *et al.*, 1991), had normal amidolytic activity on the tripeptide substrate S-2288 (H-D-isoleucyl-L-prolyl-L-arginine-*p*-nitroanilide dihydrochloride), but reduced activity toward human Glu-plasminogen in the absence of a stimulator, or in the presence of the weak stimulator fibrinogen. In the presence of fibrin, however, this variant was nearly three times more active than wild-type t-PA. If we define fibrin specificity as the ratio of the activity of t-PA in the presence of fibrin over fibrinogen, then the combined effect of reduced activity in the presence of fibrinogen and increased activity in the presence of fibrin resulted in nearly an order of magnitude more fibrin specificity for KHRR 296–299 AAAA compared with the wild-type form of t-PA.

In order to determine the structural requirements for this change in catalytic function, each of the amino acid residues from positions 296 to 299 in t-PA were substituted with several different amino acids. The enzymatic properties of the resulting variants were then characterized and compared with wild-type and the tetra-alanine substitution variant. In addition, we tested the hypothesis that the enhanced fibrin specificity of KHRR 296–299 AAAA, predicted on the basis of analysis of plasminogen activator activity, would result in less fibrinogenolysis than observed with wild-type t-PA. The results of our analyses are presented in this report.

Materials and methods

Materials

D-Phenylalanyl-L-prolyl-L-arginine chloromethyl ketone dihydrochloride was obtained from Calbiochem (San Diego, CA). H-D-valyl-L-leucyl-L-lysine-*p*-nitroanilide dihydrochloride (S-2251; KabiVitrum) and H-D-isoleucyl-L-prolyl-L-arginine-*p*-nitroanilide dihydrochloride (S-2288; KabiVitrum) were purchased from Helena Laboratories (Beaumont, TX). Human plasma was purchased from Peninsula Memorial Blood Bank, Burlingame, CA. Activase® t-PA was from Genentech, Inc., South San Francisco, CA. Human thrombin was a generous gift from Dr Walter Kisiel, University of New Mexico, Albuquerque, NM.

Mutagenesis, expression and analysis of t-PA variants

The methods used for oligonucleotide-directed mutagenesis, transient expression of the variants in serum-free media and analysis of the harvested cell culture fluids for t-PA enzymatic

activity have been described in detail elsewhere (Bennett *et al.*, 1991). The activity assays included S-2288 hydrolysis and activation of plasminogen in the absence and presence of fibrinogen and fibrin.

Plasminogen activator activity in the presence of human plasma clots

The plasminogen activator activity of the variants was measured in the presence of a human plasma clot. The variants were converted to the two chain form using plasmin–Sepharose as previously described (Bennett *et al.*, 1991). Human thrombin (10 μl of a 30 μg/ml solution) was added to each well of a microtiter plate. Cell culture fluid containing the variants (40 μl) was added to the plate and the assay was initiated by the addition of 100 μl of a mixture of acid–citrate–dextrose collected human plasma (90 μl) and 9 mM S-2251 (10 μl) to each well. Under these conditions clot formation was rapid compared with the time course of the plasminogen activation reaction. Color development was monitored at 405 nm and the absorbance at the reference wavelengths of 492 nm was subtracted from each time point to correct for the effect of turbidity. The data collection and analysis were the same as those used for the other plasminogen activator assays. Data were collected using an SLT Laboratories Model EAR 340 AT microtiter plate reader interfaced to an AST Premium/286 computer. The initial velocities of the reactions were determined using a computer program written in our laboratory which calculated the slope of the absorbance versus time squared curve for each sample. A standard curve was prepared by assaying Activase® t-PA diluted in cell culture media. The concentration of the variants based on enzymatic activity was

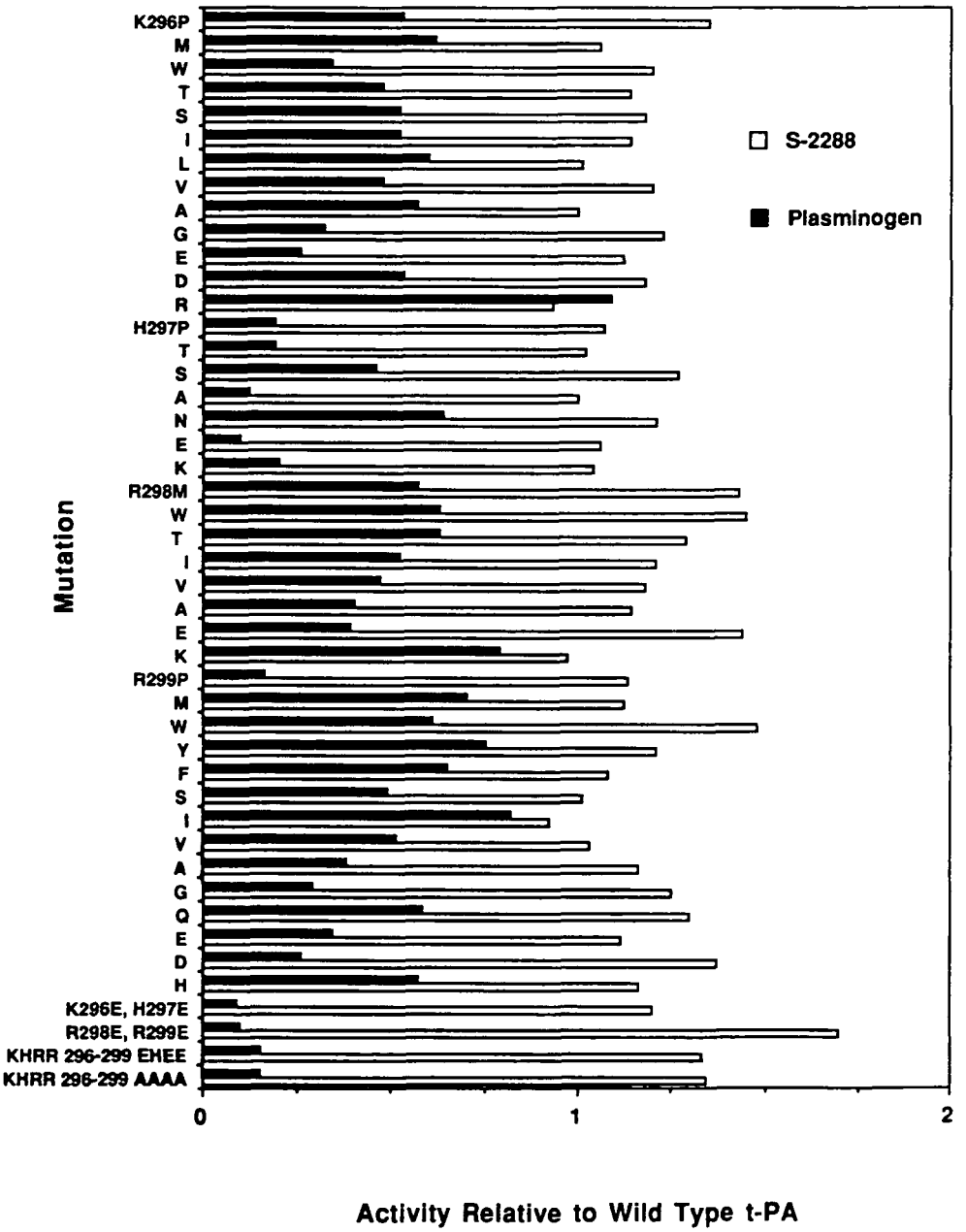

Fig. 1. Amidolytic and plasminogen activator activities of the t-PA variants relative to wild-type t-PA. Initial rates of S-2288 hydrolysis and plasminogen activation are shown. Plasminogen activator activity was measured using a coupled assay. Human Glu-plasminogen was used as the t-PA substrate and the resulting plasmin was measured using S-2251. All samples were assayed in the two chain form. The values are expressed as a ratio with wild-type recombinant t-PA, which was transfected along with the variants and which was assigned a value of 1.0.

obtained from the t-PA standard curve. The relative activities of the variants compared with Activase® t-PA were obtained by dividing the 'activity concentration' of the variant by the 'antigen concentration' obtained from the t-PA ELISA. Wild-type t-PA transfected at the same time as the variants consistently gave values of ~1 in this assay system. In order to compare more accurately results from assays performed at different times, four wild-type controls were included in every transfection. The results for the samples analyzed in each assay were normalized such that the mean of the relative activity of the wild-type internal controls was equal to 1.0.

Plasminogen activator activity in the presence of human plasma

The plasminogen activator activity of the variants was also measured in human plasma. This assay was performed as described for the plasma clot stimulated assay except that phosphate-buffered saline (PBS) was substituted for thrombin and reference wavelength subtraction was not applied.

In vitro fibrinogenolysis

Fibrinogen consumption was assessed *in vitro* by adding 200 μl of acid–citrate–dextrose collected human plasma to the wells of a microtiter plate. The plate was warmed to 37°C and 20 μl of the t-PA test sample were added. The mixtures were incubated at 37°C for 1 h then the reaction was stopped by transferring 200 μl to a 5°C chilled plate containing 20 μl of 35 μM D-phenylalanyl-L-prolyl-L-arginine chloromethyl ketone dihydrochloride in 10 mM EDTA. The plate was put through one freeze–thaw cycle and the residual fibrinogen was determined by the Clauss clotting time method (Clauss, 1957).

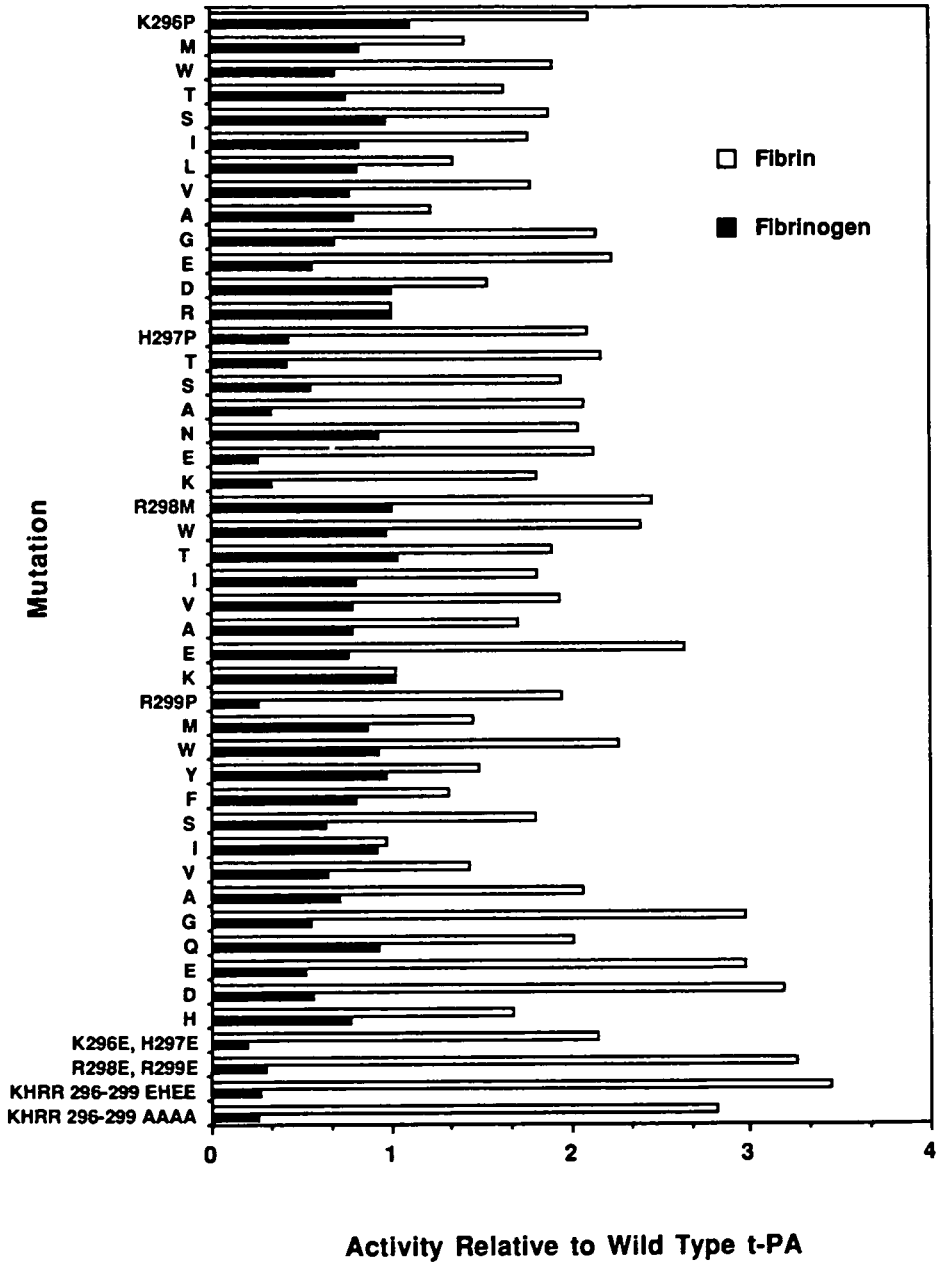

Fig. 2. Fibrin and fibrinogen stimulated plasminogen activator activity of the t-PA variants relative to that of wild-type t-PA. The plasminogen activator activity of the t-PA variants in the two chain form was determined as described in Figure 1 except that the reaction was performed in the presence of fibrinogen or fibrin.

Results

Site-directed mutagenesis was used to replace the amino acid residues KHRR at positions 296–299 in t-PA with several different amino acids. The resulting variants were transiently expressed in human 293 cells and the t-PA activity in the serum-free cell culture supernatants was evaluated. For each assay the activity of the variants is expressed relative to that of wild-type t-PA which is assigned a value of 1.0.

The results shown in Figure 1 compare the enzymatic activity of the variants on the tripeptide substrate S-2288 and on human plasminogen. When the tripeptide was used, each of the variants, including the multiple substitutions, had activity equal to or slightly greater than wild-type t-PA. With Glu-plasminogen as substrate, however, most of the variants had considerably less than wild-type activity.

One of the characteristics ascribed to a tetra-alanine substituted variant in the 296–299 region of t-PA (Bennett *et al.*, 1991) was an increased activity relative to wild-type t-PA in the presence of fibrin. This was coupled with a decreased activity in the presence of the weak stimulator fibrinogen. Taken together, these properties result in a molecule which is more fibrin specific than wild-type t-PA. Figure 2 shows the fibrin and fibrinogen stimulated plasminogen activator activities of the variants relative to wild-type t-PA. Most have greater activity than wild-type in the presence of fibrin and several have 2- to 3-fold higher activity. In contrast, the activity of the mutants in the presence of fibrinogen is less than or at best equal to that of wild-type t-PA. The activity of the variants in plasma and in clotted plasma was also evaluated. The results are shown in Figure 3. The increased differential activity observed in the presence of fibrinogen and fibrin was also evident when plasma and clotted plasma were

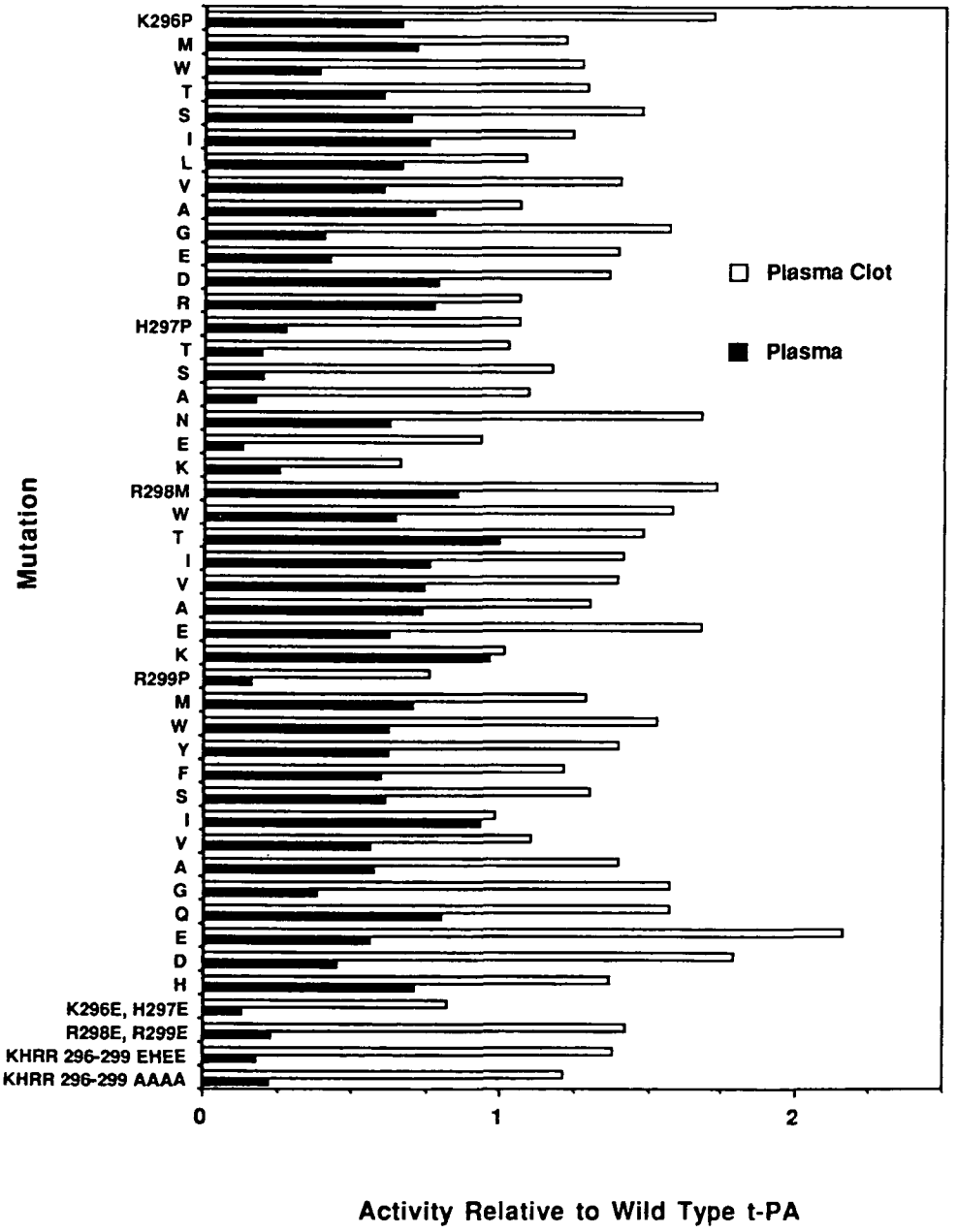

Fig. 3. Plasminogen activator activity of t-PA variants in plasma and clotted plasma. The plasminogen activator activity of the variants in human plasma and clotted human plasma was determined as described in Materials and methods. Activities are expressed relative to wild-type t-PA which was assigned a value of 1.0. All variants were assayed in the two chain form.

used, although the activities of most of the variants were attenuated in the plasma clot based assay system.

Wild-type t-PA already has substantial fibrin specificity. Under our assay conditions there is an ~6-fold rate enhancement when fibrinogen is added to the plasminogen–t-PA reaction mixture. The rate is further enhanced ~6-fold in the presence of fibrin (Bennett *et al.*, 1991). The increase in fibrin specificity of the variants is in addition to the intrinsic specificity of wild-type t-PA. It is calculated by taking the ratio of activity relative to wild-type in the presence of fibrin and fibrinogen. The plasma clot specificity of the mutants can likewise be represented relative to wild-type using the ratio of the relative activity in clotted and unclotted plasma. The results of this analysis are shown in Figure 4. Most of the variants have >2-fold enhanced fibrin and plasma clot specificity over wild-type t-PA, and several of the point mutations resulted in fibrin and plasma clot specificity from four to eight times that of the wild-type molecule.

In general, the data presented herein indicate that multiple mutations in the 296–299 region of t-PA had a greater effect on the enzymatic activity of the molecule than the individual substitutions. Some individual mutations, however, such as charge reversals and proline substitutions, had substantial effects. Conservative mutations such as K296R and R298K behaved similarly to wild-type t-PA.

The data described above were gathered by assaying the t-PA variants directly in harvested cell culture fluid without purification. Two t-PA variants, KHRR 296–299 AAAA and KHRR 296–299 EHEE, were transiently expressed in 293 cells and the proteins were purified using lysine affinity chromatography. The enzymatic activities of the purified proteins were compared with those obtained using cell culture fluid samples. The data are shown in Table I. Similar results were obtained in the various assays whether purified or non-purified samples were analyzed. Only the magnitude of the activity

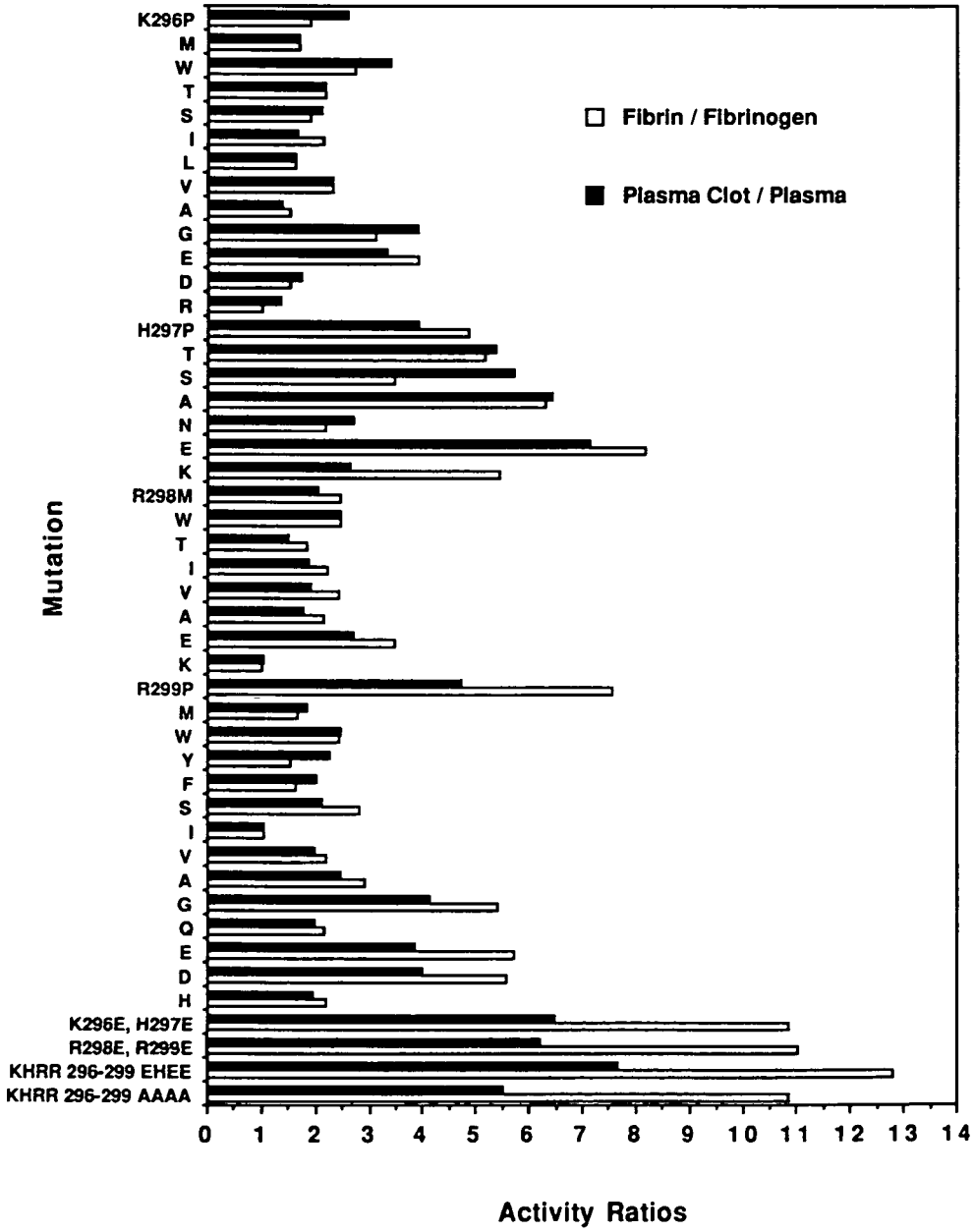

Fig. 4. The fibrin and plasma clot specificity of the t-PA variants. The fibrin specificity of the variants relative to wild-type t-PA was determined as the ratio of the relative activities shown in Figure 2. The plasma clot specificity of the variants was determined as the ratio of the values shown in Figure 3.

Table I. Comparison of the enzymatic activity of t-PA variants assayed in serum-free cell culture supernatants and after purification

	Amidolytic activity	Plasminogen activator activity				
		Unstimulated	+Fg	+Fn	Plasma	Plasma clot
KHRR 296–299 AAAA						
Cell culture fluid	1.34	0.15	0.26	2.82	0.22	1.21
Purified	1.07	0.13	0.20	1.72	0.16	0.95
KHRR 296–299 EHEE						
Cell culture fluid	1.33	0.15	0.27	3.45	0.18	1.38
Purified	1.25	0.11	0.18	2.10	0.14	1.10

The assays were carried out as described in Materials and methods. All values are relative to wild-type t-PA. Fg and Fn are fibrinogen and fibrin respectively.

increase in the presence of fibrin appeared significantly different after purification of the variant proteins. The enzymatic properties of the KHRR 296–299 AAAA are less like wild-type than any of the individual alanine substitution variants tested. Reversing the charges at three of the four positions, as was done with the KHRR 296–299 EHEE variant, had little additional effect.

From the low activity measured in plasma for the KHRR 296–299 AAAA variant one would predict that the molecule should be more fibrinogen sparing than wild-type t-PA. In order to test that hypothesis, purified wild-type t-PA (Activase® t-PA) and the purified tetra-alanine substitution variant were added to human plasma *in vitro* and the effect on fibrinogen concentration was determined as described in Materials and methods. The results are shown in Figure 5. Less than 10% of the fibrinogen was consumed in the KHRR 296–299 AAAA incubation mixture at the t-PA concentration which consumed nearly 70% of the fibrinogen when wild-type t-PA was used. In addition approximately four times more variant was needed than wild-type t-PA to consume 50% of the fibrinogen under the same conditions. These results are in good agreement with the relative activity of the KHRR variant in plasma reported in Table I. Enhanced fibrinogen sparing apparently does not come at the expense of clot lysis, since greater activity than wild-type was measured for the KHRR 296–299 AAAA variant using an *in vitro* plasma clot lysis assay (Bennett *et al.*, 1991).

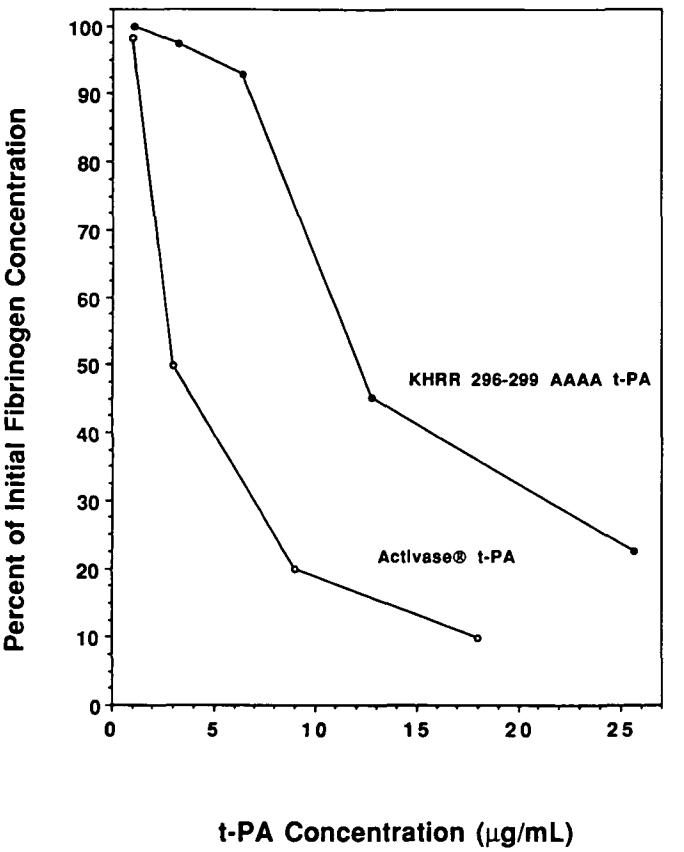

Fig. 5. *In vitro* fibrinogenolysis generated by wild-type t-PA and KHRR 296–299 AAAA t-PA. Fibrinogen consumption in 1 h at 37°C was measured in human plasma as a function of t-PA concentration. The percent of the initial concentration of fibrinogen in the mixture was determined by the method of Clauss (1957).

Discussion

The data presented here extend the observations reported by Bennett *et al.* (1991) on the impact of the region of t-PA from residue 296 to 299 on the enzymatic functions of the molecule. This positively charged region of t-PA is located near the active site and, to our knowledge, is only found on serine proteases which have plasminogen as their primary substrate [other examples are urokinase (Heyneker *et al.*, 1983; Nagai *et al.*, 1985; Riccio *et al.*, 1985) and vampire bat salivary plasminogen activator (Gardell *et al.*, 1989)]. It is striking that almost any substitution of the amino acids in this region of t-PA results in a variant which is more active in the presence of fibrin, less active in the presence of fibrinogen or in the absence of a stimulator, and thus more fibrin specific than wild-type t-PA. Further, the already substantial differential activity of wild-type t-PA in the presence of fibrin and fibrinogen (~6-fold in our hands) can be increased by an order of magnitude with just two charge reversals at positions 296 and 297 or 298 and 299. It is interesting to note that vampire bat salivary plasminogen activator, which is very fibrin specific (Gardell *et al.*, 1989, 1990, 1991), contains Q and N in the positions corresponding to K296 and H297, respectively, of t-PA.

It has been proposed (Madison *et al.*, 1989, 1990) that residues 296–302 interact with PAI-1 but not with plasminogen. Their hypothesis was based on the fact that deletion and substitution variants in this region of t-PA were resistant to PAI-1, but had normal activity towards Lys-plasminogen in the presence of the stimulator DESAFIB™. We are in agreement with these authors on the importance of this region for PAI-1 inhibition. However, the data presented herein indicate that the 296–299 region of t-PA is also important in the catalysis of plasminogen activation. Nearly all of the amino acid substitution variants we have constructed in that region have normal enzymatic activity on the tripeptide substrate S-2288, but reduced activity towards the full length form of human plasminogen, Glu-plasminogen. In addition, variants in this region of t-PA activate Glu-

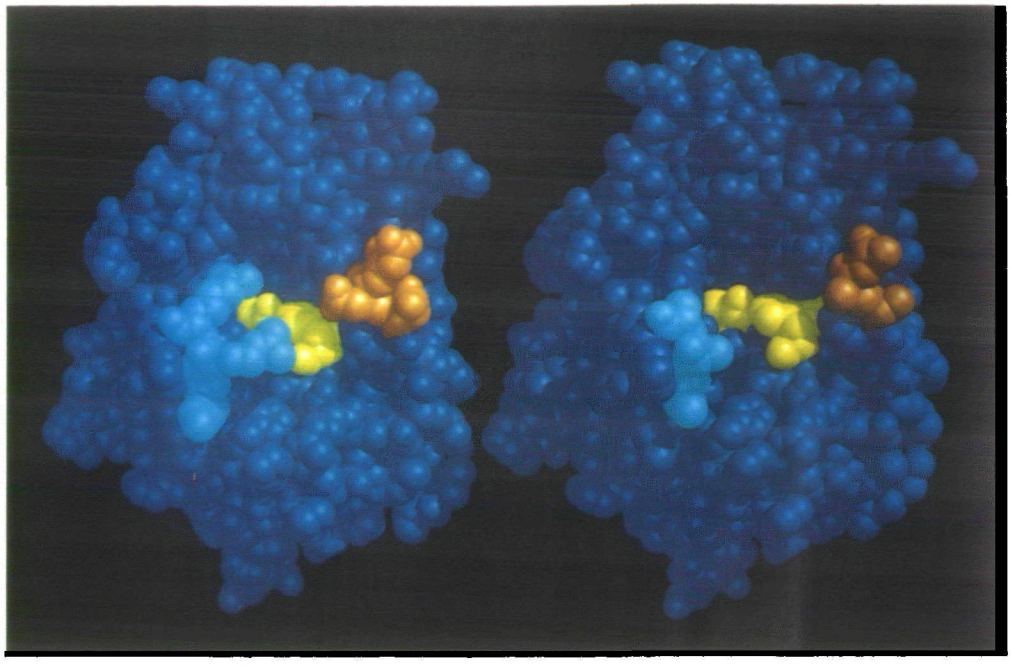

Fig. 6. Location of residues KHRR 296–299 and DDD 364–366 on a model of the t-PA protease. The structure on the left shows KHRR 296–299 (light blue) and DDD 364–366 (orange) in relation to the active site residues H322, D371 and S478 shown in yellow. In the structure on the right the native residues at positions 296–299 and 364–366 were replaced with alanine.

plasminogen differently from wild-type in the presence of two physiological stimulators, fibrin and fibrinogen, while the KHRR 296–299 area is not involved in fibrin binding (Bennett *et al.*, 1991).

Wallén *et al.* (1990) also reported that this region of the t-PA protease was involved in plasminogen activation. Their conclusion was based on the observation that limited trypsin digestion of the isolated t-PA protease resulted in cleavage of the peptide immediately after the KHRR sequence. While the amidolytic activity of the molecule on S-2288 was largely unaffected by the treatment, the activity towards plasminogen was reduced by ~90%. Wallén *et al.* interpreted their data to indicate that a binding site for plasminogen was present at or near the KHRR region in the isolated t-PA protease. In the intact molecule this is likely not the primary plasminogen binding site since Eastman *et al.* (1991) determined that the tetra-alanine substitution variant at those positions had a normal K_M but reduced k_{cat} for plasminogen in the absence of fibrin. The concept of a binding event in this region which facilitates the catalysis of plasminogen activation in the presence of fibrin is, however, an intriguing possibility, especially in light of the position of these residues in relation to another set of charged amino acids DDD 264–266 (see Figure 6). Our model of the t-PA protease (Bennett *et al.*, 1991) predicts that the positively charged KHRR 296–299 loop and the negatively charged DDD 364–366 residues oppose each other across the active site cleft in the protease. The tri-alanine substitution variant DDD 364–366 AAA also has altered enzymatic activity compared with wild-type t-PA (Bennett *et al.*, 1991). It has reduced activity on S-2288 and reduced activity on Glu-plasminogen (57 and 38% of wild-type respectively), increased activity towards plasminogen in the presence of fibrin (177% of normal) and increased fibrin specificity (2.4-fold). The similarity in enzymatic activity of alanine substitution variants in these two charged surfaces may indicate that together they help to constrain plasminogen at the active site of t-PA in the absence of fibrin and also may help in defining the substrate specificity of the t-PA protease.

We previously developed several screening techniques to evaluate the plasminogen activator activity of t-PA variants in harvested cell culture fluid samples (Bennett *et al.*, 1991). In this study we extended those analytical techniques to include the measurement of plasminogen activation in human plasma and clotted plasma. The fibrin specificity of the variants, determined with assay systems composed of purified components of the coagulation and fibrinolytic systems, was reproduced when similar assays were performed using human plasma. The attenuation of activity of the variants in the plasma versus the purified component assay systems was unexpected and emphasizes the importance of including plasma based assay systems when evaluating variants of t-PA.

The assay methods we have used appear to be reasonably accurate measures of the enzymatic properties of the unpurified variants. Once purified, the variants show essentially the same pattern of plasminogen activator activity under the various assay conditions. Further, the increased fibrin specificity of KHRR 296–299 AAAA predicted on the basis of plasminogen activator activity in these assays translated into reduced fibrinogenolysis for this variant compared with the wild-type form of t-PA.

The KHRR 296–299 region of t-PA provides an interesting locus to study the complex interactions involved in catalysis and regulation of an important plasma protease. It also provides an opportunity to engineer and potentially to improve a therapeutic protein. Clearly it is possible to construct variants of t-PA with limited amino acid substitutions in the 296–299 region which have greater enzymatic activity, more fibrin specificity and greater resistance to inhibitors than wild-type t-PA. Studies are currently under way to evaluate the effectiveness of these changes on t-PA function in *in vivo* models of thrombolysis.

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