A Region of Tissue Plasminogen Activator That Affects Plasminogen Activation Differentially with Various Fibrin(ogen)-Related Stimulators

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ABSTRACT: The dissolution of blood clots by plasmin is normally initiated in vivo by the activation of plasminogen to plasmin through the activity of tissue plasminogen activator (t-PA). The rate of plasminogen activation can be stimulated several orders of magnitude by the presence of fibrin-related proteins. Here we describe the kinetic analysis of both recombinant human t-PA (wild-type) and a t-PA variant produced by site-directed mutagenesis in which the original sequence from amino acids 296 to 299, KHRR, has been altered to AAAA. This tetra-alanine variant form of t-PA, K296A/H297A/R298A/R299A t-PA, we refer to as "KHRR" t-PA here. The plasminogen activating kinetics of wild-type t-PA (Activase alteplase) showed a catalytic efficiency which changed over 100-fold dependent on the stimulator in the assay. The lowest rate was in the absence of a stimulator. The following stimulators showed increasing ability to accelerate the catalytic efficiency of the reaction: fibrinogen, fragments of fibrinogen obtained by digestion with plasmin, fibrin, and slightly degraded fibrin. This increase in efficiency was driven primarily by decreases in the Michaelis constant (K_M) of the reaction, whereas the catalytic rate constant (k_{cat}) of the reaction did not change significantly. The "KHRR" variant of t-PA displayed novel kinetics with all stimulators tested. In the absence of a stimulator or with the poorer stimulators (fibrinogen and fibrinogen fragments), the K_M values of the reaction with Activase alteplase and "KHRR" t-PA were similar. The k_{cat} however, was lower with "KHRR" t-PA than with wild-type t-PA. This led to a decrease in the rate of plasminogen activation with "KHRR" t-PA in the presence of these stimulators. In the presence of the more potent stimulators (fibrin or degraded fibrin), the k_{cat} of the reaction with "KHRR" t-PA was actually more efficient than that observed with Activase alteplase. The K_M was also higher, indicating that the variant and plasminogen may interact less well than wild-type t-PA and plasminogen in the presence of fibrin-related stimulators. However, the kinetic constants predict that at concentrations of plasminogen above 1 μM, the variant would display a more rapid activation of plasminogen in the presence of these stimulators. These properties would suggest that "KHRR" t-PA may be a superior thrombolytic agent to Activase alteplase by virtue of its decreased ability to be stimulated by fibrinogen-related molecules found systemically (i.e., fibrinogen and fibrinogen degradation products) but have increased activity in the presence of components found in a clot (i.e., fibrin and slightly degraded fibrin).

The degradation of the fibrin network of thrombi is accomplished through the action of plasmin, which is formed by the hydrolysis of plasminogen in blood by tissue plasminogen activator (t-PA). The rate of the reaction in which t-PA activates plasminogen to plasmin is altered significantly by the presence or absence of stimulators related to fibrin (Camiolo et al., 1971; Wallen, 1977; Hoylaerts et al., 1982; Ranby, 1982; Suenson et al., 1984; Norrman et al., 1985; Suenson & Petersen, 1986). Many different stimulators have been used in vitro and give different results. Among the stimulators commonly used are fibrin, fibrin monomers, fibrinogen, and fragments of fibrinogen prepared by cyanogen bromide cleavage. These fragments may also be treated with plasmin. The presence of any of these stimulators in a reaction mixture containing t-PA and plasminogen accelerates the formation of plasmin, and each works to a varying extent.

Recently, a specific region of the t-PA molecule, residues 296–302, which comprise a unique insertion in the protease portion of t-PA which is not present in most other serine proteases, has been shown to influence two important functions of the molecule. Madison et al. (1989, 1990) demonstrated that this region governs the interaction of t-PA with its naturally occurring inhibitor, plasminogen activator inhibitor 1 (PAI-1). In addition to confirming that the 296–299 region of the protease was involved in the interaction of t-PA with PAI-1, Bennett et al. (1991) demonstrated that this region is also involved in the ability of fibrinogen and fibrin to increase the rate at which t-PA can activate plasminogen. They found that the tetra-alanine variant K296A/H297A/R298A/R299A ("KHRR") t-PA exhibited higher plasminogen activating activity with fibrin as a stimulator, but had decreased activity in the absence of a stimulator or with fibrinogen as the stimulator, when compared to wild-type t-PA. This result contrasts the observation of Madison and co-workers (Madison et al., 1989, 1990) which demonstrated that variants in the same region displayed similar kinetics to wild-type t-PA with DES-AFIB (des-AA fibrinogen, soluble fibrin I). The purpose of this study was to analyze the ability of "KHRR" t-PA and wild-type sequence t-PA to activate plasminogen in the presence of various stimulators related to fibrin and believed to be physiologically relevant. The catalytic rate constants obtained with the various stimulators may yield insight into

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Table I: k_{cat}/K_M Data[a]

	wild-type t-PA			"KHRR" t-PA		
stimulator	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M (μM^{-1} min^{-1})	K_M (μM)	k_{cat} (min^{-1})	k_{cat}/K_M (μM^{-1} min^{-1})
none	33 ± 16	0.74 ± 0.53	0.022	38 ± 12	0.13 ± 0.08	0.003
intact fibrinogen	7.4 ± 3.3	0.88 ± 0.40	0.12	7.1 ± 0.5	0.22 ± 0.10	0.031
degraded fibrinogen	3.1 ± 0.6	0.72 ± 0.61	0.23	3.6 ± 2.1	0.14 ± 0.06	0.039
fibrin	1.0 ± 0.8	1.1 ± 0.5	1.1	2.6 ± 1.9	3.3 ± 1.4	0.80
50% fibrin	0.36 ± 0.09	1.7 ± 1.4	4.7	5.7 ± 1.7	17.0 ± 7.5	3.0
DESAFIB	0.47 ± 0.23	0.24 ± 0.004	0.58	0.96 ± 0.04	0.58 ± 0.28	0.60

[a] Kinetic constants ± standard deviation were obtained for reactions with Glu-plasminogen in the presence of the stimulators indicated.

the influence of that region on the mechanism of plasminogen activation.

Materials and Methods

t-PA and Variants. Activase alteplase was obtained from Genentech, Inc., South San Francisco, CA. The construction and transient expression of K296A/H297A/R298A/R299A t-PA ("KHRR") were described previously (Bennett et al., 1991). The material used in these studies was transiently expressed in 293 (human embryonic kidney) cells using DEAE transfections (F. Wurm, unpublished results) and was purified by lysine affinity chromatography. Wild-type sequence rt-PA was also prepared using the same transient expression system and purification. All samples of t-PA and variants were converted to the two-chain form by incubation with plasmin–Sepharose (Tate et al., 1987) for 15 min. The concentrations of wild-type t-PA and the "KHRR" t-PA variant were determined by ELISA. t-PA and "KHRR" t-PA were analyzed for homogeneity and percent two-chain form by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and dithiothreitol (Laemmli, 1970; Morrissey, 1981).

Stimulators. Human fibrinogen (>95% clottable) was obtained from Calbiochem (La Jolla, CA). A 100-mg vial was reconstituted in 5 mL of water. Following dialysis into 0.05 M Tris, 0.12 M NaCl, and 0.01% Tween 80, pH 7.4, any contaminating plasminogen was removed by passage over a lysine–Sepharose column (Pharmacia, Inc., Piscataway, NJ) equilibrated in the same buffer. The clottability of the preparation was then monitored by incubating the fibrinogen with 10 units/mL (final concentration) human thrombin for 1 h at room temperature. The amount of protein remaining in the supernatant was compared to the initial concentration to determine clottability. The plasminogen-free fibrinogen obtained after passage over lysine–Sepharose (intact fibrinogen) was >92% clottable. This intact fibrinogen was used both as the intact fibrinogen stimulator and as the fibrin stimulator. In the latter case, 7.8 units/mL (final concentration) human thrombin was added to the reaction mixture so that a fibrin clot formed. To prepare 50%-degraded fibrinogen, the intact fibrinogen was incubated with plasmin–Sepharose (Higgins & Vehar, 1987) until the clottability had reached 50–60%. The details of this preparation including SDS–PAGE analysis have been previously described (Higgins & Vehar, 1987). This partially degraded fibrinogen preparation was used as the 50%-degraded fibrin preparation by adding 7.8 units/mL thrombin to the microtiter plates. To prepare degraded fibrinogen, the incubation with plasmin–Sepharose was allowed to continue overnight until the clottability of the fibrinogen was <10%. DESAFIB (des-AA-fibrinogen, soluble fibrin I) was purchased from American Diagnostica (Greenwich, CT).

Kinetic Methods. Glu-Plasminogen was purified from pooled normal plasma using a modification (Bennett et al., 1991) of the method of Deutsch and Mertz (1970). Sequence analysis confirmed that the material was >90% Glu-plasminogen. Glu-Plasminogen was used in kinetic experiments with each stimulator. In addition, Lys-plasminogen (from American Diagnostica, Greenwich, CT) was used in some of the DESAFIB experiments to correspond to the conditions used by Madison et al. (1989, 1990). In order to determine the kinetics of plasminogen activation, various concentrations of plasminogen were incubated with various stimulators [1.1 μM final concentration, except for DESAFIB which was 0.074 μM to correspond to the concentration used previously with a different variant in the same region of t-PA (Madison et al., 1989, 1990)] and S-2251 (0.85 mM final concentration). The concentrations of plasminogen used were 0.9–19 μM for reactions in the absence of a stimulator and 0.8–8.4 μM for reactions in the presence of intact fibrinogen. Reactions in the presence of fibrinogen degraded with plasmin, or intact fibrin, or 50% fibrin contained 0.17–1.7 μM plasminogen. The reaction buffer was composed of 0.05 M Tris-HCl, 0.12 M NaCl, and 0.01% Tween 80, pH 7.4, and all reactions except for some of those containing DESAFIB were carried out at 25 °C in a total volume of 160 μL in microtiter plates (CoStar, catalogue no. 3596, Cambridge, MA). The reactions containing DESAFIB were in a total volume of 120 μL, and some reactions were carried out at 37 °C. The DESAFIB reaction with Glu-plasminogen at 25 °C used plasminogen concentrations ranging from 0.02 to 0.2 μM. After 2.5 h at room temperature, the reaction was initiated by the addition of t-PA or "KHRR" t-PA. In the cases where the stimulator was fibrin or 50%-degraded fibrin, thrombin was added simultaneously with the t-PA to allow for clot formation. Previous studies have shown that the final thrombin concentration used here (7.8 units/mL) was sufficient for the clots to be formed prior to the start of absorbance readings. Plasmin formation was monitored by its ability to hydrolyze S-2251 as measured by the increasing absorbance at 405 nm. When fibrin or degraded fibrin was used as the stimulator, the absorbance at 492 nm was used to correct for light scattering due to clot formation (Bennett et al., 1991). The data were analyzed according to the methods of Nieuwenhuizen et al. (1985) as described previously (Higgins et al., 1990). In all cases, the data from the initial rates of reaction were used, and no evidence of a lag was present. However, the final replots of the data were done using both Lineweaver–Burk analysis and Eadie–Scatchard plots. Although the data were similar, all results listed in Table I were the averages and standard deviations obtained from Eadie–Scatchard plots which tend to weigh the data more evenly.

Results

Both the KHRR to tetra-alanine form of t-PA and t-PA with the wild-type sequence (Activase alteplase) were tested for their ability to activate Glu-plasminogen in the absence or presence of various stimulators. The stimulators tested included those normally expected to be present in vivo (i.e., fibrinogen, fibrin, fibrinogen fragments formed by plasmin

digestion, and fibrin which had been slightly digested with plasmin). In order to rule out any possible differences based on the difference in expression system, the assays were run with both Activase alteplase, stably expressed in a Chinese hamster ovary (CHO) cell line, and wild-type sequence t-PA expressed in the same system as "KHRR" t-PA (transiently transfected 293 cells). In all cases, there was no significant difference in the kinetics with Activase alteplase and 293 cell expressed wild-type t-PA. The results listed below were obtained with Activase alteplase, hereafter referred to as wild-type t-PA. The raw data showed that the increase in absorbance at 405 nm was linear with time squared as would be expected from a coupled reaction, in which the activation of plasminogen to plasmin by t-PA is monitored with a synthetic substrate to plasmin. Replots of the velocity of the reaction with time squared (the γ function) in relation to the plasminogen concentration were done using both Eadie–Scatchard analyses and Lineweaver–Burk analyses. Because Eadie–Scatchard analyses weighed the rates (and their associated errors) obtained with the various concentrations of plasminogen more equally from a statistical viewpoint, this analysis was utilized to obtain the K_M and k_{cat} functions listed in Table I. In all cases, these numbers are the average of experiments run on at least 2 separate days with duplicate runs on each day. It is evident from these data that the two forms of t-PA demonstrated significant differences in their ability to activate plasminogen in the presence of stimulators. The rate of plasminogen activation in the absence of a stimulator is over 6-fold slower with "KHRR" t-PA than with wild-type t-PA primarily because the k_{cat} of the reaction is significantly lower. The same trend is observed in the presence of fibrinogen as a stimulator, or with plasmin-degraded fibrinogen. In all cases, the slower rate of plasminogen activation with "KHRR" t-PA is due to a decrease in the k_{cat} of the reaction. If the stimulator is either fibrin or fibrin which has been slightly degraded with plasmin (made from fibrinogen which is 50% clottable), the catalytic efficiency of "KHRR" t-PA is much more similar to that observed with wild-type t-PA. However, the analyses of the catalytic rate constants with these two stimulators indicate that both the k_{cat} and the K_M of the reactions are affected. With "KHRR" t-PA , both are raised significantly, but by similar amounts, compared to wild-type t-PA.

Because Madison et al. (1989, 1990) monitored the activity of t-PA variants in a similar region of the molecule using kinetics with DESAFIB as the stimulator and saw no difference when compared to wild-type t-PA, we tested wild-type t-PA and the "KHRR" t-PA variant under the conditions of their assay using Lys-plasminogen and DESAFIB at 37 °C. The only changes were that EDTA was omitted in the assay buffer and S-2251 rather than Spectrozyme PL was used as the plasmin substrate. We found that the rate of plasminogen activation was in all cases greater with "KHRR" t-PA than with wild-type t-PA. This was also found to be the case in assays run at 25 °C and regardless of whether Glu-plasminogen or Lys-plasminogen was used as the substrate.

In addition, the data in Table I demonstrate that the presence of a stimulator can alter the rate at which wild-type t-PA activates plasminogen by over 100-fold. The slowest rates are those in which no stimulator is present. However, there is still more than an order of magnitude difference between the rates with fibrinogen (the worst stimulator) and degraded fibrin (the best). In most cases, the increase in the rate of the reaction comes primarily from the K_M term, indicating that the interaction of the enzyme (t-PA) and substrate (plasminogen) occurs with greater ease with the stimulators more closely resembling fibrin. On the other hand, with "KHRR" t-PA, the difference in the K_M term is slightly less dramatic, and it is more than offset by k_{cat} terms which vary by over 100-fold.

Discussion

The kinetic analyses of plasminogen activation by t-PA described here demonstrate that the stimulator in the reaction can have a very significant effect on the reaction rate. The kinetic constants listed in Table I agree well with those obtained by others (Ranby, 1982; Hoylaerts et al., 1982; Nieuwenhuizen et al., 1985; Higgins et al., 1990; Jones & Meunier, 1990), in spite of slight differences in methodology between the studies. The catalytic efficiency of the reaction (k_{cat}/K_M) varies 200-fold when comparing the rates in the absence of a stimulator with the rate in the presence of the best stimulator, 50%-degraded fibrin. These changes are governed primarily by the K_M of the reaction. The k_{cat} of plasminogen activation in the presence of DESAFIB is considerably lower than in the presence of fibrin or degraded fibrin. This may be because of the lack of polymerization of the DESAFIB preparations. Suenson and Peterson (1986; Suenson et al., 1990) have previously shown the importance of polymerized fibrin to obtaining maximal stimulation.

With respect to the various types of stimulators studied here, 50%-degraded fibrin appears to favor most the interaction between wild-type t-PA and plasminogen as evidenced by the highest catalytic efficiency (k_{cat}/K_M). These data correspond to the results of others who demonstrated that t-PA-mediated fibrinolysis exhibited a kinetic transition with a correlation between a decreased K_M of the reaction and the appearance of slightly degraded fibrin (Norrman et al., 1985). Fibrin which has been degraded slightly with plasmin has been shown to possess additional binding sites with lower dissociation constants for both plasminogen (Suenson et al., 1984; Tran-Thang et al., 1984, 1986; Bok & Mangel, 1985; Harpel et al., 1985) and t-PA (Higgins & Vehar, 1987; DeVries et al., 1989). The slight decrease with K_M observed in the kinetics may be due to the improved interaction between plasminogen and t-PA when either the enzyme and/or the substrate are bound to these new sites. In addition, conformational changes may occur in one or both of these molecules upon binding that contribute to the reaction rate and increase the k_{cat}/K_M.

These results with degraded fibrin are also the most difficult to interpret. Although 50% of the fibrinogen is clotted through the action of thrombin, the remaining 50% of the fibrinogen (presumably degraded to fragments) remains in solution, leading to the possibility of two competing reactions. However, because the plasminogen-activating activity of t-PA is significantly slower in the presence of degraded fibrinogen, this reaction may not contribute significantly to the overall rate with 50% fibrin. Analysis of the data did not demonstrate any significant deviations from Michaelis–Menten kinetics.

A comparison of the kinetic constants obtained for the "KHRR" variant of t-PA with those for wild-type t-PA demonstrates that one or more of the basic residues in the loop of the t-PA protease from residues 296 to 302 strongly influence the kinetic profile exhibited by t-PA. Whereas the ratios of k_{cat}/K_M with no stimulator and the best stimulator vary ~200-fold with wild-type sequence t-PA, they vary ~1000-fold with the "KHRR" t-PA variant. With less than ideal stimulators (none, fibrinogen, and fibrinogen which had been degraded to small fragments with plasmin), replacing the basic residues with alanine caused a significant decrease in the k_{cat} of the reaction and little change in the K_M. It appears that the t-PA/plasminogen/stimulator complex forms

equally well with "KHRR" and wild-type t-PA in the presence of these stimulators but the "KHRR" t-PA in the complex is not as effective in hydrolyzing the R561–V562 plasminogen bond as is wild-type t-PA. Unlike wild-type t-PA which shows little, if any, stimulator-dependent changes in k_{cat}, the k_{cat} of "KHRR" t-PA increases 100-fold between the absence of a stimulator and the best stimulator (slightly degraded fibrin). Although the catalytic efficiencies (k_{cat}/K_M) of "KHRR" t-PA and wild-type t-PA in the presence of fibrin or degraded fibrin are similar, the absolute rate obtained is very dependent on the concentration of plasminogen. Using the observed kinetic constants, it can be calculated that with fibrin as the stimulator, "KHRR" t-PA will have a significantly superior rate of plasminogen activation at Glu-plasminogen concentrations about 1 μM and above, while with degraded fibrin as the stimulator, the rate of plasminogen activation by "KHRR" t-PA will be higher than that observed for wild-type t-PA at plasminogen concentrations greater than 0.23 μM. Since the physiological concentration of plasminogen is $\sim$1 μM and may be even higher on the clot surface, one would predict that "KHRR" t-PA would demonstrate a rate of plasminogen activation superior to that of wild-type t-PA in vivo.

The data with DESAFIB as the stimulator contrast with those of Madison and co-workers (Madison et al., 1989, 1990), who noted that other variants in the same region do not significantly affect the kinetic constants of the reaction. The kinetic constants described here predict a slightly higher rate of plasminogen activation with "KHRR" t-PA at all plasminogen concentrations. It is unlikely that "KHRR" t-PA is a unique variant in this region, since several other variants in the 296–299 region also demonstrate increased activity with fibrin and decreased activity with fibrinogen when compared to wild-type t-PA (N. Paoni and W. Bennett, unpublished results). We have not, however, tested the variants described by Madison and co-workers (Madison et al., 1989, 1990) and cannot exclude differences between variants.

It is clear from these data that the "KHRR" variant of t-PA has several properties which may lead to an improvement as a thrombolytic agent. The basal level of activity in the presence of normal circulating fibrinogen is significantly lower than that of wild-type t-PA, whereas in the presence of fibrin it shows more activity at physiological concentrations of plasminogen. Ideally, these in vitro results will translate into less systemic activation and fibrinogen breakdown, yet more rapid lysis at the site of a thrombi. Given the difficulty in using in vitro experiments to predict results in plasma, in whole blood, or in animals, in vivo experiments are required to test this hypothesis.

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