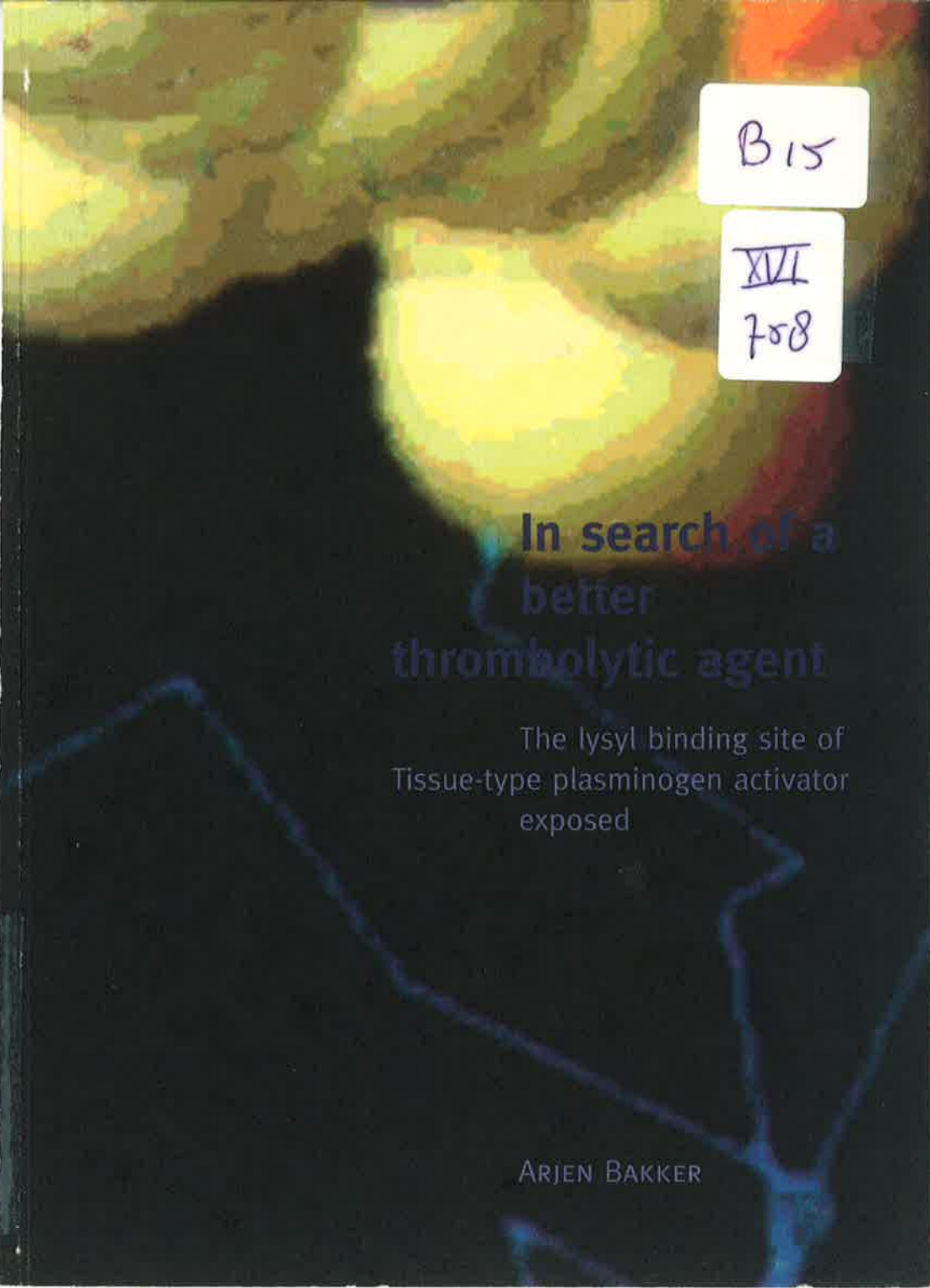

In search of a better thrombolytic agent

The lysyl binding site of Tissue-type plasminogen activator exposed

Arjen Bakker

IN SEARCH OF A BETTER THROMBOLYTIC AGENT:

THE LYSYL BINDING SITE OF TISSUE-TYPE PLASMINOGEN ACTIVATOR EXPOSED

Stellingen behorende bij het proefschrift
In search of a better thrombolytic agent: the lysyl binding site of tissue-type plasminogen activator exposed.
door Arjen Harmen Franciscus Bakker.

1. De hypothese, dat de structureel-autonome domeinen van weefsel-type plasminogeen activator ook functioneel-autonoom zijn, wordt steeds onwaarschijnlijker (dit proefschrift).

2. Dat de binding van weefsel-type plasminogeen activator aan een zich vormend fibrine netwerk verstoord kan worden door de lysine analoog 6-aminohexaanzuur, betekent nog niet dat fibrine binding verloopt via de lysyl bindingsplaats van tissue-type plasminogen activator en een lysine residu van het fibrine netwerk (dit proefschrift).

3. De fibrine afhankelijke plasminogeen activatie van weefsel-type plasminogeen activator wordt slechts gedeeltelijk verklaard door binding van weefsel-type plasminogeen activator aan fibrine (dit proefschrift).

4. Novokhatny en medewerkers gaan te gemakkelijk voorbij aan de mogelijkheid dat de oplosbaarheid van weefsel-type plasminogeen activator afhankelijk kan zijn van zijn conformatie. (Novokhatny, V.V., Ingham, K. C., and Medved, L. V. (1991) J. Biol. Chem. 266, 12994 - 13002).

5. Wanneer een scatchard analyse niet resulteert in een rechte betekent dit, dat aan de uitgangspunten van deze analyse niet wordt voldaan (Nesheim, M., Fredenburgh, J. C., and Larsen, G. R. (1990) J. Biol. Chem. 265, 21541 - 21548). Na de constatering dat hun scatchard analyse geen rechte oplevert, kiezen Nesheim en medewerkers dan ook ten onrechte voor onafhankelijke niet-equivalente bindingsplaatsen.

6. Door de mogelijke in-trans beïnvloeding is het niet mogelijk om op basis van moleculaire compositie goede promotoren van slechte promotoren te onderscheiden.

7. De waarneming, dat aan het CD28 respons element, aanwezig in het promotor/ enhancer gebied van het IL-2 gen, een eiwit bindt, dat beter verdrongen wordt door het HIV κB element dan door het CD28 respons element zelf, wil nog niet zeggen dat dit eiwit beter bindt aan het HIV κB element (Verweij, L.C., Geerts, M., and Aarden, L. C. (1991) J. Biol. Chem. 266, 14179 - 14182)

8. Het vastleggen van biologische units zoals de kallikreine Unit: "one kallikrein unit is equal to that quantity of kallikrein that, on intravenous injection into dogs, produces the same effect on blood pressure as 5 ml of human urine from healthy subjects" (Ogsten, D. (1984) Antifibrinolytic

drugs p. 52) heeft veel overeenkomst met de oude verlaten gewoonte om de afmeting van een lichaamsdeel als lengte-eenheid te gebruiken.

9. In tegenstelling tot in het verkeer zijn "ongelijkvloerse" kruisingen in de natuur vaak doodlopende wegen.

10. En toch kunnen mannen kinderen krijgen (Statistisch jaarboek (1994) bladzijde 500, tabel 26, bevallingen en complicaties van zwangerschap....3 mannen).

11. Als morfogenetische velden bestaan dan zouden deze velden overgedragen kunnen worden door morfonen, die voorgesteld kunnen worden als snel rondraaiende vectoren waarvan het kwadraat van de vectorlengte de waarschijnlijkheid aangeeft om morfonen op een bepaalde plek te vinden.

12. Gezien het huidige "hulpcircuit" rond de werkeloze, zal opheffing van de werkeloosheid alleen maar resulteren in meer werkeloosheid.

13. De manier waarop een vooraanstaand team van vulkanologen de eigen hypothese weerlegd heeft, zou meer navolging moeten krijgen binnen de wetenschap (Nature (1993) 361, 193).

14. Francis Crick promoveerde op 35 jarige leeftijd, Johannes Diderik van der Waals legde zijn doctoraal examen op 34 jarige leeftijd af. Tegenwoordig zouden zij binnen het wetenschappelijk bedrijf hoogstens als "subtoppers" gekwalificeerd worden.

15. Als de huidige ontwikkeling in vocht absorberende luiers zich voortzet, dan wordt het in de toekomst noodzakelijk babies te voorzien van vochtinbrengende mutsjes, om uitdroging te voorkomen.

IN SEARCH OF A BETTER THROMBOLYTIC AGENT:

THE LYSYL BINDING SITE OF TISSUE-TYPE PLASMINOGEN ACTIVATOR EXPOSED

Proefschrift

ter verkrijging van de graad van Doctor
aan de Rijksuniversiteit te Leiden,
op gezag van de Rector Magnificus Dr. L. Leertouwer,
hoogleraar in de faculteit der Godgeleerdheid,
volgens besluit van het College van Dekanen
te verdedigen op donderdag 29 juni 1995
te klokke 14.15 uur

door

Arjen Harmen Franciscus Bakker

geboren te Den Haag in 1960

Promotiecommissie:

Promotor:	Prof. dr. P. Brakman
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Front cover shows part of the lysyl binding site of Tissue-type plasminogen activator at low resolution. Design by Marieke Griffioen, Moniek Mulder en Arjen Bakker.

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aan Ellen
voor Moniek en
na Tietje

Contents

Chapter 1

General Introduction

1. Introduction

Thrombosis, the unwanted presence of fibrin clots in veins and arteries, is still a major health threat in Western society. A fibrin clot, within a blood vessel, will hamper the blood flow, and deprive downstream tissue of O_2. Depending on the duration of this deprivation extensive tissue necrosis occurs and life itself may be jeopardized. Therefore a therapy to remove such a fibrin clot swiftly and specifically, would be of great therapeutic value.
The knowledge that the body possesses a system which selectively degrades fibrin clots (= fibrinolytic system), initiated the search for clot degrading therapies (= thrombolytic therapy), based on this system.
The fibrinolytic system consists of at least three components 1- a proenzyme plasminogen (PLG), 2 - activators like urokinase-type plasminogen activator (u-PA) and tissue-type plasminogen activator (t-PA), which transform plasminogen into the fibrin degrading enzyme plasmin (PLM) and 3 - inhibitors like $\alpha 2$ plasmin inhibitor ($\alpha 2$-PI) , and plasminogen activator inhibitors (PAI 1 , PAI 2 and PAI 3), which limit the activity of this enzyme system.
Thrombolytic therapy today makes use of plasminogen activators. One of the problems encountered during thrombolytic therapy, is that the plasmin generation is not localized to the fibrin clot. Since plasmin is an enzyme with broad specificity, too much plasmin formed in the blood circulation will degrade other blood proteins, like fibrinogen. Degradation of fibrinogen will affect the fibrin formation and predispose the system to bleeding (= lytic state), also creating a potentially life-threatening problem. Therefore considerable interest still exists in the development of thrombolytic agents which will not induce a lytic state.
This introduction describes how the fibrinolytic system establishes fibrin specificity under physiological conditions. It focuses on the plasminogen activators t-PA and u-PA, and their role in the localisation of the plasmin(ogen) activity towards the fibrin clot. From these observations, a strategy evolved, potentially useful for the construction of a new thrombolytic agent. This thrombolytic agent combines the positive features of existing thrombolytic agents t-PA (fibrin specificity) and u-PA (proenzyme characteristic).

2. From fibrinogen to fibrin.

When blood is isolated, without any precautions, it transforms from a liquid into a gel, the blood clot. This phenomenon could be defined as ex vivo thrombosis since it is thought that similar processes are responsible for thrombosis within the blood vessel. According to Blombäck it was Malpighi who in 1686 isolated a white fibrous substance from a blood clot, through washing it with water, and called it fibrin (Blombäck, 1967) In 1847, Virchow (Virchow, 1847) proposed that fibrin is present in a soluble form in blood and named the precursor fibrinogen. Hammarsten and Dennis de Commercy unequivocally demonstrated the existence of fibrinogen by its purification. Hammarsten felt that for the transformation of a highly purified fibrinogen preparation into a fibrin gel, only the presence of one protein in the "fibrin ferment" was necessary. Furthermore, he noticed the presence of soluble proteinaceous substances in the fluid after the transformation of the fibrinogen to fibrin and proposed that these were hydrolytic cleavage products of fibrinogen (Blombäck, 1967). In 1945 Laki and Mommaerts (Laki and Mommaerts, 1945) extended the view of Hammarsten. They proposed that the fibrin formation was a two step reaction. First a hydrolytic cleavage of fibrinogen is necessary to form "self-assembly units" which arrange themselves in a fibrin network. Basically this is still the present day view of "thrombus" formation.

In the presence of thrombin (the "fibrin ferment" of Hammarsten) two pairs of short peptides are cleaved from fibrinogen resulting in the formation of a fibrin monomer, which spontaneously forms a gel-like structure, the fibrin clot (figure 1). The two short peptides (the proteinaceous product of Hammarsten) can be recovered from the fluid phase.

The arrangement of the fibrin monomers determine the final structural characteristics of the fibrin network. Variable conditions during fibrin monomer assembly, will result in fibrin networks with different physical (accessibility) and chemical properties (creation of new interaction sites, review Furlan, 1988). Upon polymerisation new sites within the fibrin network are revealed by a conformational change of the fibrin(ogen) molecule. But new sites are also created by the new contacts between different fibrin

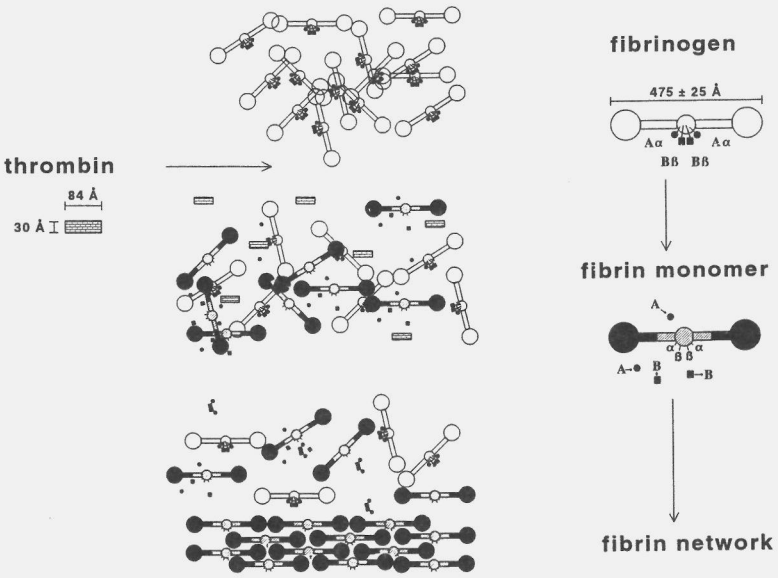


figure 1: Formation of the fibrin network

Fibrinogen consists of six polypeptide chains, two α chains of 625 amino acid residues, two β chains of 461 residues and 2 γ chains of 427 aminoacid residues. One half fibrinogen molecule consists of one α one β and one γ chain The α chain is linked to the β via 1 disulphide bond. The α chain is linked to the γ chain via 4 disulphide bonds. Two half fibrinogen molecules are linked together by 1 disulphide bond between the α chains and two disulphide bonds between the γ chains. Based on electron microscopy studies a low resolution model of the fibrin molecule was proposed by Hall and Slayter. The enzyme thrombin preferentially hydrolysis R - G peptide bonds from the amino terminus of the α and β chain respectively. Four peptides are released. Two A peptides after hydrolyses of the Aα16R-17G and two B peptide after hydrolysis of the Bβ14R-15G. These peptides are referred to as fibrino peptides A (ADSGEGDFLAEGGVR) and fibrino peptides B (<EGVNDNEEGFFSAR). The formed fibrin monomers spontaneously arrange themselves in a fibrin network. Thrombin not only activates fibrinogen but also another enzyme transglutaminase factor XIII. This enzyme covalently links two γ chains of different fibrin monomers together via a ϵ (γ glutamyl) lysyl bond formation, creating a rigid network. The transformation of fibrinogen to fibrin results in the expression of " new binding sites " which are thought to play an important role in the initiation of fibrinolysis.

monomers. Additional modification of the fibrin network such as covalent crosslinking of the fibrin monomers (by the thrombin activated transglutaminase (FXIII)) also gives rise to new binding sites in the fibrin gel. These new sites are believed to play an important role in the initiation of fibrinolysis.

3. Plasminogen.

3.a. Short history.

Konttinen (Konttinen, 1968), who thoroughly described the history of fibrinolysis, claims that the members of the Hippocratic school (4th century BC) had already noticed the fluidity of post mortem blood. Later on, in 1761 Morgagni described the slow change of blood from a gel into a liquid state. The speed of lysis of "solid" blood depended on the manner of death. Suffocation was always followed by intense lysis. The longer the deprivation of the oxygen before cessation of blood circulation the more rapid lysis was. In 1906 Morawitz noticed that mixing this post-mortem liquefied blood with normal blood destroyed the coagulation property of this normal blood. Once the post mortem blood was reliquefied it could not be clotted again. From the unclottability Dastre, inferred that the fibrin was digested instead of being simply redissolved, although he did not yet presume the existence of a specific fibrinolytic enzyme. It was probably Schmitz who suggested (Schmitz, 1936), that the destruction of fibrin is caused by the enzyme plasma-trypsin. In 1945 (Christensen and MacLeod, 1945) the term plasmin was proposed for the fibrin degrading enzyme in conformity with common usage of proteases, where the prefix indicates the source of the enzyme. By analogy with trypsin, the precursor of plasmin was named plasminogen and the inhibitor of plasmin was named antiplasmin, nowadays α_2 Plasmin Inhibitor. In 1953, plasminogen the inactive precursor was isolated in crystalline form by Kline (Kline, 1953). In 1970, Deutsch and Mertz introduced a simple purification method (Deutsch and Mertz, 1970).

3.a.1. Structural analysis

Plasminogen (figure 2) is a glycoprotein of 791 aminoacid residues secreted by the liver. The plasma concentration is about

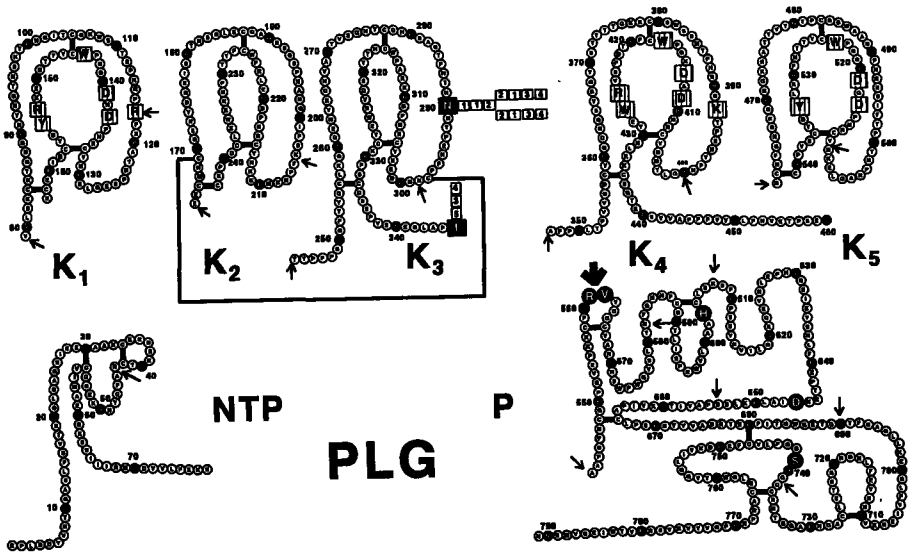


Figure 2: structure of plasmin(ogen)

Plasmin(ogen) (PLG) consists of seven domains from the N-terminus, the N-terminal peptide (NTP), five homologous kringle domains (K1-K5) and a protease domain (P). The small arrows correspond with the exon-intron boundaries in the gene. Plasmin(ogen) is a glycoprotein and contains 0-linked as well as N-linked oligosaccharide. The 0-linked carbohydrate chain is always present on residue 346 whereas a N-linked carbohydrate structure can be present on residue 290 leading to two different plasminogen forms designated plg I containing carbohydrate on residue 290 and plg II lacking this carbohydrate. The various glycosylation sites are indicated by a filled square and the carbohydrates attached by open numbered squares. 1=glcNAc, 2=man,3=gal, 4=NANA, 5=galNAc, 6=fuc The given primary carbohydrate structure is highly variable. The structure shown must therefore be considered a possible primary structure. The active site residues serine, aspartic acid and histidine are indicated by black filled circles. The sites of hydrolysis leading to formation of plasmin is indicated by a large arrow. The amino acid residues involved in the lysyl binding of the various kringles are represented by hatched boxes.

2 μM. Its molecular weight varies between 90000 -94000. At the present time the three dimensional structure at atomic resolution has not been established. A model of the three dimensional structure, at low resolution, of plasminogen has been proposed by Ponting et al (see page 20).
Several groups have participated in elucidating the primary structure of human plasminogen (Sottrup-Jensen et al. 1975; Sottrup-Jensen et al., 1978; Wiman and Wallén, 1975 ; Wiman, 1977). The aminoacid sequence reported was confirmed (one additional I between I^{65} and I^{67} was found) by c-DNA cloning (Malinowski et al., 1984, Forsgren et al., 1987) and genomic cloning (Petersen et al., 1990).
Determination of the primary structure of plasminogen revealed that plasminogen consists of five structural domains. From the amino - terminus 5 kringle domains can be discerned (K1, K2, K3, K4 and K5) which have high homology with the two kringle domains of prothrombin (Magnusson et al., 1975). The protease part (P) of plasminogen resembles trypsin. Plasminogen is referred to as K1K2K3K4K5P.
Several molecular forms of plasmino(gen) have been described. The conversion of the single chain peptide into a two chain peptide occurs by hydrolysis of the R^{561}-V^{562} bond. The resultant plasmin contains a heavy chain (E^{1}-R^{561}) linked by two disulphide bonds, most likely C^{548}-C^{666} and C^{558}-C^{566} to a light chain (V^{562}-N^{791}). Plasmin, as described above, is highly labile and undergoes auto-catalytic cleavage of the R^{68}-M^{69}, K^{77}-K^{78}, or K^{78}-V^{79} peptide bonds in the amino-terminus of the heavy chain (Wallén and Wiman 1972 ; Sottrup-Jensen et al., 1978). Differential glycosylation result in two forms of plasmin(ogen). Glu-plasminogen I (glu I) has an O-linked carbohydrate on T^{346} and an N linked carbohydrate on N^{288}. Glu-plasminogen II (glu II) only has the O linked carbohydrate. When analyzed on isoelectric
focusing every glycosylation consists of six additional forms (Collen, 1975).

3.a.2. Degradation of a fibrin clot.

In the presence of plasmin several peptide bonds (there are 29 identified plasmin cleavage sites in fibrin(ogen) of which 12 R-x and 17 K-x , Furlan, 1988) of fibrin(ogen) are hydrolyzed,

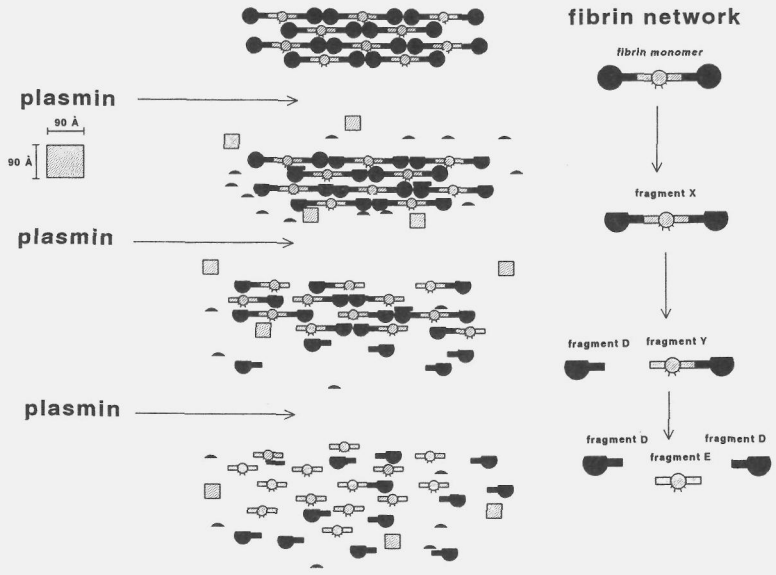


Figure 3: Degradation of fibrin(ogen) by plasmin.

The degradation of the fibrin surface by plasmin is a highly regulated process. There are 29 plasmin digestion sites within a fibrin(ogen) molecule. Plasmin digestion will result in 17 carboxyl terminal lysyl residues and 12 carboxyl terminal arginyl residues. But not all sites are equally accessible. The plasmin most sensitive sites are found on the carboxyl terminal part of the α chain. The resulting fragment which is formed after release of these protein fragments is referred to as fragment X. This fragment X, is subsequently degraded by plasmin into a fragment Y and a fragment D. Fragment Y is further processed to another fragment D and a fragment E.
The heterogeneity of the fragments generated during plasmin digestion is large. For instance, at least three fragments E have been characterised : E_1 G-α17 to K-α78, G-β15 to K-β121 and Y-γ1 to K-γ62, E_2: G-α17 to K-α78, K-β54 to L-β120 and Y-γ1 to K-γ62, E_3: V-α20 to K-α78, K-β54 to L-β120 and Y-γ1 to K-γ53. Crosslinking in the gamma chain by transglutaminase results in the formation of DD fragments. This fragment binds tightly to the fragments E1 and E2 forming DDE complexes.

resulting in destruction of the fibrin network or fibrin(ogen). The degradation of fibrin(ogen) or a non cross linked fibrin clot, is a

highly regulated process (figure 3). First the carboxyl-terminus of the α chain is digested at several places, giving rise to a mixture of products called X (240 kD - 320 kD). Next the fragment X is cleaved by plasmin and splits into the fragment Y (150 kD) and fragment D (70 kD - 95 kD). Fragment Y is also digested by plasmin, resulting in another fragment D and a fragment E (80 kD). Incubation of plasmin with covalently cross linked fibrin results in other plasmin degradation products, since two or more fibrin monomers are covalently linked together (for example: D dimers and DDE).

3.a.3. Localisation towards the fibrin clot.

In principle plasmin has no preference for fibrin, and in vitro fibrin(ogen), the precursor of fibrin, is also a good substrate. In vivo, however, plasmin activity is highly fibrin specific. For the localisation of plasmin activity towards the fibrin surface the presence of the plasmin inhibitor (α_2 plasmin inhibitor) is essential. (Collen, 1976 ; Moroi and Aoki, 1976: Müllertz and Clemmensen, 1976). A simple experiment demonstrated this fact. Incubation of plasmin (0.7 μM) α_2 plasmin inhibitor (1 μM) and fibrinogen (6 μM) with thrombin resulted in a stable fibrin clot, while in the presence of equal amounts of plasmin without plasmin inhibitor the fibrin was lysed within 1 minute (Wiman and Collen, 1978).
The authors suggested that α_2 plasmin inhibitor limits the activity of plasmin in the blood to the fibrin surface. Fibrin-bound plasmin is relatively protected against inactivation while plasmin in solution is rapidly inactivated. The K_d between plasmin and α_2 - PI is 2×10^{-10} M, and based on the kinetic constants the half life of free plasmin in circulation was calculated to be between $25 - 30 \times 10^{-3}$ sec. (Wiman and Collen, 1978; Wiman et al., 1979).
Another way to localise plasmin activity towards the fibrin surface is to form plasmin on the fibrin surface. Theoretically, this may be achieved by the activation of fibrin bound plasminogen by a plasminogen activator as suggested by Alkjaersig et al.(Alkjaersig et al., 1959). However, a consistent description of the molecular interactions between a fibrin clot and glu-plasminogen can not be given. In vitro, association of glu-plasminogen with a forming fibrin clot, (Wu et al., 1990) pre-formed fibrin (Suenson and

Thorsen, 1981; Bok and Mangel, 1985) and fibrin degradation products (Lucas et al., 1983; Cederholm-Williams, 1981) has been reported. On the other hand, several authors did not observe binding of glu-plasminogen to a forming fibrin clot (Nesheim et al., 1990) or preformed fibrin (Shah and Dhall, 1983) preformed non cross linked fibrin or to fibrin degradation products (Lucas et al., 1983). In vivo, no association of glu-plasminogen to fibrin clots has been observed (Juhan-Vague et al., 1981). The amount of plasminogen in serum before and after clot formation is not significantly changed but on this point also disagreement exists (Hedner et al., 1966; Fantl, 1962; Rakoczi et al., 1978). Since the system under study is highly variable, it seems likely that experimental conditions used to study the fibrin binding of glu-plasminogen have a profound influence on the final outcome. The variation in experimental procedures, of which some are listed below, could affect the observation. 1- condition used to transform fibrinogen into fibrin (structure of the fibrin clot) 2 - the use of a preformed fibrin clot versus a forming fibrin clot (transiently expressed binding sites, cross-linked versus non-cross-linked) 3 - the use of plasminogen-free fibrin(ogen) versus fibrinogen containing plasmin(ogen) (competition) 4 - the system used to study binding (perfusion versus reaction vessel) 5 - prevention of the modification of the fibrin clot (generation of carboxyl terminal lysyl residues) by plasmin, 6 - the plasminogen used (glu-plasminogen versus lys-plasminogen and glu plasminogen I versus glu-plasminogen II), 7 - the method of detection of the bound glu-plasminogen (direct with labelled plasminogen versus indirect determination of plasmin).

Several investigators hypothesized, on the basis of competition experiments with lysine analogues like ϵ amino caproic acid (EACA), that lysyl mediated interactions are involved in the interaction of plasminogen with fibrin (Landman 1973; Thorsen, 1975; Wiman and Wallén, 1977; Rackoczi et al., 1976). The localisation of fibrin binding sites in the plasminogen molecule was therefore similar to the localisation of the lysyl binding sites. Sottrup-Jensen suggested in 1978 that the lysyl binding sites of plasmin(ogen) were probably localised in the kringle domains (Sottrup-Jensen et al., 1978). Markus and coworkers using elastase digestion products of glu-plasminogen, localised one high affinity (K_a = 852 mM^{-1} (K_d = 1.2 μM)) and one low affinity lysyl

binding site in the K1-K3 portion of the heavy chain. Furthermore the K4 contained a lower affinity (K_a = 158 mM^{-1} (K_d = 6.3 μM)) lysyl binding site. In the K5P part of plasminogen no lysyl binding site was observed (Markus et al., 1982) However, Christensen did describe a lysyl binding site in the K5P part of plasminogen. The binding of the K5P to aminohexyl Sepharose (comparable with an intra chain lysyl residue within a protein molecule) was weak (K_d = 24 μM (Christensen, 1984). Fluorescence titration studies with the K5P and lysine analogue EACA, confirmed her observations (K_d = 64 μM, Novokhatny et al., 1989). At the present time the lysyl binding site in the K1 (Hoover et al., 1993), the K4 (Mulichak et al., 1991; Wu et al., 1991) and the K5 (Thewes et al., 1990) have been functionally as well as structurally extensivly analyzed. (see references and cross-references).

Several authors have studied the binding of glu-plasminogen fragments to fibrin(ogen) and fibrin fragment. All agree on binding of lys-plasminogen (the plasmin modified form of glu-plasminogen) to fibrin although there is no consensus on the stoichiometry. (Bok and Mangel, 1985; Lucas et al., 1983). In contrast, no agreement exists on the binding of the K5P fragment to fibrin. Cederholm-Williams studied the binding of plasminogen fragments (K123, K4 and K5P) to fibrinogen, fibrin and fibrin α chain linked to Sepharose. No binding of K5P was observed, while the amino terminal fragment K1K2K3 and K4 were retained on the column and could be eluted with EACA.(Cederholm-Williams, 1981). Wu and coworkers showed that K5P did interact with fibrin (They only used plasminogen form II). Furthermore isolated K1K2K3K4K5 was an efficient competitor of fibrin binding of glu-plasminogen, lys-plasminogen and K5P while K1K2K3 was not. EACA (concentration around 0.5 mM) and L-lysine (concentration around 10-50 mM) inhibited the binding of K5P, glu-plasminogen and lys-plasminogen to a forming fibrin clot. Since both K1K2K3 and K1K2K3K4K5 bound to a forming fibrin clot at least two plasmin(ogen) binding sites must exist (Wu et al.,1990). It is difficult to state unequivocally that the lysyl binding sites in glu-plasminogen are directly involved in fibrin binding. Glu-plasminogen is a highly "flexible" molecule. It has been suggested by several authors, that the binding of EACA to glu-plasminogen induces a conformational change in glu-plasminogen (Collen, 1975;

Markus et al., 1978; Markus et al., 1979; Christensen, 1984). This change in conformation could result in the disappearance of previously exposed fibrin binding sites of plasmin(ogen). Therefore competition by EACA in fibrin binding experiments could be the result of a conformationally changed molecule.

3.a.4. Proposed three dimensional structure

Recently Ponting and coworkers proposed that native glu-plasminogen can adopt three dissimilar conformations (Ponting et al., 1992). The lysyl binding sites in the K5 and the K4 domain are hypothesized to play an important role in the stabilisation of these conformations. The α conformation, a compact spiral form containing intramolecular interactions between NTP (amino-terminal peptide, probably residue K^{51} (Urano et al., 1991)), and the K5 domain and between the K1K2K3 part of plasminogen and the K4 domain. The β form only contains the intra-molecular interaction between the K1K2K3 and the K4 (which of the lysyl residue involved is not known), In the γ form in both the K1K2K3-K4 and the NTP-K5 interactions are disturbed, resulting in a open conformation. The effects of ligands like EACA on the conformation of glu-plasminogen are the result of a competition between EACA molecules and lysyl residues within plasminogen for the lysyl binding sites. It is believed that in vivo also these conformations play an important role in the initiation and prolongation of fibrinolysis. The initial binding of glu-plasminogen to a fibrin surface is mediated via the K5 domain and an inter chain lysyl residue of the fibrin network. The binding of glu-plasminogen to a partially degraded fibrin network is mediated via the K4 domain and a carboxyl-terminal lysyl residue of the fibrin network.

3.a.5. Role of plasmin(ogen) in fibrinolysis

Localisation of the plasmin activity towards the fibrin surface is achieved by the rapid inactivation of fluid phase plasmin. Furthermore, the activation of fibrin bound glu-plasminogen could also result in the localisation of plasmin activity towards the fibrin clot. However, binding of glu-plasminogen to an intact fibrin clot is low or absent. In contrast, fragments of glu-plasminogen bind to fragments of fibrin(ogen) and this interaction is disturbed by lysine

analogues. This indicates that fibrin binding sites in plasminogen are present, and possibly identical with the lysyl binding sites. However, these fibrin/lysyl binding sites are not freely accessible in the glu-plasminogen molecule. This is in agreement with the proposed structural model of glu-plasminogen, where the lysyl binding sites play an important role in stabilisation of conformations.

4. The plasminogen activators.

Chesterman and coworkers, (Chesterman et al., 1972) proposed that it were the plasminogen activators that localized the plasminogen activation towards the fibrin clot. In man, two plasminogen activators, urokinase-type plasminogen activator and tissue-type plasminogen activator are known at the present time.

4.a. Urokinase-type plasminogen activator.

4.a.1. A short history

In 1947 Macfarlane and Pilling described fibrin degrading activity in urine. 5 years later Sobel and coworkers showed that it was the presence of a plasminogen activator in urine that was responsible for fibrinolysis. (MacFarlane and Pilling, 1947; Sobel et al., 1952). However purification of urokinase was difficult and time consuming (Pye et al., 1977). Its concentration in urine is low and vary considerably. One of the more successful approaches to isolate u-PA from urine was concentrating the protein by shaking and collecting the foam. (Celander et al., 1955). In 1965 Lesuk and coworkers prepared crystalline urokinase (Lesuk et al., 1965). Central in the history of urokinase stand Bernik and Nolan and coworkers (Bernik, 1973; Nolan et al., 1977), who found that the fibrinolytic activity of urokinase produced by kidney cells was increased after the addition of proteolytic enzymes, indicating that u-PA is an proenzyme. This result was confirmed by others. (Wun et al., 1982; Nielsen et al., 1982)

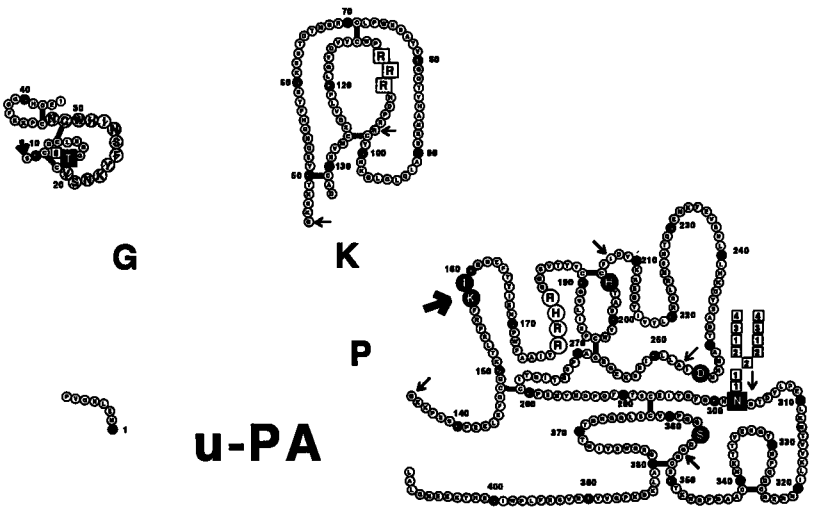


Figure 4: the structure of u-PA.

Urokinase-type plasminogen activator (u-PA) consists apart from a small N-terminal peptide (N) of three major domains a growth factor like domain (G), a single kringle domain (K) and a protease domain (P). In urokinase an N-linked carbohydrate chain is present on residue 302 and a single 0-linked fucose on residue 18. The various glycosylation sites are indicated by a filled square and the carbohydrates attached by open numbered squares. 1=glcNAc, 2=man, 3=gal, 4=NANA, 5=galNAc. 6=fuc. The given primary carbohydrate structure is highly variable, the structure shown must therefore be considered as a possible primary structure. The active site residues serine, aspartic acid and histidine are indicated by black filled circles. The site of hydrolysis leading to formation of active-two chain u-PA is indicated by a large arrow. A heparin binding site in u-PA is indicated by large open boxes. The residues involved in the interaction of u-PA with its receptor are indicated by large hatched circles. The secondary interaction sites of u-PA with PAI-1 are indicated by large circles.

4.a.2. Structural analysis

Urokinase (figure 4) is a serine protease. It is a glycoprotein of 411 aminoacids, predominantly synthesised in the kidney. The molecular weight of u-PA is about 54 kD. At the present time no three dimensional structure at atomic resolution has been established. The concentration in blood is approximately 65 $\pm$ 25 pM (Tissot et al., 1982; Binnema et al., 1986). In 1982 the primary structure of human urokinase was determined (Günzler et al., 1982 ; Steffens et al., 1982) The amino acid sequence was confirmed by c-DNA cloning (Verde et al., 1984) and by genomic cloning (Riccio et al., 1985).
After the determination of the primary structure it became clear that u-PA consists of a domain like structure. From the amino terminus three domains may be discerned; a growth factor domain (G) with homologies to the epidermal growth factor, a kringle domain (K) with homologies to kringle structures in plasminogen and a protease domain (P) having considerable homology having other serine-proteinases. The protein is referred to as GKP.
Several molecular forms of u-PA have been described. The first originates from the cleavage of the single chain high molecular weight form (54 kD) of urokinase by plasmin at position K^{158}-I^{159} to form a two chain enzyme. The tcu-PA chains are believed to be held together by a single disulphide bond between C^{148}-C^{279}. This two chain form of u-PA has a much higher reactivity to chromogenic substrates than single chain u-PA . The 54 kD single chain form does not react with diisopropyfluorophosphate and glutamyl-glycyl-arginyl-chloromethyl-ketone. Furthermore scu-PA is stable in plasma and does not form SDS stable scu-PA inhibitor complexes (Gurewich et al., 1984; Pannell and Gurewich 1986). The limited proteolytic digestion in the linker peptide at position K^{135}-K^{136} by plasmin releases the amino-terminal part of the protein, resulting in low molecular weight tcu-PA or scu-PA (33 kD, LMW) Another molecular form is the result of the proteolytic degradation of the R^{156}-F^{157} bond by thrombin (Conforti and Loskutoff, 1985; Ichinose et al., 1986; Gurewich and Pannell 1987). The inactive LMW u-PA, thus created can be reactivated with cathepsin C. The two-chain high molecular weight u-PA form found in urine has been shown to have the F^{157} residue as carboxyl terminus. (Steffens et al., 1982). The K^{158} residue is missing, probably due to the

action of a carboxypeptidase B in urine.

4.a.3. Localisation towards the fibrin clot.

In plasma single chain urokinase-type plasminogen activator (scu-PA) is stable and does not activate plasminogen (Gurewich and Pannell, 1987). In the presence of a fibrin clot in plasma, scu-PA induces the generation of plasmin on the fibrin clot. In contrast, tcu-PA will not induce clot lysis (Gurewich et al., 1984; Zamarron et al., 1984). The localisation of plasmin activity towards the fibrin surface of scu-PA is not accomplished by direct binding of scu-PA to the fibrin clot. Single chain u-PA (scu-PA) nor two chain urokinase-type plasminogen activator (tcu-PA) does interact with fibrin in a purified system. (Pannell et al., 1990; Lijnen et al., 1986; Nelles et al., 1987). The observation that scu-PA (5 nM) induces fibrin clot lysis in citrated plasma, with a lag phase (less the 10 % of the clot was lysed in the first 90 minutes), suggested that first plasmin had to be available to activate scu-PA to tcu-PA. The latter could efficiently activate plasminogen in the vicinity of the fibrin clot. Several investigators reported that scu-PA is a pro-enzyme and that it first had to be converted to tcu-PA by limited proteolysis. (Wun etal., 1982; Kasai et al., 1985; Petersen et al., 1988; Husain, 1991;). However, Lijnen and coworkers showed that scu-PA can convert plasminogen to plasmin. This reaction obeys Michaelis-Menten kinetics with K_M = 0.83 μM and k_{cat} = 0.0009 s^{-1} (substrate plasminogen: 0.125 - 1 μM with 25 nM of scu-PA). The analysis is complicated by the presence of modification reactions i.e. the formation of tcu-PA by the generated plasmin. Nelles and coworkers (Nelles et al., 1987) observed, that an urokinase mutant which no longer could be converted to the two chain form (scu-PA(K158G) or scu-PA(K158E)) still possessed plasminogen activating ability. The kinetic constants found for scu-PA(K158G) (K_M = 62 μM, k_{cat}= 0.011 s^{-1}) for scu-PA(K158E) (K_M = 87 μM, K_{cat}= 0.011 s^{-1}) and for scu-PA (K_M = 0.8 μM, k_{cat}= 0.002 s^{-1}), led to the hypothesis that single chain u-PA is in fact a plasminogen activator, although with large K_M value. This high K_M is interesting, since the lysyl binding sites in plasminogen have been implicated in the activation of plasminogen by u-PA (Markus et al.,1978; Markus et al., 1979). Using glu-PLG(S741A) (Busby et al., 1991) which no longer can be converted to

activeplasmin, and scu-PA(K158E) , Lijnen and coworkers established that scu-PA(K158E) can hydrolyse the R^{561}-V^{562} peptide bond of the plasminogen mutant although with low efficiency (1 % of the tcu-PA(K158E) activity, V8 protease can hydrolize the E^{158}-I^{159} peptide bond resulting in an active two chain u-PA molecule with comparable kinetic parameters as the wildtype u-PA ; Lijnen et al., 1990).
Treatment of a fibrin clot with plasmin (20 pM) shortened the lag phase of pro-uPA mediated lysis in plasma. The effect of plasmin could be reversed by treatment with carboxypeptidase B (CPB), which removes carboxy terminal lysyl residues (0.1 μM , Pannell et al., 1988). In 1991 Liu and Gurewich demonstrated that the E2 fragment of fibrin(ogen) played an important role in the plasmin generation by scu-uPA. The presence of carboxy-terminal lysyl residues was a prerequisite for this conversion. The plasminogen activation of scu-PA(K158A) (1 nM), a mutant that no longer could be converted to the tcu-PA form, was promoted by fragment E2 (1 μM), but not by fragment FCB-2 (1 μM) or soluble fibrin monomer (1 μM). In contrast plasmin formation by 0.5 nM tcu-PA was not promoted by one of these fragments. In the presence of Carboxypeptidase B, the potentiating effect was abolished. (Liu and Gurewich, 1991). Recently these results were confirmed in a solid phase assay. (Fleury et al., 1993). The conversion of glu-PLG(S741A) by scu-PA(K158E) in plasma milieu was "accelerated" on a partially degraded fibrin surface, and the presence of carboxyl terminal lysyl residues was essential for this "accelerated" conversion. Based on these observations Fleury and coworkers conclude that the conversion of scu-PA to tcu-PA and of glu-plasminogen to lys-plasminogen play a minor role in scu-PA mediated plasmin formation. Furthermore, generation of carboxy-terminal lysyl residues on the partially degraded fibrin, plays an important regulating role in scu-PA mediated plasmin generation. On an intact fibrin surface, without carboxy-terminal lysyl residues, no conversion of plasminogen to plasmin by scu-PA occurs. Since carboxy-terminal lysyl residues are most likely the result of plasmin activity, the plasminogen activation by scu-PA will only take place after plasmin generation. Therefore, this pathway of plasmin activation could play an important role in the prolongation phase of fibrinolysis.

4.a.4. Role of u-PA in fibrinolysis

The localisation of the plasmin activity towards the fibrin surface is achieved in two ways: 1 - plasmin is rapidly inactivated in the fluid phase by the plasmin inhibitor $\alpha 2$ Plasmin Inhibitor, whereas on the fibrin surface plasmin is relatively protected from inactivation. 2 - scu-PA seems to recognise a conformation of glu-plasminogen bound to partially degraded fibrin (fragment E2). Carboxy-terminal lysyl residues play an important role in this process. It is therefore likely that scu-PA mainly plays a role in the prolongation of fibrinolysis and that for the initiation of fibrinolysis other pathways exist.

4.b. Tissue-type plasminogen activator

4.b.1. Short history

At the beginning of the 20th century blood clots were used as a substrate during cultivation of tissue explants. It was found that certain epithelial tissues and certain tumours caused the liquefaction of blood clots (Fischer, 1946). At the end of the 1940's Astrup and Permin showed that the lytic effect of tissue cells on the plasma clot is caused by the transformation of plasminogen in the fibrin substrate by an activator from tissue fragments (Astrup and Permin, 1947). At first this tissue activator was assumed to remove reversibly a blocking substance from plasminogen, thereby converting it into plasmin (Astrup, 1951). Based on the effect of trypsin on plasminogen Astrup later suggested that activators of plasminogen act through an enzymatic reaction (Astrup, 1951). Tissue activator differed from the known plasminogen activator from human urine: while urokinase- type plasminogen activator only adsorbed slightly or not at all to fibrin, tissue activator adsorbed strongly (Thorsen, 1972). Astrup and Müllertz recognized the physiological significance of this finding. A selective binding of the lytic agent would provide a mechanism enabling the organism to enhance fibrinolysis locally and to achieve a relative decrease in effect of circulating inhibitors (Astrup, 1956; Müllertz, 1956). It was immediately realised that this fibrin binding property would make tissue activator a better thrombolytic agent than urokinase plasminogen activator or plasmin itself. However in 1978

Astrup wrote " vascular activators of human origin can now be prepared in quantities sufficient for animal experiments but may never be applied to the treatment of patients (Astrup, T. (1978) in Chemical Fibrinolysis and Thrombolysis vol 3 eds. Davidson, R.M., Rowan, R.M., Samuma, M. M., and Desnoyers, P. C. Raven Press, New York pp 1 - 57 17, page 32). Indeed purification of tissue activator proved difficult. The levels of vascular activator in blood are low, typically 1 - 5 ng/ml. Perfusing human cadavers with 3 M KSCN (Aoki and von Kaulla, 1971 , Binder et al., 1979) or extracting tissue activator from uterine tissue (Rijken et al., 1979; Wallén et al., 1981) seemed not very promising, considering the amounts purified relating to the amounts of starting material. The finding that a human melanoma cel-line produced tissue activator in reasonable quantities made the use of t-PA in thrombolytic therapy possible (Wilson and Dowdle, 1978; Rijken and Collen, 1981; Wallén et al., 1983; Nielsen et al.,.1983). Thus only three years after Astrups sceptical remark preliminary studies showed the clinical potential of tissue activator as a thrombolytic agent in humans (reviewed by Collen and Lijnen, 1984)

4.b.2. Structural analysis

t-PA (figure 5) is a serine protease of 527 - 530 aminoacid residues in length. The molecular weights reported vary, depending on the method used to determine it between 65 - 75 kD. It is synthesised by endothelial cells (Todd, 1959). The blood concentration is about 0.1 nM. The t-PA molecule differs from many other existing serine proteases.The single chain t-PA molecule has considerable serine protease activity. This was explained by an alternative salt bridge between K^{416} and D^{477} residues near the S^{478} residue of the active site (Petersen et al., 1989). At the present time the three dimensional structure at atomic resolution has not been determined.
The primary amino acid sequence was deduced from c-DNA cloning (Pennica et al., 1983) and later confirmed by peptide mapping (Pohl et al.,1984) and genomic cloning (Ny et al., 1984; Fischer et al., 1985; Friezner-Degen et al., 1986). From the primary structure five protein modules can be discerned: The finger domain (F) resembling the type I fibronectin finger , the growth

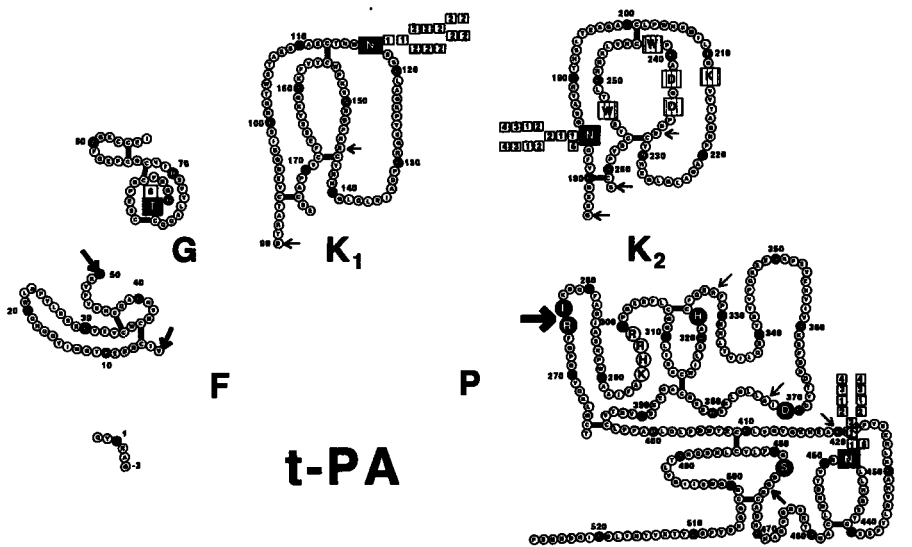


Figure 5: The structure of t-PA.

Tissue-type plasminogen activator (t-PA) consists of a small N-terminal peptide and five major domains, a finger like domain (F) a growth factor-like domain (G), two non identical kringle domains (K1 and K2) and a protease domain (P). Four carbohydrate chains can be attached to t-PA. A single 0-linked fucose on residue 61, two different N-linked carbohydrates on residues 117 and 448 and a third optional N-linked oligosaccharide on residue 184. The presence or absence of this latter oligosaccharide leads to two forms t-PA I and t-PA II in the latter form residue 184 is not glycosylated. The various glycosylation sites are indicated by a filled square and the carbohydrates attached by open numbered squares. 1=glcNAc, 2=man, 3=gal, 4=NANA, 5=galNAc, 6=fuc. The given primary carbohydrate structure is highly variable. The structure shown must therefore be considered as a possible primary structure. The active site residues serine, aspartic acid and histidine are indicated by black filled circles. The site of hydrolysis leading to formation of two chain t-PA is indicated by a large arrow. The secondary interaction sites of t-PA with PAI-1 are indicated by large circles. The amino acid residues involved in lysyl binding of t-PA are represented by hatched boxes.

factor domain (G) resembling the epidermal growth factor , two kringle domains (K1) and (K2) respectively, resembling the kringle domains in prothrombin and the protease domain (P), resembling the protease trypsin. The protein t-PA is referred to as FGK1K2P.
For the t-PA molecule several molecular forms have been described. First the single chain molecule can bcleaved between amino acid residue R^{278}-I^{279} resulting in a two chain molecule which is held via one disulphide bond between C^{277}-C^{398}. Upon reduction with mercapto-ethanol this complex will give a heavy chain of approximately 38 kD and the light chain of 31 kD. After re-oxidising, the fibrin binding feature is localised in the heavy chain. The light chain contains the active site triad S^{481}, D^{327}, H^{330}. Other molecular forms are due to differential glycosylation. There are three N linked glycosylation sites N^{121}, N^{187} and N^{451} and one O linked glycosylation site T^{64}. Type I t-PA isolated from melanoma or CHO cells expresses the simple type high mannose oligosaccharide at N^{120}, and a complex type oligosaccharide at N^{187} and N^{451}. Besides the N linked oligosaccharide t-PA from CHO and melanoma and human embryonic kidney cells have a fucose attached to T^{64}. Type II t-PA does not possess an oligosaccharide at N^{187}. Furthermore, an amino acid residue variation on the N terminus of the molecule, 50 % of the molecules start with GARSYN and are referred to as the L form and 50 % of the molecules start with SYN and are referred to as the S form.

4.b.3. Localisation towards the fibrin clot

The observation that t-PA is adsorbed on fibrin initiated research on the description of the fibrin binding capacity of t-PA (Thorsen et al., 1972). In 1982 the first K_d for the interaction of the fibrin binding site in one chain and two chain melanoma t-PA with fibrin (K_d= 0.14 mg/ml) was reported (Rijken et al., 1982). Four years later Rijken and coworkers separated the light and heavy chain of melanoma t-PA. After re-oxidising only the heavy chain (FGK1K2) interacted with fibrin Sepharose (Rijken and Groeneveld, 1986). This observation was extended by Ichinose who showed that the thermolytic fragment only containing the K2 domain bound to lysyl Sepharose (lysyl binding sites are thought to be equivalent with fibrin binding sites; Ichinose et al., 1986). In

1985 the first recombinant mutant t-PA molecule without the F domain was made. (Kagitani et al., 1985). It still possessed fibrin binding capacity. One year later, the first complete analysis with domain deletion mutants was presented by two groups. (Verheijen et al., 1986; Zonneveld et al., 1986). Both sets of mutants stressed the importance of the F domain and the K2 domain in fibrin binding. The K2 mediated interaction could be completely blocked by EACA. (Zonneveld et al., 1986). These results suggested that the F and the K2 domain of t-PA are involved in fibrin binding. Nesheim and coworkers determined the stoichiometry between the F and the K2 mediated interactions and the t-PA binding sites on a forming fibrin clot. The fibrin binding of FGK1K2P (N117Q, V245M) and K1K2P (N117Q, V245M) in the presence and the absence of EACA were studied (Nesheim et al., 1990). Based on their curved scatchard analysis they proposed two independent interaction sites on the fibrin clot with different K_d 's. Based on the binding of FGK1K2P (N117Q, V245M) in the presence of EACA, a F mediated interaction (K_d = 260 nM n= 0.60) was described, and based on the binding of K1K2P(N117Q, V245M), a K2 mediated binding was described (K_d = 690 nM n = 1.34). These results further suggest that three t-PA molecules are bound to two molecules of fibrin monomer. Two molecules of t-PA bound to two molecules of fibrin monomer and one molecule of t-PA recognizes a structure only present on two monomers. Horrevoets and coworkers also proposed two fibrin interaction sites in t-PA, in the F and the K2 domain respectively (K_d (FGK1K2P) = 360 nM n = 1, K_d (FGK1P) = 1100 nM, n = 2 and K_d (GK1K2P) = 1400 nM, no saturable binding Horrevoets et al., 1994). In contrast to the results of Nesheim and coworkers, only one t-PA binding site on a fibrin monomer was observed.
Besides the involvement of the F and the K2 domain of t-PA in fibrin binding other domains were found to play a role. The interaction of t-PA with fibrin-Sepharose could be blocked with the peptide GxRP (Kaczmarek et al., 1993). The sequence is GxRP is found in the K1 domain of t-PA and it was proposed that t-PA interacts with the "GPRP site" localized on the D part of fibrin. This was supported by the K1 mutant (t-PA (P125A R129Q R130S)) which showed a lower affinity for a forming fibrin clot (Ikenaka et al., 1992). Furthermore Bennet and coworkers found that substitution of charged amino acid residues for alanine

residues, in the K1 domain of two chain t-PA, also resulted in diminished fibrin binding. (Bennet et al., 1991). Their alanine scan of t-PA revealed that fibrin binding in t-PA is spread over all domains, and that surprisingly, the K2 domain is not involved. Weening-Verhoeff and coworkers found that deletion of the lysyl binding site, t-PA (D236N) or t-PA (D238N), hardly influenced the fibrin binding site (Weening-Verhoeff et al., 1990). These results question the direct involvement of the K2 domain in fibrin binding.

Limited digestion of a fibrin matrix increases the amount (1.5 fold) of t-PA bound. Part (1.25 fold) of this increased binding can be explained by the presence of carboxy terminal lysyl residues and was proposed to be K2 mediated. (Vries et al., 1989). Bosma and coworkers determined the binding of t-PA to fibrin fragments (Bosma et al., 1988). They found a strong binding of t-PA to FCB-2 (K_d = 140 nM), while no binding towards fibrinogen, X, Y and E was observed. The interaction of t-PA with FCB-2 is mediated by the K2 domain of t-PA and could be disturbed with EACA (Munk et al., 1989). Besides the FCB-2 part of firbrin(ogen) also the FCB-5 part is involved in t-PA binding (Yonekawa et al., 1992). Hasan et al. (Hasan et al., 1992) studied the interaction of the one and two chain variants with cross-linked fibrin network. (DD)E and DD and E3 retained t-PA binding function of the parent fibrin molecule. Fragment E1 did not interact with t-PA. EACA even at 50 mM concentration had only a minimal effect on the binding of one and two chain t-PA to (DD) and (DDE). It is unlikely that the lysyl binding site in the K2 domain of t-PA mediates this interaction. From the above mentioned results it becomes clear that the binding of t-PA to fibrin is a complicated process. As with the binding of glu-plasminogen, experimental conditions used may determine the final outcome (see page 17).

In the presence of fibrin, t-PA becomes a 100 - 1000 times more efficient plasminogen activator (Binder and Spragg, 1980). The underlying mechanism of this rate enhancing effect was proposed by Hoylaerts and coworkers (Hoylaerts et al., 1982). In the absence of fibrin t-PA does not activate glu-plasminogen (K_M = 65 μM) since the concentration of glu-plasminogen in blood is approximately 2 μM. In the presence of fibrin (K_M = 0.16 μM) t-PA becomes a good plasminogen activator. The catalytic efficiency of t-PA for glu plasminogen is hardly influenced by the presence of

fibrin (k_{cat} = 0.06 s^{-1} in the absence versus 0.1 s^{-1} in the presence). To explain their kinetic parameters they proposed that on the fibrin surface a cyclic ternary complex was formed: t-PA and plasminogen had to be bound to fibrin for efficient plasminogen activation. Using a mix of CNBr digestion products of fibrinogen, which resembles the plasmin degradation products of fibrin(ogen) D and E, Zamarron and coworkers obtained comparable kinetic constants (for melanoma t-PA, without CNBr fragments: K_M = 83 μM k_{cat} = 0.07s^{-1} with CNBr fragments: K_M = 0.18 μM k_{cat} = 0.28 s^{-1}; Zamarron et al., 1984). Rånby showed that the activation of glu-plasminogen by t-PA followed Michaelis Menten kinetics although the kinetic constants differed from the ones mentioned before (for one chain t-PA: K_M = 4.9 μM, k_{cat} = 0.0013 s^{-1} ; for two chain t-PA K_M = 7.6 μM, k_{cat} = 0.0078 s^{-1} Rånby (1982)) Plasminogen activation in the presence of fibrin did not obey Michaelis-Menten kinetics, and thus contradicted the observations made by Hoylaerts and Zamarron. Rånby proposed that plasminogen activation proceeds via some complex containing t-PA and two molecules of plasminogen. Nieuwenhuizen and coworkers noticed in their experiments with FCB-2 and plasminogen a curvature which could be prevented by incubating plasminogen with FCB-2 for 2 to 3 hours (Nieuwenhuizen et al., 1985). They thought that in contrast to Zamarron, first glu-plasminogen associated with FCB-2 and that subsequently this complex was activated by t-PA via a ternary complex. Besides this slow association, it was observed that the K_M and k_{cat} for glu-plasminogen conversion by t-PA was depended on the plasminogen concentration used. Using FCB-2 as a stimulator, Binder and coworkers confirmed the existence of a lag-phase (Beckman et al., 1988). They argued that this lag-phase was probably due to a covalent modification of FCB-2, since plasmin treatment of FCB-2 abolished it (if t-PA (0.017 nM) , FCB-2 (2.1 μM) and plg (0.23 μM) are incubated a lag phase of 15 minutes is observed before the start of plasmin production. Treatment of FCB-2 with 3 nM plasmin resulted in the abolition of this lag phase). Therefore, plasminogen activation in this system is a two step process, first the plasmin formed modifies the FCB-2 fragment, creating carboxyl terminal lysyl residues. These new carboxyl-terminal lysyl residues binds glu-plasminogen. This bound form of plasminogen is subsequently rapidly transformed by t-PA into plasmin. For intact

fibrin a comparable mechanism was suggested by Norrman and coworkers (first phase: K_M = 1 μM k_{cat} 0.13 s^{-1} second phase: K_M = 0.06 μM k_{cat} 0.13 s^{-1} Norrman et al., 1985). When a two step model was applied to their kinetic data, both steps followed Michaelis-Menten kinetics. Transition from the first to the second phase also occurred when the system was exposed to plasmin. The appearance of X fragments and the initiation of the second phase were associated in time. These results explained one of the observations of Nieuwenhuizen and coworkers. Probably during the 2 hours incubation of FCB-2 with plasminogen, a certain amount of plasmin was formed which generated modified FCB-2. An explanation for the second observation of Nieuwenhuizen and coworkers and Rånby (K_M and the k_{cat} of the glu-plasminogen to plasmin conversion by t-PA are dependent on the glu-plasminogen concentration) was suggested by Geppert and Binder (Geppert and Binder, 1992). At higher plasminogen (> 0.1 μM) concentration, t-PA binds plasminogen via a second site in the FGK1K2 part of t-PA (Kd = 0.1 μM) thereby reducing the catalytic efficiency (plasminogen concentration < 0.1 μM : K_M = 0.0625 μM k_{cat} = 0.03 s^{-1}) plasminogen concentration > 0.1 μM : K_M = 14 μM k_{cat} = 0.15 s^{-1}) The fibrin-bound plasminogen and fibrin-bound t-PA molecules no longer can interact via this allosteric site.

Several investigators have coupled the fibrin binding feature of t-PA mutants and the length of the lag phase (Norrman et al., 1985; Larsen et al 1988, Horrevoets et al., 1994), indicating that for this initiation the binding of t-PA towards fibrin is obligatory. Depending on the assay conditions used, i.e. plasminogen concentration and t-PA concentration a short or extended lag phase may be observed. Comparing the fibrin(ogen) digestion products of plasmin and CNBr, Verheijen et al. (Verheijen et al., 1983) showed that stimulation efficiency differed for mini-plasminogen (K5P) but not for glu-plasminogen (K1K2K3K4K5P). The CNBr fragment of fibrinogen, FCB-2 turned out to be a good stimulator with glu-plasminogen (K1K2K3K4K5P) as substrate, while no stimulation was observed when mini-plasminogen (K5P) was used as a substrate. Performing comparable experiments with a 1000 fold higher concentration of t-PA, Wu et al (Wu et al., 1990) showed that in contrast to the observation of Verheijen and coworkers. CNBr fragments stimulate the plasminogen to plasmin conversion of

K5P by t-PA. It seems likely that the amount of plasmin generated under the conditions used by Verheijen and coworkers was not enough to modify the CNBr fragments thereby abolishing the second phase of the fibrinolysis creating an eternal lag phase. Using a thousand higher concentration of t-PA, enough mini-plasmin could be generated to modify CNBr fragments and initiate the second phase. Not only the fibrin surface can be modified by plasmin, but also the substrate (glu-plasminogen to lys plasminogen) and the enzyme (one chain t-PA versus two chain t-PA). In principle all these modification reactions could be responsible for creation of a lag phase. Lijnen et al. (Lijnen et al., 1990) studied the initiation phase with t-PA and a plasminogen mutant which no longer could be converted by plasmin (Glu-plasminogen (S741A)).
High concentrations of plasminogen (3-6 μM) and t-PA (50 - 400 nM range) were used. From the observation that in the presence of CNBr fragments native plasminogen was by a factor 20 more efficiently converted than the plasminogen mutant, they concluded that probably the P domain of plasminogen played an important role in the stability of the cyclic ternary complex. Alternatively, they reasoned, that the conversion of one-chain to two-chain t-PA which could not be formed in the assays with mutant plasmin, was responsible for the observed difference. Using the t-PA (R275E) mutant which no longer could be converted to the two-chain form by plasmin, the authors showed that the one-chain to two-chain t-PA conversion resulted in a 12 fold increase in plasminogen activating activity, independent of the CNBr fragments present (Lijnen et al., 1990).

Domain deletion mutants of t-PA showed the importance of the K2 domain in the process of fibrin dependent plasminogen activation (Zonneveld et al., 1986; Verheijen et al., 1986.). It was reported (Munk et al., 1989), that the binding of the t-PA mutant K2P to FCB 2 fragments can be inhibited by EACA. Furthermore the binding of K2P to aminohexyl (Kd = 80 μM) and lysyl Sepharose (70 μM) did not differ signifficantly suggesting that t-PA recognizes carboxyl terminal lysyl residues and intra chain lysyl residues of fibrin(ogen) with comparable affinity. Deleting the lysyl binding site in t-PA reduced fibrin dependent plasminogen activation by only a factor 2.5 (Weening-Verhoeff et al., 1990). Bennet and coworkers found no effect of deleting the lysyl binding

site on fibrin dependent plasminogen activation and clot lysis. (Bennet et al., 1991).

4.b.5. Role of t-PA in fibrinolysis.

Under physiological conditions the plasmin activity is localised to the fibrin surface. In the first place by the rapid plasminogen inactivator $\alpha 2$ plasmin inhibitor. Plasmin in the fluid phase is rapidly inactivated whereas plasmin on the fibrin surface is relatively protected against inactivation. Secondly, the activation of plasminogen is localised on the fibrin surface. scu-PA converts glu-plasminogen, associated with the carboxy-terminal lysyl residues on fibrin fragment E2 very efficiently. Since this fragment only is present after extended plasmin degradation of the fibrin clot, it seems that scu-PA is involved in the prolongation of fibrinolysis. t-PA has affinity for a forming fibrin clot and can activate plasminogen in the vicinity of a fibrin clot. t-PA is therefore a good candidate for initiating fibrinolysis on the fibrin surface. Besides this potential role in the initiation of fibrinolysis t-PA is involved in the prolongation of fibrinolysis as well. For this prolongation, carboxy-terminal residues on the fibrin fragment D are obligatory.

4.c. Combining the positive features of u-PA and t-PA

Under physiological conditions plasmin is a highly fibrin specific enzyme. Under codition of thrombolytic therapy, when the free concentration of plasminogen activator is increases 2 to 3 in orders of magnitude the processes involved in localisation of the plasmin activity to the fibrin surface are circumvented (Francis and Wood, 1988 ; Wood, 1988; Bell, 1989;). t-PA which is not a pro-enzyme will convert glu-plasminogen in the circulation to plasmin (Francis and Wood, 1988). First this plasmin is inhibited by α_2 plasmin inhibitor. But when this inhibitor pool is depleted plasmin will degrade fibrin(ogen) and induce a lytic state (Bell, 1989). Scu-PA has proenzyme characteristics and will only marginally activate plasminogen in the circulation. However, it has no affinity for fibrin and when converted to active u-PA, will not be localised to the fibrin clot (Werf et al., 1987; Collen and Werf, 1987) Am. J. Cardiol. 60, 431 - 434; PRIMI Trial Study Group, 1989).

Combination of these two characteristics, fibrin specificity and pro-enzyme feature could therefore result in a potentially improved thrombolytic agent when the lytic state is considered.

The determination of the primary structures of plasminogen, t-PA, u-PA made it apparent that these proteins contained conserved regions also present in other proteins (see figure 2, 4 and 5; Patthy, 1985). It was suggested that during evolution new proteins are build from old ones using the same building blocks over and over again. This model was partially based on the exon shuffling theory of Gilbert (Gilbert et al., 1986) and the splice junction theory of Craik (Craik et al., 1983). Trexler and Patthy showed that kringle domains consist of highly conserved regions and variable regions (Trexler and Patthy, 1983). They reasoned that the conserved regions contained the information for the three dimensional-structure of the kringle domain and the variable regions endow the kringle domain with certain functional features. Indeed isolated kringle domains can be denatured and renatured without loss of function (Trexler et al., 1984). The knowledge that the lysyl binding sites in glu-plasminogen are involved in the regulation of fibrinolysis and located in the kringle domains resulted in the idea that these structural autonomous folding units could function as functional autonomous units (Patthy et al., 1984). This hypothesis was strengthened by the observation with t-PA. Denaturation / renaturation experiments with the FGK1K2 part and the P part resulted in functional protein domains: the FGK1K2 part interacted with fibrin Sepharose and the P part contained the serine protease activity (Rijken and Groeneveld, 1982). The first domain deletion mutants confirmed this view. The fibrin binding features of t-PA were localised in the finger (F) and kringle 2 domain (K2) respectively and the protease domain (P) was responsible for serine protease activity (Zonneveld et al., 1986; Verheijen et al., 1986). In addition the presence of the kringle 2 domain was essential for fibrin dependent plasminogen activation The P domain alone, did not show fibrin dependent plasminogen activation whereas the K2P did.
The knowledge that plasminogen activators are mosaic proteins, and that the domains are functionally autonomous should make it possible to introduce a function by transposing domains provided that the function and the domain are known. Since the kringle 2

domain in t-PA plays an essential role in fibrin specificity and a kringle domain is present in the u-PA molecule we focused on the kringle domain.
Recently the three-dimensional structure of the K2 domain of t-PA and the kringle domain of u-PA were described. (Vos et al., 1992; Li et al., 1994). The primary structures and the three dimensional foldings are shown (figure 6).
In both molecules there exists a hydrophobic core in which the disulphide bridges are embedded. As a result a characteristic triple looped structure is formed. The overall folding is comparable although the conformation in the K2 between N26-Q33 (loop III) is different. In the u-PA kringle a helix is formed.
The ligand binding site of the K2 domain in the crystal structure and in solution are similar. (Cleary et al., 1989; Beyon et al., 1989 ; Beyon and Llinás, 1991; Vos et al., 1992). The negatively charged residues are the D^{57} and D^{59} which interact with the ϵ - amino group of EACA and the positively charged K^{33} residue is responsible for the interaction with the α carboxylic group of EACA. The hydrophobic interaction is mediated by the two W^{63} and W^{74} residues, which form a V shaped trough. Within this trough the hexyl group of EACA is positioned. Site-directed mutagenesis of the K2 domain shows the importance of the above mentioned residues in lysyl binding. The affinity of the K2 protein for various ligands have been determined (Serrano et al., 1992; Serrano and Castellino, 1992 a: Serrano and Castellino, 1992 b; Serrano and Castellino, 1993). The best ligand was 7-amino-heptanoic acid (K_d = 6 μM). Using site directed mutagenesis, they identified the K^{33} residue as the most important cationic centre of the ligand binding site. Substitution of the K^{33} residue for an R^{33} residue hardly influences the affinity for the various ligands. However, substituting the K^{33} residue for an E^{33} residue reduces binding for 7-amino-heptanoic acid by a factor 153 (Kd = 921 μM), but hardly changed the binding to L-Lysine (K_d= 85 μM). It seems that the α-amino group of the L-lysine ligand can compensate the repulsion of the two negative charges. Indeed di-amino-hexanoicacid binds this modified kringle 8 times better (K_d = 13 μM). It is interesting to see that the specificity of the ligand binding pocket can be changed. The u-PA kringle does not bind to lysine analogues. Instead Stephens et al. observed that this kringle domains interacts with heparin. (Stephens et al., 1992). In

comparison with the K2 kringle, within the u-PA kringle one of the amino acid residues forming the ionic centre, residue D^{57}, is a R^{57} in the u-PA kringle domain. The W^{62} residue which is part of the hydrophobic cleft is shielded by the R^{57} residue and cannot interact with ligands. The other residue involved in the hydrophobic core, residue W^{74}, is a V^{74} residue in the u-PA kringle domain. The amino acid residue K^{33} in the K2 domain of t-PA which is involved in the interaction with the negatively charged α carboxylic acid of the EACA molecule is a Q^{33} residue in the u-PA kringle. The most noticeable change is the presence of three arginine residues in the u-PA kringle the residues R^{57}, R^{58}, and R^{59}. These three arginyl residues form a cationic triad which may interact with anionic molecules like heparin.
The observation that the ligand binding site of t-PA K2 and the u-PA kringle are structurally very similar, should make it possible to change the specificity of this u-PA kringle domain.

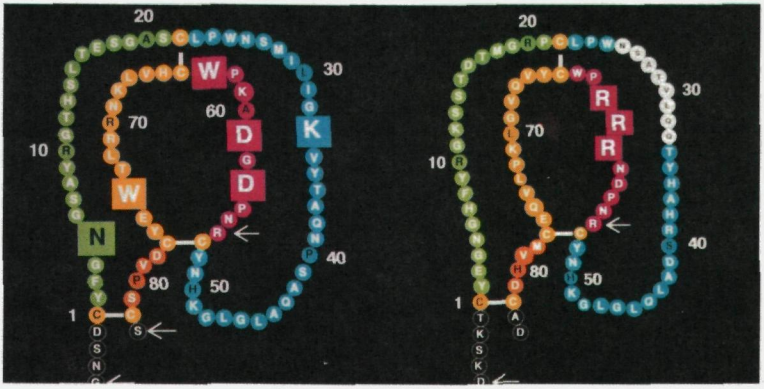
5. Summary

Under physiological conditions, the localisation of plasmin to the fibrin clot is the result of different processes. Firstly, plasmin which is not bound to a fibrin clot is rapidly inactivated by α_2 - Plasmin Inhibitor. Secondly, plasminogen is selectively activated on the fibrin surface by plasminogen activators. For the initiation of fibrinolysis, t-PA seems to play an important role, while for the prolongation both t-PA and scu-PA are essential. Because both sct-PA and scu-PA (although to a far lesser extent) can activateplasminogen in the absence of a fibrin clot, the second process of plasmin localisation to the fibrin clot, is plasminogen activator concentration dependent.
Under therapeutical conditions, when the free plasminogen activator concentration is 2 to 3 orders of magnitude higher than the physiological concentration, all the mechanisms involved in the localisation of plasmin activity to the fibrin clot are circumvented. t-PA will activate glu-plasminogen in the absence of fibrin, while scu-PA once activated will not be localised to the fibrin clot. A combination of both features (fibrin specificity of t-PA and pro-enzyme character of scu-PA) could result in a plasminogen activator which no longer induces a lytic state.
The notion that u-PA and t-PA consist of different domains which

Figure 6: The K2 domain of t-PA and the K of u-PA compared.

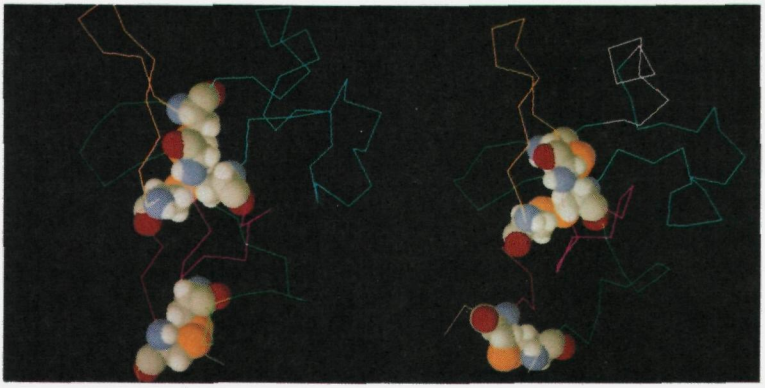
Kringles are highly conserved protein domains. In (a) and (b) the primary structures of the kringle 2 (K2, (a)) domain of t-PA and the kringle domain (K, (b)) of u-PA are presented. Both consist of 82 amino acid residues. The primary structure resembles the danish biscuits which are called "kringles". The residues involved in ligand binding of both kringles are boxed. For K2 the ligand is lysyl/arginyl redidue, for the K domain the ligand is heparin. The disulphide bonds are white.

a b



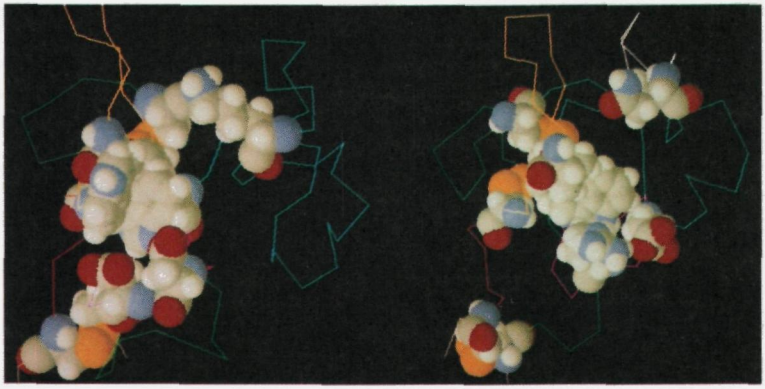
In (c) and (d) the three-dimensional structures of the K2 (c) and the K (d) are shown. The coloured amino acid residues in the primary structure correspond with the colours of the peptide backbone in the three-dimensional structure. The atoms of the cysteine residues which form the 3 disulphide bonds are space filled. (nitrogen = blue, oxygen = red, hydrogen = white , carbon = greyish and sulphur = yellow). The two disulphide bonds (formed by four sulphur groups which are thought to be

c d



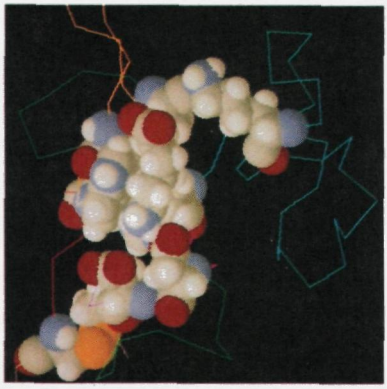
important in the stabilisation of the kringle structure are buried in the centre of the kringle and are hardly visible. The overall folding of both kringles is comparable. In both kringles the characteristic three loop kringle structure (light blue, yellow and magenta) can be seen. The structure which differs most between the K2 and the K is shown in white in the u-PA kringle. In (e) and (g) the ligand binding sites of the K2 domain without (e) and with the lysine analogue amino caproic acid (EACA) (g) are shown. The two W (W64 , W74) residues form a V shaped trough. At the upper site of this V shaped trough, four oxygens

e f



atoms (red) of the negatively charged carboxylic groups of the two D (D57, D59) residues are positioned. At the bottom of this V shaped trough the nitrogen (blue) of the positively charged amino group of the K33 residue is positioned.
In figure (g) an EACA molecule is positioned in the ligand binding site. The positively charged amino-group of the EACA molecule (nitrogen is blue) is in close contact with negatively charged carboxylic acid groups of the D57 and D59 residues. The hydrophobic hexyl part of the EACA molecule (carbon is greyish) is nicely positioned

g



in the V shaped hydrophobic trough formed by the W64 and W74 residues. the positively charged amino-group of the K33 residue is probably too far away from the negatively charged carboxylic acid group of the EACA molecule to be deeply involved in the stabilisation of the EACA-K2 interaction. This might explain why the lysyl analogue 7-amino-heptanoic acid is a better ligand for the K2 (Serrano and Castellino, 1992b). The extra methylene group should bring the negatively charged carboxylic residue of

7-amino-heptanoic acid in closer contact with the positively charged amino group of the K^{33} residue. In (f) the u-PA kringle is shown with the amino acid residues which form the ligand binding pocket in the K2 domain. There is only part of the hydrophobic V shaped through present, the W^{74} residue of the K2 is a V^{74} residue in u-PA kringle. The negatively charged D^{59} residue in the K2 domain is a positively charged R^{59} residue in the u-PA kringle. The positively charged K^{33} residue in the K2 is a neutral Q^{33} residue in the u-PA kringle. Coordinates for the three dimensional structures of the two kringles were obtained from the Brookhaven data bank (kringle 2 domain of t-PA (Beyon and Llinás, 1991) and kringle domain of u-PA (Li et al., 1992) and these structures were visualised with the program Rasmol (R. Sayle, Glaxo Research and Development, UK).

are structurally and functionally autonomous, should make it possible to transfer functions of t-PA (fibrin specificity) to scu-PA (proenzyme).

The lysyl binding site present in the kringle domains of t-PA (K2) plays an important role in fibrin specificity of t-PA. Since the kringle structure of u-PA and t-PA and the binding pocket are very similar we focused on this domain.

6. Aim of the study

We set out to improve the fibrinolytic properties of the plasminogen activators by combining the positive features of t-PA (fibrin specificity) and u-PA (pro-enzyme). Our strategy was based on two assumptions:

1 - plasminogen activators consist of autonomous building blocks which also function autonomously,

2 - The lysyl binding site of tissue-type plasminogen activator present in the kringle 2 domain plays an essential role in the fibrin specificity of t-PA, most likely via a direct interaction between a lysyl residue of the fibrin network and the lysyl binding site.

In chapter 2 we demonstrated that the introduction of a lysyl binding site in the kringle 1 domain of the t-PA activator mutant molecule (K1P) is possible, resulting in the introduction of a lysyl binding site, a fibrin binding site and fibrin dependent plasminogen activation. This result confirms the two above mentioned premises for at least the t-PA like molecules.

In chapter 3 this work is extended towards the u-PA molecule. However two problems were encountered, which challenge the view that structurally autonomous domains are functionally autonomous domains, at least in hybrid plasminogen activators. In chapter 4, we

investigated whether in a native plasminogen activator (t-PA) domains are truly functionally autonomous. It is shown that the position of the lysyl binding site within the t-PA molecule influences the functional properties of the resulting molecule. In chapter 5 a new role for the lysyl binding site of the K2 domain in the t-PA molecule is presented. It questions the view that the lysyl binding site in t-PA is directly involved in fibrin binding. In chapter 6 old and new strategies to circumvent the drawbacks of the existing thrombolytic agents u-PA and t-PA are discussed.

7. References

Alkjaersig, N., Fletcher, A. P., and Sherry, S.(1959). J. Clin. Invest. 38: 1086 -1095
Aoki, N. and Kaulla, von K. N., (1971) Am. J. Clin. Pathol. 55, 171 - 179
Astrup, T., and Permin, P. M. Nature (1947) 159, 681
Astrup, T. (1951) Biochem. J. 50, 5 - 9
Astrup, T.(1956) Blood 11, 781 - 806
Beckman, R., Geiger, M., and Binder, B. R. (1988) J. Biol. Chem. 263, 7176 - 7180
Bell, W.R. (1989) Therapy - Bleeding and Rethrombosis. in Dose - Response and patency (Sherry, S., ed.) pp 59 - 75, Current medical literature, Ltd, London.
Bennet, W. F., Paoni, N. F., Keyt, B.A., Botstein, D., Jones, A.J.S., Presta, L., Wurm, F. M., Zoller, M. J. (1991). J. Biol. Chem. 266, 5191 - 5201
Bernik, M. B. (1973). J. Clin. Invest. 52, 823 - 834;
Beyon, L. I., Kelley, R. F., and Llinás, M. (1989) Biochemistry 28, 9350 -.9360
Beyon, L. I., and Llinás, M., J. Mol. Biol. (1991) 222, 1035 - 1051
Binnema, D.J., van Iersel, J.J.L., Dooijewaard, G. (1986). Thromb. Res 43, 569 -577
Binder, B. R., Sragg, J., and Austen, K.F. (1979) J. Biol. Chem. 254, 1998 -2003
Binder, B.R. and Spragg, J. (1980) in Protides of the biological fluids (Peeters, H., ed.) pp 391-394, Pergamon Press, Oxford.
Blombäck, B. (1967) in Blood Clotting Enzymology (ed Seegers) pp. 143 - 215, Academic Press, New York
Bok, R.A., and Mangel, W.F., (1985) Biochemistry 24, 3279-3286
Bosma, P. J., Rijken, D. C., and Nieuwenhuizen, W. (1988) Eur. J. Biochem. 172, 399 -404
Busby, S. J., Mulvihill, E., Rao, D., Kumar, A., Lioubin, P., Heipel, M., Sprecher, C., Halfpap, L., Prunkard, D., Gambee, J., and Foster, D.C. (1991) J. Biol. Chem. 266, 15286 - 15292)
Cederholm-Williams, C. E. (1981) in Progress in Fibrinolysis (Davidson J. F., Nilsson, I.M., and Asted B.,eds) vol V, 114 - 118, Churchill Livingstone, New York
Celander, D. R., Langlinais, R. P., and Guest, M. M. (1955) Arch. Biochem. Biophys. 55, 286 - 295
Cleary, S., Mulkerrin, M. G., Kelley, R.F. (1989) Biochemistry 28, 1884 - 1891
Chesterman, C.N., Allington, M. J. and Sharp, A. (1972) Nature New Biology 238, 15 -17
Christensen, L. R., and Mc Cleod, C. M. (1945) J. Gen. Physiol. 28, 559 - 563
Christensen, U.(1984) Biochem.J. 223, 413 - 421
Collen, D. (1975) Thrombos. Diathes. haemorrh. 34, 396 - 401
Collen, D. (1976) Eur. J. Biochem. 69, 209 - 216
Collen, D., and Lijnen, H.R. (1984) Athriosclerosis 57 , 579 - 585
Collen, D., and van der Werf, F. (1987) Am. J. Cardiol. 60, 431 - 434
Conforti, G. and Loskutoff, D. F. (1985) Throm. Haemost. 54, 171 (abstr)
Craik, C.S., Rutter, W.J., and Fletterick, R. (1983) Science 220, 1125 - 1129
Deutsch, D.G., and Mertz, E.T. (1970) Science 170, 1095 - 1096
Fantl, P .(1962) Science 135, 787 - 788
Fischer, A. (1946) Nature 157, 442
Fischer, R., Waller, E.K., Grossi, G., Thompson, D., Tizard, R., and Schleuning, W. D. (1985) J. Biol. Chem. 260, 11223 - 11230
Fleury, V., Lijnen, H.R., Anglés-Cano (1993) J. Biol. Chem. 268, 18554 - 18559.
Forsgren, M., Räden, B., Israelsson, M., Larsson, K., and Hedén, L. O. (1987) FEBS Lett. 213, 254-260

Francis, J. L., and Wood, D. (1988) Recent advances in Thrombolytic agents in Fibrinogen, Fibrin stabilisation and Fibrinolysis (Francis, J. L., ed.) pp 326 - 353, Ellis Horwood Ltd, Chichester, England
Friezner Degen, S. J., Rajput, B., and Reich, E. (1986) J. Biol. Chem. 261, 6972 - 6985 Furlan, M. (1988) in Fibrinogen, Fibrin Stabilisation, and Fibrinolysis (Francis, J.L., ed) pp 17 - 62, Ellis Horwood Ltd, England
Geppert, A. G., and Binder, R.B., (1992) Archives of Biochemistry and Biophysics 297, 205 - 212
Gilbert, W., Marchionni, M., and Mcknight, G. (1986) Cell. 46, 151 - 154
Günzler, W. A., Steffens, G.J., Oetting, F., Kim, S. A., Frankus, E., and Flohé L. (1982) Hoppe-Seylers's Physiol. Chem. 363, 1155 -1165
Gurewich, V., and Pannell, R. (1987) Blood 69, 769 - 772
Gurewich, V., Pannell, R., Louie, S., Kelley, P, Suddith, R. L., and Greenlee, R. (1984) J Clin. Invest. 73, 1731 - 1739
Hasan, A.A.K., Chang, W.S., and Budzynsski, A. Z. (1992) Blood 79, 2313 -2321)
Hedner, U., Nilsson, I. M., and Robertson, B. (1966) Throm. Diath. Haemorrh. 16, 38 -50
Hoover, G.J., Menhart, N., Martin, A., Warder, S., and Castellino, F.J. (1993) Biochemistry 32, 10936 - 10943
Horrevoets, A.J.G., Smilde, A., Vries, de C., and Pannekoek, H. (1994) J. Biol. Chem. 269, 12639 - 12644
Hoylaerts, M., Rijken, D.C., Lijnen, H.R., and Collen, D. (1982) J Biol. Chem. 257, 2912 - 2919
Husain, S. S., (1991) Biochemistry 30, 5797 - 5805
Ichinose A., Fujikawa, K., Suyama, T. (1986) J. Biol. Chem. 261, 3486 - 3492
Ichinose, A., Takio, K., and Fujikawa, K. (1986) J. Clin. Invest. 78, 163 - 169
Ikenaka, Y., Yajima, K., Yahara, H., Maruyama, H., Matsumoto, K., Okada, K., Ueshia, S., and Matsuo, O. (1992) Blood Coagulation and Fibrinolysis 3, 381 - 387
Juhan-Vague, I., Cala, M.F., Roux, F., Juhan, C., Durand-Dessemon, de Laforte, C. and Serradimigni, A. (1981) Thrombos. Haemostas. 42, 154 - 157
Kagitani, H., Tagawa, M., Hatanaka, K., Ikari, T., Saito, A., Bando, H., Okada, K., Matsuo, O. (1985) FEBS 189, 145 -149
Kaczmarek, E., Lee, M. H., and McDonagh, J. (1993) J. Biol. Chem. 268, 2474 - 2479
Kasai, S., Arimura, H., Nishida, M., and Suyama, T. (1985) J. Biol. Chem. 260, 12377 -12381
Kirshenbaum, J.M., Bahr, R. D., Flaherty, J. T., Gurewich, V., Levine, H. J., Loscalzo, J. Schumacher, R. R., Topol, E. J., Wahr, D. W., Braunwald, E. and the Pro-Urokinase for Myocardial Infarction Study Group (1991) Am. J. Cardiol. 68, 1564 - 1569
Kline, D. L. (1953) J. Biol. Chem 204, 949 - 951
Konttinen, Y. P. (1968) in Fibrinolysis pp 17 - 42, Oy Star Ab, Finland
Laki, K., and Mommaerts, W. F. H. M. (1945) Nature 156, 664
Landman, H. (1973) Thromb. Diath. Haemorrh. 29, 253 - 275;
Larsen, G., Henson, K., and Blue, Y. (1988) J Biol. Chem. 263, 1023 - 1029
Lesuk, A., Terminiello, L., and Travy, J. H. (1965) Science 147, 880 - 885
Li, X., Bokman, A.M., Llinàs, M., Smith, R.A.G., Dobson, C.M. (1994) J. Mol. Biol. 235, 1548 - 1559
Lijnen, H. R., Zamarron, C., Blaber, M., Winkler, M.E., and Collen, D. (1986) J. Biol. Chem. 261, 1253 - 1258
Lijnen, H.R., Hoef, van B., and Collen, D. (1990) thrombosis research suppl. X, 45 - 54
Lijnen, H. R., Hoef, van B, Cock, de F., and Collen, D. (1990) Thrombosis and Haemostasis (1990) 64, 61-69
Lijnen, H.R., van Hoef, B., Nelles, L., and Collen, D. (1990) J. Biol. Chem. 265, 5232 -5236
Liu, J., and Gurewich, V. (1991) J. Clin. Invest. 88, 2012 - 2017
Lucas, A. M., Fretto, L.J., and McKee, A. (1983) J. Biol. Chem. 258, 4249 - 4256
Magnusson, S., Peterson, T.E., Sottrup-Jensen,L., and Claeys, H. (1975) in Proteases and Biological Control (Rifkin, D.E., and Shaw, E., eds) pp. 123 - 149, Cold Spring Harbor Laboratories, Cold Spring Harbor, NY.
Malinowski, D. P., Sadler, J.E., and Davie, E.W. (1984) Biochemistry 23, 423 - 4250
Markus, G., de Pasquale, L.J., and Wissler, F.C.(1978) J. Biol. Chem., 253, 727 - 732
Markus, G., Evers, J.L. and Hobika, G.H. (1979) J.Biol.Chem. 253, 733 - 739
Markus, G., Camiolo, S. M., Sottrup-Jensen,L., and Magnusson, S. (1982) Progress in Fibrinolysis (Davidson, R.M., Rowan, R. M., Samama, M.M., and Desnoyers, P. C., eds) Vol. V pp 125 - 128, Raven Press, New York
Mac Farlane, R. G. and Pilling, J. (1947) Nature 159, 779 - 780
Moroi, M., and Aoki, N. J. (1976) Biol. Chem. 251, 5956 - 5965
Mulichak, A.M., Tulinsky, A., and Ravichandran, K.G. (1991) Biochemistry 30, 10576 -10588
Müllertz, S. Acta Physiologica Scandinaviea 28, 29-35
Munk de, G. A. W., Caspers, M. P. M., Chang, G. T. G., Pouwels, P. H., Enger-Valk, B. E., and Verheijen, J.H. (1989) Biochemistry 28, 7318 - 7325
Nelles, L., Lijnen, R., Collen, D., and Holmes, W. E. (1987) J. Biol. Chem. 262, 5682 -5689

Nielsen, I., S., Hansen, J.G., Skriver, L., Wilson, E. L., Kaltoft, K., Zeuthen, J., Danø, K. (1982) Biochemistry 21, 6410 - 6415
Nielsen, L.S., Hansen, J.G., Andreasen, P.A., Skniver, L., Danø, K., and Zeuthen, J. (1983) EMBO. J. 2, 115 - 119
Nieuwenhuizen, W., Voskuilen, M., Traas, D. W., Hoegee-de Nobel, B., and Verheijen, J.H. (1985) in Fibrinogen - structural variants and interactions (Henschen, A., Hessel, B., McDonagh, J., and Saldeen, T., eds) pp 331 - 342, Walter de Gruyter, Berlijn
Nolan, C., Hall, L.S., Barlow, G. H., Tribby II, E. (1977). Biochem. Biophys. Acta. 496: 384 - 400
Norrman, B., Wallén, P., and Ránby, M. (1985) Eur. J. Biochem. 149, 193 - 200
Novokhatny, V.V., Matsuka, Y.V., and Kudinov, S. A. (1989) Thrombosis Research 53, 243 - 252
Ny, T., Elgh, F., and Lund, B. (1984) Proc. Natl. Acad. Sci. U.S.A. 81, 5355 - 5359
Pannell, R., Gurewich, V. (1986). Blood 76, 1215 - 1223
Pannell, R., Black, J., and Gurewich, V. (1988) J. Clin. Invest. 81, 853 - 859
Pannell, R., Anglés-Cano, E., Gurewich,V. (1990) Thromb. Haemostasis. 64, 556 - 558
Patthy, L. (1985) Cell. 41, 657 - 663
Patthy, L., Trexler, M., Váli, Z., Bányai, L., and Váradi, A. (1984) FEBS 171, 131 - 136
Pennica, D., Holmes, W. E., Kohr, W. J. Harkins, R. N., Vehar, G. A., Ward, C. A., Bennet, W. F., Yelverton, E., Seeberg, P. H., Heynecker, H. L., Goeddel, D. V., and Collen, D. (1983) Nature 301, 214 - 221
Petersen, L.C., Lund, L.R., Nielsen,. L.S., Danø, K., and Skriver, L. (1988) J. Biol. Chem. 263, 11189 - 11195
Petersen, L.C, Boel, E., Johannessen M., and Foster, D. (1989) Thromb. Haemostas 62, 322 (abs))
Pohl, G., Kallstrom, M., Bergsdorf, N., Wallén, P., and Jornvall, H. (1984) Biochemistry 23, 3701 - 3707
Ponting, C.P., Marshall, J. M. and Cederholm-Williams, S.A. (1992) Blood Coagulation and Fibrinolysis 3, 605 - 614
PRIMI Trial Study Group (1989) The Lancet 22, 863 - 868
Pye, E. Maciag, T., Kelly, P., and Iyengar, M.R. (1977) in Thrombosis and Urokinase (Paoletti, R., and Sherry, S. eds) pp 43 - 59, Academic Press, London
Rackoczi, I., Wiman, B., and Collen,D. (1978) Biochem. Biophys. Acta 540 : 295 - 300
Ránby (1982) Biochimica et Biophysica Acta 704, 461 - 469
Riccio, A., Grimaldi, G., Verde, P., Sebastio, G., Boast, S., and Blasi, F. (1985) NAR 13, 2759 - 2770
Rijken, D.C., and Collen, D. (1981) J. Biol. Chem. 256, 7035 - 7041
Rijken, D.C., Wijngaards, G., Zaal-de Jong, M., van Welbergen, J. (1979) Biochem. Biophys. Act 580, 140 - 153
Rijken, D. C. and Groeneveld, E. (1986) J. Biol. Chem. 261, 3098 - 3120
Schmitz, A (1936) Z. Physsiol. Chem. 244, 89 - 93
Serrano, de V. S., Sehl, L. C., and Castellino, F. J. (1992) Archives of Biochemistry and Biophysics 292, 206 -212
Serrano, de V. S., and Castellino, F. J. (1992a) Biochemistry 31, 3326 - 3335
Serrano, de V. S., and Castellino, F. J. (1992b) Biochemistry 31, 11698 - 11706
Serrano, de V. S., and Castellino, F. J. (1993) Biochemistry 32, 3540 - 3548
Shah, G. A, and Dhall, D.P. (1983) Thrombosis Research 32, 67 - 72
Sobel, G.W., Mohler, S.R., Jones, N. W., Dowdy, A.B.C., and Guest, M. M. (1952) Am. J. Physiol. 171, 768 - 769
Sottrup-Jensen, L., Claeys, H., Zajdel, M., Petersen, T.E., and Magnusson S. (1978) in Progress in Chemical Fibrinolysis and Thrombolysis V (Davidson, R.M., Rowan, R. M., Samama, M.M., and Desnoyers, P. C., eds) Vol V, pp 191 - 209, Raven Press, New York Sottrup-Jensen, L., Zajdel, M., Claeys, H.,Petersen, T.E., and Magnusson S. (1978) In Progress in Chemical Fibrinolysis and Thrombolysis (Davidson, R.M., Rowan, R. M., Samama, M.M., and Desnoyers, P. C., eds) Vol III pp 191 - 209, Raven Press New York
Sottrup-Jensen, L., Zajdel,M., Claeys,H., Petersen, T.E., and Magnusson S. (1975) Proc. Natl. Acad. Sci. USA 72, 2577 - 2581
Sottrup-Jensen L, Petersen T. E., Magnusson, S. Atl;as of Protein Sequence and structure, vol 5, suppl 3. Dayhoff M O (ed). National Biochemical Research Foundation, Washington D.C. 1978, p 91
Stephens, R.W., Bokman, A. M., Myöhänen, H.T., Reisberg,T., Tapiovaara, H., Pedersen, N., Grødahl-Hansen, J., Llinàs, M., and Vaheri, A. (1992) Biochemistry 31, 7572 - 7579
Steffens, G.J., Günzler, W. A., Oetting, F., Kim, S. A., Frankus, E., and Flohé L. (1982) Hoppe-Seylers's Physiol. Chem. 363, 1043 - 1058
Suenson, E., and Thorsen, S. (1981) Biochem. J. 197, 619 - 628
Thewes, T., Constantine, K., Beyon, I. L., and Llinás, M. (1990) J. Biol. Chem. 265, 3906 - 3915
Thorsen, S., Glas-Greenwalt, P., Astrup, T. (1972) Thrombos. Diathes. haemorrh. 28, 65 -74
Thorsen, S. (1975) Biochem. Biophys. Acta 393, 55 - 65

Tissot, J.d., Schneider,P., Hauert, J., Ruegg, M., Kruithof, E.K., and Bachmann, F. (1982) J. Clin. Invest. 70, 1320 - 1323
Todd, A. S. (1959) J. Path.Bact. 78, 281 - 283
Trexler, M., and Patthy, L. (1983) Proc. Natl. Acad. Sci. USA 80, 2457 - 2461
Urano, T., Takada, Y., and Takada, A.(1991) Thrombosis Research 61, 349 - 359
Verde, P., Stoppelli, M.P., Galeffi, P., DiNocera, P.P, and Blasi, F. (1984) Proc. Natl. Acad. Sci. USA 81, 4727 - 4731
Verheijen, J.H., Nieuwenhuizen, W., Traas, D.W., Chang, G.T.G., Hoegee, E. (1983) Thrombosis Research 32, 87 - 92
Verheijen, J. H., Caspers, M. P. M., Chang, G.T.G., Munk, de G. A. W. Pouwels, P. H. and Enger-Valk, B. E. (1986) EMBO J. 5, 3525 - 3530;
Virchow, R. (1847) Arch. Pathol. Physiol. Virchows 1, 547
Vos de , A. M., Ultsch, H. M., Kelley, F. R., Padmanabhan, K., Tulinsky, A., Westbrook, M. L., Kossiakoff, A.A. (1992) Biochemistry 31, 271 - 279
Vries, de C., Veerman, H., and Pannekoek, H. (1989). J. Biol. Chem. 264, 12604 - 12610 Wallén, P. and, Wiman B.(1972) Biochem. Biophys. Acta. 257, 122 - 134
Wallén, P, Pohl, G., Bergsdorf, N., Rånby, M., Ny, T., and Jörnvall, H. (1983) Eur. J. Biochem. 132, 681 - 686
Wallén, P, Rånby, M., Bergsdorf, N.,and Kok, P. (1981) Prog. Fibrinolysis 5, 16 - 23
Weening-Verhoeff, E. J. D., Quax, P. H. A., van Leeuwen, R.T.J., Rehberg, E.F., Marotti, K. R., and Verheijen, J. H. (1990) Prot. Engng. 4, 191 - 198
Werf, van de F., Nobuhara, M., and Collen, D. (1987) Am. J. Cardiol. 104, 345 - 348; Wiman, B., and Wallén, P. (1977) Thromb. Res. 10, 213 - 222
Wilson, E. L., and Dowdle, E. B. (1978) Int. J. Cancer 22, 390 - 396
Wiman, B. and Collen, D. (1978) Nature 272, 549 - 550
Wiman, B., and Collen, D. (1978) Eur. J. Biochem. 18, 19 - 26
Wiman, B., Lijnen, H. R., and Collen, D. (1979) Biochemica et Biophysica Acta. 579, 142 - 154
Wood, D. (1988) Thrombolysis in acute myocardial infarction: a review of clinical trials in relation to mortality and side - effects. in Fibrinogen, Fibrin Stabilisation, and Fibrinolysis (Francis, J. L., ed.) pp 354 - 371, Ellis Horwood Ltd, Chichester, England
Wu, H., Chang, B., Wu, D., Chang, L., Gong, C., Lou,K., and Shi, G. (1990) J. Biol. Chem. 265, 19658 - 19664)
Wu, T., Padmanabhan, K., Tulinsky, A., and Mulichak, A. M. (1991) Biochemistry 30, 10598 - 10594
Wun, T., Ossowski, L., and Reich, E. (1982) J. Biol. Chem. 257, 7262 - 7268
Yonekawa, O., Voskuilen, M., and Nieuwenhuizen, W. Biochem. J. (1992) 283, 187 - 191 Zamarron, C., Lijnen, H.R., and Collen, D. (1984) J. Biol. Chem. 259, 2080 - 2083)
Zamarron, C. Lijnen, H. R., van Hoef, B., and Collen, D. (1984) Thromb. Haemostasis 52, 19 - 23
Zonneveld, van A., Veerman, H., and Pannekoek, H. (1986), J. Biol. Chem. 261, 14214 -14218
Zonneveld, van A., Veerman, H., and Pannekoek, H. (1986) Proc. Natl. Acad. Sci. USA 83, 4670 - 4674

Chapter 2

Introduction of Lysyl and Clot Binding Properties in the Kringle One Domain of Tissue-type Plasminogen Activator

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Summary

Despite the high overall similarity in primary structure between kringle one (K1) and kringle two (K2) of tissue-type plasminogen activator (t-PA) there exists an enormous functional difference. It is thought that, in contrast to K1, K2 mediates lysyl binding and fibrin binding and is involved in stimulation of plasminogen activation by fibrin or derivatives like CNBr-fragments of fibrinogen. Hypothesizing that sequence differences are responsible for differences in function, we compared the amino acid sequences of K1 and K2 with a consensus kringle sequence. Six consecutive amino acids, unique to K2 of t-PA were found; i.e. from Asn^{248} to Trp^{253}. To test whether these residues are involved in lysyl binding, fibrin binding and fibrin dependent plasminogen activation, we constructed a set of t-PA mutant proteins containing only a kringle and the protease (P) domain: K2P, K1P and k1P. In the latter molecule the original amino acid residues Ala^{160} to Ser^{165} from K1 were substituted by Asn^{248} to Trp^{253} from K2. As expected, K2P showed enhanced plasminogen activation in the presence of CNBr-fragments of fibrinogen, bound to lysyl Sepharose and to a forming fibrin clot. K1P did not show any of these features. In contrast, k1P could be stimulated by CNBr fragments of fibrinogen and bound to lysyl Sepharose and a forming fibrin clot. These results indicate that at least a part of the functional differences between K1 and K2 of t-PA can be localised to a stretch of six amino acids residues from Asn^{248} to Trp^{253} present in K2.

Introduction

Tissue-type plasminogen activator is a fibrin-dependent plasminogen activator (1). In the presence of fibrin t-PA efficiently converts plasminogen to plasmin, the protease responsible for the degradation of fibrin (2). Based on the primary structure, t-PA is believed to consist of structurally and functionally autonomous building blocks. From the N-terminus t-PA comprises a finger domain (F) a growth factor domain (G) two kringle domains (K1 and K2) and a protease domain (P) (3,4). Domain deletion mutants of t-PA stressed the importance of the K2 domain in fibrin dependent features such as the stimulation of plasminogen activation in the presence of CNBr fragments of fibrinogen (5,6). A lysyl

binding site in t-PA and the involvement of this site in fibrin binding and CNBr fibrinogen fragment dependent plasminogen activation was described and localised in the K2P domain (7-11). Amino acid residue substitution studies with t-PA (amino acid residue numbering based on the t-PA sequence according to (4)) demonstrated that the negatively charged cluster in K2 involving the two Asp residues Asp^{236} and Asp^{238} mediates lysyl binding. Substitution of either Asp by Asn or Ala impaired binding to lysyl Sepharose (12,13). Amino acid residue substitution and Nuclear Magnetic Resonance studies on isolated K2 (amino acid residue numbering based on the Kringle sequence according to (25)) confirmed the involvement of these two Asp residues (Asp^{55} and Asp^{57}) in lysyl binding (14,15). It was further shown that Lys^{33} functions as a cationic center and Trp^{72} mediates the hydrophobic interaction involved in binding of lysyl residue to K2. (16-18). Despite the high overall similarity in primary structure between the K2 and the K1 (52 %) a function of K1 is at present unknown although some authors have claimed that the K1 and the K2 have the same features and are exchangeable (19-21).

Generally, kringle domains are thought to function as autonomously folding modules specialized in protein-protein interaction (22). Based on homology studies on various kringle domains it was hypothesized that a kringle domain consists of conserved sequences necessary for the three dimensional structure and variable specific sequences involved in a specialised function i.e. binding to other proteins (23). Crystallographic studies of three kringles from other kringle containing proteins confirmed the conserved three dimensional structure of the kringle domains (24-27). Therefore in theory it should be possible to localise these specific sequences responsible for the fibrin dependent features of t-PA mediated by K2 and introduce these sequences into another kringle, and thus introduce a new function in this kringle. Here, we report the identification of such a K2 specific sequence Asn^{248} to Trp^{253} in K2. When this K2 specific sequence is put into the K1, in the corresponding location, it endows the K1 with the expected fibrin dependent features.

Experimental procedures

primary amino acid comparison of kringle domains

The following primary structures were used: Tissue-type plasminogen activator kringle 1 and kringle 2 (3,4,28), Urokinase-type plasminogen activator kringle (29,30,31), Plasminogen kringles 1 to 5 (32,33), Factor XII kringle (34-36), Hepatocyte Growth Factor kringle 1 to 4 (37), Prothrombin kringle 1 and 2 (38-40). Amino acid residue numbering for t-PA is according to reference 4, amino acid residue numbering in kringles is according to reference 25. Primary amino acid sequence comparison was performed using the program PROSIS (Pharmacia LKB Biotechnology AB).

construction of mutant proteins

The following mutants were constructed t-PA\del(*S1-G176*) referred to as K2P and t-PA\del(*S1-R275*)ins(t-PA: *R87-G176*) referred to as K1P and t-PA\del(*S1-R275*)ins(K1:*R89-G176*(del(*A160-S165*)-ins(t-PA:*N248-W253*)) referred to as k1P (nomenclature of used t-PA mutant proteins according to reference 41). The previously described expression plasmid peV$_2$/t-PA (10) was used to construct the K1P and the K2P. For the construction of the k1P , the peV$_2$/FGK1P (42) in which primer 32 and primer 42 were exchanged for the primer 106: 5'TACGTCTTTAAGAACCGCAGGCTGACGTGGGAG and 107: 5' GCAGAACTCCACGTCAGCCTGCGGTTCTT was used. This molecule designated PeV$_2$/FGk1P contains instead of the Ala^{160} to Ser^{165} of K1 the Asn^{248} to Trp^{253} of K2. Mutant DNAs were constructed using the recombinant circle polymerase chain reaction (43). The mutant DNAs were constructed according to the exon/intron boundary of t-PA. Primers used to construct K2P:
5' GGAAACAGTGACTGCTACTT (TPA18),
5' TCTTACCAAGGAAACAGTGACTGCTCTACTTT (TPA17),
5' TTGGTAAGATCTGGCTCCTC (TPA7)
5' TCTGGCTCCTCTTCTGAATC (TPA8)
primers to construct K1P and k1P :
5' GATACCAGGGCCACGTGCTA (TPA 16)
5' TCTTACCAAGATACCAGGGCCACGTGCTAC (TPA15)
5' CTCAGAGCAGGCAGGGGTGC (TPA14)
5' GCAGGTGGACTCAGAGCAGGCAGGGGTGC (TPA13)
5' TCCACCTGCGGCCTGAGACA (TPA3)
5' GGCCTGAGACAGTACAGCCA (TPA4)
Briefly: 2 ng of CsCl purified expression plasmid peV$_2$/t-PA or peV$_2$/FGk1P were amplified in a total volume of 100 ul: containing 1-2 μM primer (Isogen, Amsterdam, The Netherlands), 10 μl of Taq amplification buffer (Amersham, Houten, The Netherlands) 5 Units Taq polymerase (Amersham, Houten, The Netherlands) and 20μM dNTP (Promega, Leiden, The Netherlands) and 20 μl of Nujol mineral oil (Perkin Elmer, Gouda, The Netherlands). In total 20 amplification cycles were completed on the Perkin Elmer thermocycler 480. One cycle consisted of 2 min denaturation at 94 °C, 1 min reannealing at 55 °C and 5 min extension at 72 °C. The amplification products were isolated as described (43). Denaturation-renaturation reactions were performed in a total volume of 40 ul containing 10mM Tris-HCl pH 8.0 , 1 mM EDTA and 100 mM NaCl and 20 ul Nujol mineral oil (Perkin Elmer, Gouda, The Netherlands) . The protocol consisted of 10 min denaturation /renaturation steps at respectively 100 °C , 90 °C, 80 °C, 70 °C followed by 1 h incubation at 55°C and 2 h at room temperature. 10 ul of the denaturation/renaturation mix was used to transform E.coli JM109 (Promega, Leiden, The Netherlands). Recombinants were screened with restriction enzyme digestion . Mutation frequencies varied between 60% and 90 %. Nucleotide sequences of the mutant plasmids were checked by plasmid dideoxy sequencing (44) using the T7 sequenase kit (Promega, Leiden, The Netherlands).

LB6 cell transfections

t-PA expression plasmids were used to transfect mouse L cells (LB6) by calcium phosphate co-precipitation with peV$_2$/Neo which contains the gene for Aminoglycoside Phoshotransferase 3' (45). Cells that incorporated the plasmids and thus were Neo-resistant were selected in Dulbecco's MEM supplemented with 10 % fetal calf serum (Boehringer/Mannheim, Germany), L-glutamine (Gibco, Breda, The Neterlands), 100 U penicillin/ml and 100 ug/ml streptomycin (Gibco, Breda, The Netherlands) and 1.2 mg/ml of the neomycin analogue geneticin (Gibco, Breda, The Netherlands). For purification of the recombinant proteins, cells were cultured in Dulbecco's MEM supplemented with 100 KIU/ml (Bayer, Leverkusen, Germany) and 10 mM ϵ- amino caproic acid (Merck, Darmstadt,Germany) to prevent plasmin activity, 0.3 g/l human serum albumin (CLB, Amsterdam,The

Netherlands) L-glutamine (Gibco, Breda, The Netherlands), 100 U penicillin/ml and 100 ug/ml streptomycin (Gibco, Breda, The Netherlands) and 1.2 mg/ml geneticin (Gibco, Breda, the Netherlands). Recombinant t-PA mutants were purified by immunoaffinity chromatography using a monoclonal antibody ESP-2 (Bioscot, Edinburgh, Scotland) against the protease domain of t-PA coupled to agarose. A 0.5 ml aliquot of anti t-PA - Sepharose suspension was placed on a disposable PD-10 gel filtration column (Pharmacia, Woerden, The Netherlands). The tandem column was equilibrated with 0.1 M Tris-HCl pH 7.5, 0.01% (v/v) Tween 80. Conditioned medium was loaded onto the column followed by sequential washing with the buffer mentioned above. The column was washed with 2 column volumes of a buffer containing 0.1 M Tris-HCl pH 7.5, 1.0 M NaCl and 0.01% (v/v) Tween 80. Subsequently the column was reequilibrated with the same buffer without NaCl. The t-PA mutant was eluted from the column with a buffer containing 0.1 M Tris-HCl pH 7.5, 0.01 % (v/v) Tween 80 and 3.0 M KSCN (Merck, Darmstadt, Germany). Column fractions were tested for plasminogen activation activity : those fractions containing activity were pooled for further characterization.

Gel electrophoresis

Polyacrylamide gel electrophoresis in the presence of SDS was performed under non-reducing conditions on 10 % acrylamide gels with 5% stacking gels using the Laemmli system (46). After electrophoresis, gels were washed in 2.5% (v/v) Triton X-100 to remove SDS and placed on plasminogen-containing fibrin agarose layers (47). Upon incubation plasminogen activators appear as clear lysis zones on an opaque background.

Labeling of t-PA analogues

For labeling of rt-PA or the t-PA analogues an active site directed inhibitor of t-PA was used. The inhibitor 4-aminobenzoyl-Gly-Arg-CH_2Cl (a kind gift of Dr. E. Shaw) was iodinated with [^{125}I] and purified as described (48). rt-PA and t-PA analogues in 0.1 M Tris-HCl, pH 7.5, and 0.1% v/v Tween 80 were incubated with 2 μM iodinated inhibitor for 4 h at room temperature. Radiolabeled t-PA and t-PA analogues were purified on a 1 ml column of Zn chelate-Sepharose and extensively washed with 0.02 M Tris-HCl,pH 7.4, 1M NaCl, and 0.01% v/v Tween 80, and eluted with the same buffer containing 100 mM imidazole (Merck, Darmstadt, Germany). Specific activity of the labeled t-PA or t-PA analogues were approximately 26000 cpm/pmol.

Determination of the relative apparent K_d of the lysyl binding site

The affinity of the t-PA mutants for lysyl Sepharose was determined as described before (10). Radiolabeled rt-PA or t-PA analogues (0.4 nM, final concentration) were incubated in a volume of 200 μl 0.1 M Tris-HCl pH 7.5, 0.01 % (v/v) Tween 80, 100 mM NaCl with different amounts of lysyl Sepharose/Sepharose mixtures. After 3 hour at 4°C, the Sepharose was spun down and the amount of radioactivity in the supernatant was determined with a γ-counter. t-PA bound was expressed as a fraction of the total amount t-PA added to the lysyl Sepharose. The data were fitted to $([t\text{-}PA]_{bound}/[t\text{-}PA]_{free})^{-1}$ = (apparent K_d/ volume of lysyl Sepharose present) + 1. The slope and the standard deviation of the slope were determined using the linear least square regression analysis algorithm of Lotus 3.1 (Lotus Developmental cooperation, Cambridge, United Kingdom). The apparent K_d is expressed as μl. The relative apparent K_d = apparent K_d of the t-PA analogue / apparent K_d of rt-PA.

Binding to a forming fibrin clot.

Fibrin binding was performed as described before (10). Radiolabeled t-PA analogues (0.1 nM, final concentration) was mixed with plasminogen-free fibrinogen (Kabi Diagnostica Ab, Mölndalh, Sweden) in the presence of 500 KIE/ml trasylol (Bayer, Leverkusen, Germany). Clotting was performed with 2 NIH/ml thrombin (Leo, Ballerup, Danmark). After 1 hour incubation by 37°C, clots were centrifuged and radioactivity in the supernatant was determined with a γ-counter. t-PA bound was expressed as the fraction of the total amount of t-PA analogue added to the fibrinogen solution.

Conversion of single chain t-PA to two chain t-PA

Conversion of the single chain form t-PA analogues to the two chain form was performed as described previously (49). In short, 10 μl plasmin-Sepharose slurry (1.95 mg plasmin/2.2 g Sepharose (wet weight)) was washed twice with 10 mM Tris-HCl, pH 7.5 and 0.01% (v/v) Tween 80. The buffer was removed and replaced with an aliquot of t-PA analogue in the same buffer. The reaction was carried out with constant mixing at 37 °C. Conversion of t-PA analogues from the single chain to the two chain form was confirmed by spectrophotometric activity determination with S-2366 (L-Pyro-Glu-L-Pro-L-Arg-p-nitroanilide hydrochloride) and S-2288 (H-D-Ile-L-Pro-L-Arg-p-nitroanilide dihydrochloride) (KabiVitrum, Stockholm, Sweden).

Determination of the t-PA analogue concentration.

Spectrophotometric assays were performed as previously described (50). Briefly, the reaction mixture (250 μl total volume) contained plasmin-treated t-PA analogues, 100 mM Tris-HCl (pH 7,4), 0.1% (v/v) Tween 80 and 1.0 mM S-2288 (H-D-Ile-L-Pro-L-Arg-p-nitroanilide) (KabiVitrum, Stockholm, Sweden). The absorbance of the reaction mixtures was measured at 405 nm in an eight-channel microtiter plate reader against suitable blanks without termination of the reaction. The t-PA analogue sample was tested at four different dilutions. The absorbance at 405 nm was plotted against time ($\Delta A/\Delta t$) for each dilution. These slopes were plotted against four different dilutions of t-PA analogues, representing the absorbance change per time per volume t-PA analogue added ($\Delta A/\Delta t)/V$).
The change in absorbance at 405 nm per hour for a known amount of t-PA standard (($\Delta A/\Delta t$)/pmol = 6.87×10^4 $h^{-1}pmol^{-1}$) was compared with the ($\Delta A/\Delta t$)/V of the t-PA analogues. Assuming that the amidolytic activity for the P domain of the t-PA standard is similar to the amidolytic activivty of the P domain of the t-PA analogues, the concentration of t-PA was caculated.

Determination of the stimulation factor.

Spectrophotometric assays were performed as previously described (51). Briefly, the reaction mixture (250 μl total volume) contained various amounts of plasmin-treated t-PA analogues, 100 mM Tris-HCl (pH 7,4), 0.1% (v/v) Tween 80, 0.12 μM Glu-plasminogen and 0.7 mM S-2251 (H-D-Val-L-Leu-L-Lys-p-nitroanilide) (KabiVitrum, Stockholm, Sweden). In certain cases, 120 ug/ml of CNBr-digested fibrinogen were included. The absorbance of the reaction mixtures was measured at 405 nm in an eight-channel microtiter plate reader against suitable blanks without termination of the reaction. The t-PA anologue sample was tested at 10, 20 and 40 pM (final concentration) of active enzyme both for reaction mixtures containing fibrinogen fragments and for reactions mixtures without fragments. Fibrinogen fragments were prepared as described (51). The enhancement factors were determined as follows: the change in absorbance was monitored over time for each t-PA analogue in the presence and in the absence of CNBr digest of fibrinogen. For each enzyme concentration a slope was calculated, representing the absorbance change over time-squared ($\Delta A/\Delta t^2$). These slopes, in turn, were plotted against enzyme concentration, representing the absorbance change per time-squared per molar concentration of enzyme ($\Delta A/\Delta t^2$)/M). The ratio of the slope in the presence of fibrinogen fragments to the slope in the absence of fragments is the enhancement factor. This ratio reflects the extent to which fibrinogen fragments enhance the activity of the particular t-PA analogue preparation.

Results

Previous experiments by us and others have demonstrated the involvement of the K2 domain of t-PA in fibrin dependent plasminogen activation, lysyl binding and fibrin binding. The K1 domain has none of these features (5,6). Comparison of 15 different kringles with alignment of the Cysteine residues shows highly conserved regions probably responsible for the 3 D structure of the kringles (Figure 1, lower part).
Two regions Trp^{25} to Cys^{52} designated Kringle Specific Sequence, KSS 1 and Cys^{64} to Cys^{77} designated Kringle Specific Sequence 2 with less similarity were found. As suggested before (23,24) these are the sequences in the kringle domain which endow the kringles with a certain function . Next we compared KSS1 and the KSS2 within the K1 and K2 of t-PA in more detail (Figure 1, upper part). A K2 specific stretch of six amino acid residues Asn^{248} to Trp^{253} within region KSS 2 was found. We hypothesized that this stretch

of six amino acid residues is responsible for the fibrin dependent features of K2. To test wheter or not the six amino acids are responsible for the functional differences between K2 and K1 we decided to replace the Ala^{160} to Ser^{165} of K1 for Asn^{248} to Trp^{253} from K2. To exclude the interference of other domains of the t-PA

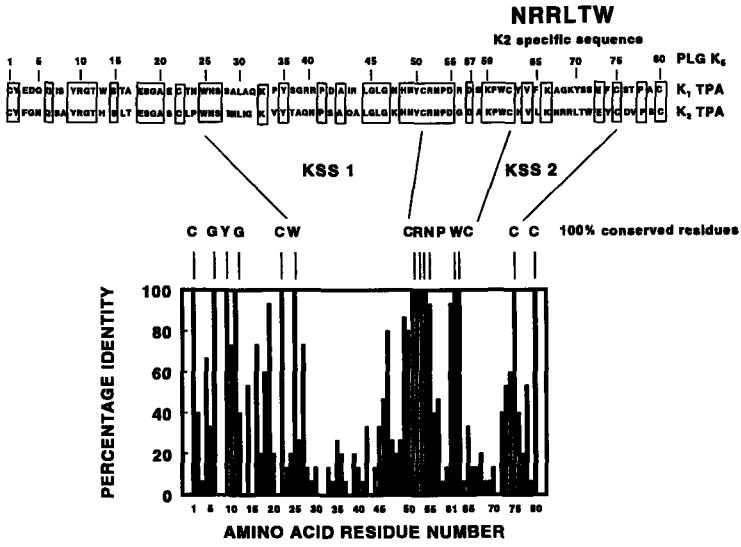

Figure 1 : Determination of a kringle primary consensus sequence.

The primary structures of 15 kringles (see Experimental procedures) were aligned on the Cysteine residues. Amino acid deletions or insertions were considered as residues not identical with any of the amino acids residues. The result is plotted as the percentage identity on the Y-axis versus the position of the amino acid residue number (according to reference (25)) on the X-asis. Two Kringle Specific Sequences (KSS) were found designated KSS1 and KSS2 respectively. Within the KSS2 a unique amino acid sequence for the K2 not present in K1 of t-PA was found.

molecule we constructed the K2P, K1P and k1P. The latter contains instead of the normal K1 molecule a modified K1 designated k1 in which Ala^{160} to Ser^{165} of K1 is replaced by the Asn^{248} to Trp^{253} of K2. DNA's were constructed and expressed in LB6 cells. Zymography showed the integrity of the affinity purified proteins (Figure 2).

The K1P and k1P had a lower electrophoretic mobility than K2P. All the mutants were secreted as single chain molecules which could be transformed to two chain molecules by limited plasmin

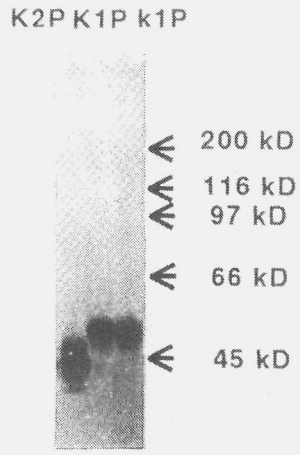

Figure 2: Fibrin zymography of t-PA analogues.

K2P, K1P, and k1P were isolated from culture media by affinity chromatography. Gel electrophoresis and zymography were performed as described under Experimental procedures. High molecular weight standards were run in a separate lane (not shown).

digestion (results not shown).More than 90 % of the K2P molecules bound to lysyl Sepharose (Table 1, column 2). K1P showed no affinity for lysyl Sepharose.
In contrast, k1P which only differs from K1P by six aminoacids showed a clear affinity for lysyl Sepharose. However binding of k1P to the lysyl Sepharose column (30 $\pm$ 15 %) did not reach the level of the K2P, indicating that possibly a low affinity binding site was created or that only part of the k1P molecules had a lysyl binding site. Furthermore, the fraction of k1P which binds to lysyl Sepharose varied for different batches of k1P. When the non binding fraction of k1P was applied for the second time to a lysyl Sepharose column no binding was observed. The lysyl binding fraction when applied for a second time to a lysyl Sepharose column reached K2P levels of binding. This result suggest that there are two populations of the k1P. The determination of the relative apparent K_d for lysyl binding showed that the affinity of the k1P for lysyl Sepharose is about a factor 10 lower than that of K2P (Table 1, column 3), indicating that for creation of a K2P-

TABLE 1: Characteristics of rt-PA and t-PA analogues.

rt-PA or t-PA analogue	fraction bound to lysine sepharose	relative apparent K_d	plasminogen activator activity per pmol protein $\Delta Ah^{-2}pmol^{-1}$ no CNBR fragments of fibrinogen	plasminogen activator activity per pmol protein $\Delta Ah^{-2}pmol^{-1}$ with CNBr fragments of fibrinogen	stimulation factor
rt-PA	0.88 ± 0.11	1	0.079 ± 0.009	10.8 ± 0.6	135 ± 17
K2P	0.98 ± 0.11	1.3 ± 0.2	0.12 ± 0.02	4.0 ± 0.6	34 ± 8
K1P	0.2 ± 0.2	-	0.087 ± 0.009	0.055 ± 0.005	0.63 ± 0.09
k1P lysine bound fraction	0.93 ± 0.08	11 ± 2	0.068 ± 0.006	0.9 ± 0.1	14 ± 2
k1P non lysine bound fraction	0.2 ± 0.2	-	0.036 ± 0.005	0.09 ± 0.01	2.7 ± 0.5

column 2: binding to lysine sepharose. Radiolabeled rt-PA or t-PA analogues were applied to 1 ml columns of lysine sepharose, flow-through was collected. The columns were washed with 2.5 ml buffer containing 50 mM of the lysine analogue ε- amino caproic acid. The radioactivity present in the flow-through, the wash fraction, the elution fraction and remaining on the columns was determined. The radioactivity present in the elution fraction is expressed as percentage of the total radioactivity and was determined for three different t-PA analogue batches (for details, see Experimental procedures).

column 3: determination of the relative apparent K_d . Radiolabeled rt-PA and t-PA analogues were incubated in a volume of 200 ul buffer with different amounts of lysine sepharose/sepharose mixtures. rt-PA or t-PA analogue bound was expressed as a fraction of total rt-PA ort-PA analogue added. The apparent K_d was determined as described in the Experimental procedures. The relative apparent K_d (= apparent K_d of the t-PA analogue divided by the apparent K_d of rt-PA) was determined for three different batches of t-PA analogue. - = no binding observed (for details see Experimental procedures).

column 4, 5: determination of the plasminogen activator activity of the t-PA analogues in the presence and in the absence of CNBr fragments of fibrinogen. The amount of two chain t-PA analogue was determined using the substrate H-D-Isoleucyl-L-prolyl-L-arginine-p-nitroanilide-dihydrochloride (S2288) as described in the Experimental procedures. Plasminogen activator activity of rt-PA and the t-PA analogues, in the presence and absence of CNBr fragments of fibrinogen were determined as described in the Experimental procedures and expressed as $h^{-2}pmol^{-1}$. Three different t-PA analogue batches were tested (for details see Experimental procedures).

column 6: determination of the stimulation factors. Stimulation factors were calculated as follows : the $(\Delta A/\Delta t')$/pmol in the presence of CNBr fragments of fibrinogen divided by $(\Delta A/\Delta t')$/pmol in the absence of CNBr fragments of fibrinogen. Three different batches of t-PA analogue were tested (for details see Experimental procedures).

like lysyl binding site more aminoacid residue changes in K1 are required.
K2P binds to a forming fibrin clot (Figure 3). In the presence of the lysine analogue ϵ-amino caproic acid, binding was disturbed suggesting that a lysyl binding site is involved, supporting previous results (9,10,11). K1P did not have fibrin binding properties. The k1P fraction which bound to lysyl Sepharose also bound to a forming fibrin clot like K2P. ϵ-amino caproic acid blocked the binding suggesting that binding occurs via the newly introduced lysyl binding site in k1P. The fraction of k1P which did not bind to lysyl Sepharose did not bind to a forming fibrin clot either.

The enhancement factors for plasminogen activation in the presence of CNBr-digested fibrinogen catalysed by K2P, K1P and k1P were determined (Table 1, column 4, 5, 6). A stimulation factor of 30 was observed for K2P while no stimulation was found for K1P . The fraction of the k1P which bound to lysyl Sepharose showed a stimulation factor of 14. The non lysyl binding fraction of k1P was stimulated only 3 fold. The basal plasminogen activitor activities of K1P, K2P and the lysyl bound population of k1P were similar, where as that of the non lysyl bound fraction of k1P was about a factor 2 lower.

Discussion

We set out to localize the amino acid residues in K2 of t-PA which are involved in the fibrin dependent plasminogen activation. Based on the high homology between K1 and K2 we reasoned that it should be possible to locate these amino acid residues by substituting individual amino acid residues or stretches of amino acids residues in K1. To exclude the interference of other domains we first constructed the K1P and K2P molecules. As expected the K1P molecule did not exhibit the fibrin dependent features which are characteristic for K2P. Next we set up a strategy to localize the amino acids involved in the fibrin dependent plasminogen activation. We first compared the sequence of 15 kringles. Based on identity, we could distinguish two types of sequences in the kringle : conserved and kringle specific. Two kringle specific sequences were found, designated KSS1 and KSS2. When the primary structures of the K1 and K2 of t-PA within KSS1 and KSS 2 were compared, a unique stretch of six amino acid

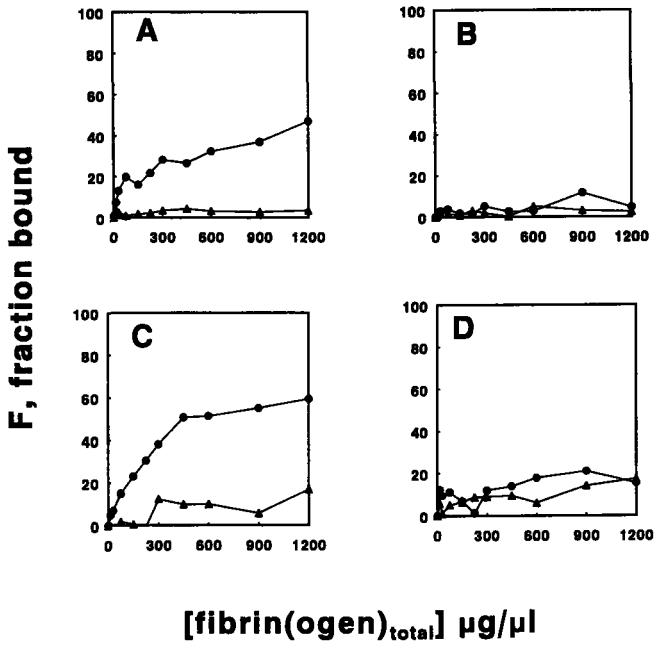

Figure 3: Binding of t-PA and t-PA analogues to a forming fibrin clot in the presence and absence of the lysine analogue ϵ-amino caproic acid.

Radiolabeled t-PA analogues K2P (panel A), K1P (panel B), k1P lysyl binding fraction (panel C), k1P non lysyl binding fraction (panel D) were mixed with various amounts of fibrinogen and clotted with thrombin in the presence (▲) or absence (●) of ϵ-amino caproic acid. Clots were centrifuged and the amount of label in the supernatant was determined using a γ-counter. Bound t-PA was then calculated on the basis of control experiments without fibrinogen or thrombin present. The fraction of bound t-PA is plotted on the Y-axis, the concentration of fibrinogen is plotted on the X-axis expressed as µg/ml (for details see Experimental procedures).

residues, Asn^{248} to Trp^{253} in K2 was found. Introduction of Asn^{248} to Trp^{253} in K1P conferred some of the K2 specific fibrin dependent features to K1. We further noticed that introduction of Asn^{248} to Trp^{253} resulted in two populations of k1P. The population of k1P which bound to lysyl Sepharose differed per batch. Although the plasmin inhibitor trasylol was present during the whole culturing period it can not be excluded that some protease activity was present and modified the k1P and possibly to a lesser extent the K2P. The k1P which bound to lysyl Sepharose, also bound to a forming fibrin clot and was stimulated by CNBr fragments of fibrinogen suggesting that the six newly introduced amino acid residues are involved in these three properties.
As inferred from amino acid substitution, the amino acids involved in lysyl binding are the Asp^{236} ,Asp^{238}, Lys^{211} and Trp^{253} (12,13,16). Of these Trp^{253} in K2 is the only amino acid not present in K1. At present we do not know if this Trp^{253} in the stretch Asn^{248} to Trp^{253} is sufficient or that more residues are required for the introduction of the fibrin dependent features in K1. A reported attempt to introduce the high affinity lysyl binding site of plasminogen K4 in the K1 of prothrombin by substituting Asp^{57}, Gln^{34}, Trp^{72} for Ser^{57}, Glu^{34} ,Arg^{72} was not succesful indicating that for the prothrombin K1 more amino acid residue substitutions are needed for an operational lysyl binding site (52). Our results strongly support the view that kringles which can been seen as mini proteins mediating protein protein interaction (23), consist of constant regions and Kringle Specific Sequences. The conserved sequences are responsible for the three dimensional structure, whereas the Kringle Specific Sequences endow the kringle with a certain function. The Kringle Specific Sequence 2 (KSS2) within the K2 of t-PA seems to be autonomous and transferrable, at least within the t-PA molecule. Introduction of the KSS2 into other kringles for instance the kringle of Urokinase-type plasminogen activator could extend this view.

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References

1. Collen, D. (1980) Thromb. Haemostasis 43, 77-89
2. Hoylaerts, M., Rijken, D.C., Lijnen, H.R., and Collen, D. (1982) J.Biol.Chem. 257, 2912-2919
3. Pennica, P., Holmes, W.E., Kohr, W.J., Harkins, R. N., Vehar, G.A., Ward, C.A., Bennett, W.F., Yelverton, E., Seeburg, P.H., Heyneker, H.L., and Goeddel, D.V. (1983) Nature 301, 214-221
4. Ny, T., Elgh, F., and Lund, B. (1984) Proc. Natl. Acad. Sci. USA 81, 5355-5359
5. Verheijen, J.H., Caspers, M.P.M., Chang, G.T.G., Munk, de G.A.W., Pouwels, P.H., and Enger-Valk, B.E. (1986) EMBO J. 5, 3525-3530
6. Zonneveld, van A.J., Veerman, H., and Pannekoek, H. (1986) Proc. Natl. Acad. Sci. USA 83, 4670-4674
7. Radcliffe, R., and Heinze, T. (1978) Arch. Biochem. Biophys. 189, 185-194
8. Ichinose, A., Takio, K., and Fujikawa, K. (1986) J. Clin. Invest. 78, 163-169
9. Zonneveld, van A.J., Veerman, H., and Pannekoek, H. (1986) J.Biol.Chem. 261, 14214-14218
10. Munk, de G.A.W., Caspers, M.P.M., Chang, G.T.G., Pouwels, P.H., Enger-Valk, B.E.,and Verheijen, J.H. (1989) Biochemistry 28, 7318-7325
11. Nesheim, M., Fredenburgh, J.H.,and Larsen, G.R. (1990) J.Biol.Chem. 265, 21541-21548
12 Weening-Verhoeff, E.J.D., Quax, P.H.A., van Leeuwen, R.T.J., Rehberg, E.F., Marotti, K.R.,and Verheijen, J.H. (1990) Protein Eng. 4, 191-198
13. Bennett, W.F., Paoni, N.F., Keyt, B.A., Botstein, D., Jones, A.J.S., Presta, L., Wurm, F.M.,and Zoller, M.J. (1991) J.Biol.Chem. 266, 5191-5201
14. Byeon, I.J.L., Kelley, R.F.,and Llinás, M. (1889) Biochemistry 28, 9350-9360
15. Byeon, I.J.L.,and Llinás, M. (1991) J.Mol.Biol. 222, 1035-1051
16. Vos, de A.M., Ultsch, M.H., Kelley, R.F., Padmanabhan, K., Tulinsky, A., Westbrook, M.L., and Kossiakoff, A.A. (1992) Biochemistry 31, 270-279
17. Serrano, V.S., Sehl, L.C., and Castellino, F.J. (1992) Arch. Biochem. Biophys. 292, 206-212
18. Serrano, de V.S., and Castellino, F.J. (1992) Biochemistry 31, 11698-11706
19. Gething, M.J., Adler, B., Boose, J.A., Gerard, R.D., Madison E.L., McGookey D., Meidell, R.S., Roman, L.M., and Sambrook, J. (1988) EMBO J. 9, 2731-2740
20. Stern, A., Mattes, R., Buckel, P., Weidle, U.H. (1989) Gene 79, 333-344
21. Stern, A., and Weidle, U.H. (1990) Gene 87, 305-308
22. Patthy, L., Trexler, M., Banyai, L., and Varadi, A. (1984) FEBS Lett. 171, 131-136
23. Trexler, M., and Patthy, L. (1983) Proc. Natl. Acad. Sci. USA 80, 2457-2461
24. Park, H.C., and Tulinsky, A. (1986) Biochemistry 25, 3977-3982
25. Tulinsky, A., Park, C., and Skrzypczak-Jankun, E. (1988) J. Mol. Biol. 202, 885-901
26. Mulichak, A.M., and Tulinsky, A. (1990) Blood Coag. Fibrinol. 1, 673-679
27. Mulichak, A.M., Tulinsky, A., and Ravichandran, K.G. (1991) Biochemistry 30, 10576-10588
28. Pohl, G., Kallstrom, M., Bergsdorf, N., Wallén, P., and Jornvall, H. (1984) Biochemistry 23, 3701-3707
29. Gunzler, W. A., Steffens, G.J., Otting, F., Kim S. M.A., Frankus E., and Flohe, L. (1982) Hoppe-Seyler's Z. Physiol. Chem. 363, 1155-1165.
30. Steffens, G.J., Gunzler, W. A., Otting, Frankus E., and Flohe, L. (1982) Hoppe-Seyler's Z. Physiol. Chem. 363, 1043-1058.
31. Riccio, A., Grimaldi, G., Verde, P., Sebastio, G., Boast, S., and Blasi, F.(1985) Nucleic Acids Res. 13, 2759-2771.
32 Sottrup-Jensen, L., Zajdel, M., Claeys, H., Petersen, T. E., and Magnusson, S.(1978) in Progress in Chemical Fibrinolysis and Thrombolysis, Vol. 3 (Davidson et al., eds) pp191-209, Raven Press, New York.
33. Forsgren, M., Raden, B., Israelsson, M., Larsson, K., and Heden L.O. (1987) FEBS Lett. 213, 254-260
34. Que, B.G., and Davie, E.W. (1986) Biochemistry 25, 1525-1528
35. McMullen, B.A., and Fujikawa, K. (1985) J.Biol.Chem. 260, 5328-5341
36. Cool, D.E., and MacGillivray, R.T.A. (1987) J.Biol.Chem. 262, 13662-13673
37. Nakamura, T., Nishizawa, T., Hagiya, M., Seki, T., Shimonishi, M., Sugimura, A., Tashiro, K., and Shimizu, S. (1989) Nature 342, 440-443
38. Magnusson, S., Petersen, T. E., Sottrup-Jensen, L., and Claeys, H.(1975) in Proteases and Biological Controls (Reich, E., Rifkin, D. B., and Shaw, E., eds),pp. 123-149, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.
39. Friezner Degen, S.J., MacGillivray, R.T.A., and Davie, E.W. (1983) Biochemistry 22, 2087-2097
40. Friezner Degen, S.J., and Davie, E.W. (1987) Biochemistry 26, 6165-6177
41. Pannekoek, H., Lijnen, H.R., and Loskutoff, D.J. (1990) Thromb. Haemostasis 64, 600-603
42. Rehberg, E.F., Theriault, N.Y., Carter, J.B., Palermo, D.P., Hubert, E.V., Bergum, P.W., Wunderlich, C.J., Erickson, L.A., Marotti, K.R. (1989) Protein Eng. 2, 371-377

43. Jones, D.H., and Howard, B.H. (1990) Biotechniques 8, 178-183
44. Sanger, F., Nicklen, S., and Coulson, A.R. (1977) Proc. Natl. Acad. Sci. USA 74, 5463-5467
45. Graham, F.L., and van der EB, A.J. (1973) Virology 52, 456-467
46. Laemmli, U.K. (1970) Nature 227, 680-685
47. Granelli-Piperno, A., and Reich, E. (1978) J. Exp. Med. 148, 223-234
48. Rauber, P., Wikstrom, P., and Shaw, E. (1988) Anal. Biochem. 168, 259-264
49. Rånby, M., Bergsdorf, N., Nilsson, T. (1982) Thromb. Res. 27, 175-183
50. Verheijen, J.H., Y.F. de Jong, and Chang, G.T.G. (1985) Thromb. Res. 39, 281-288
51. Verheijen, J.H., Nieuwenhuizen, W., and Wijngaards G. (1982) Throm. Res. 27, 377-385
52. Trexler, M., Gereben, O., Banyai, L., and Patthy, L. (1992) Fibrinolysis suppl. 6, 88

Chapter 3

Domain-Domain Interaction in Hybrids of Tissue-type Plasminogen activator and Urokinase-type plasminogen activator.

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Summary

Fibrin dependent plasminogen activation by tissue-type plasminogen activator (t-PA) is in part associated with the presence of the kringle 2 domain in t-PA. Within this kringle 2 domain a lysyl binding site has been described. The plasminogen to plasmin conversion by urokinase-type plasminogen activator (u-PA) is in contrast to that of t-PA not enhanced in the presence of fibrin. Within the u-PA kringle domain no lysyl binding site is found. To study whether introduction of a lysyl binding site in the u-PA kringle domain will make u-PA a fibrin dependent plasminogen activator we replaced three stretches of amino acid residues of the u-PA kringle domain (A^{28}-Q^{33}, D^{55}-N^{57} and G^{67}-V^{72}) by three stretches of amino acids from the corresponding positions of the kringle 2 domain of t-PA (M^{28}-K^{33}, D^{55}-D^{57}, N^{67}-W^{72}). These changes resulted in the creation of the lysyl binding site consensus of the kringle 2 domain (K^{33}, D^{55}, D^{57}, W^{62} and W^{72}) in the u-PA kringle. However, the resulting u-PA mutant did not interact with lysyl Sepharose nor did it display fibrin enhanced plasminogen activation in the presence of soluble fibrin mimic. When the kringle domain of u-PA was replaced by the kringle 2 domain of t-PA essentially comparable results were obtained. The hybrid protein hardly interacted with lysyl Sepharose and the plasminogen activation was not enhanced in the presence of fibrin mimic. However, the amino terminal fragment isolated from this hybrid molecule (consisting of growth factor domain and kringle 2 domain) did interact with lysyl Sepharose, suggesting that in the hybrid molecule a functional lysyl binding site is present but not operational. Indeed, lysine analogue (ϵ amino caproic acid) sensitive binding of isolated t-PA kringle 2 domain to u-PA could be observed. The modified u-PA kringle, the wild-type u-PA kringle and the kringle 2 of the u-PA hybrid were also placed N-terminal of the protease domain of t-PA. As expected, the t-PA mutant consisting of the kringle 2 domain and the protease domain bound to lysyl Sepharose and showed fibrin dependent plasminogen activation. The hybrid molecule consisting of u-PA kringle placed N-terminal of the t-PA protease did not display these features. However, introduction of the modified u-PA kringle N-terminal of the t-PA protease domain did only result in a very weak interaction with a lysyl Sepharose column. Despite the high overall similarity

in primary structure of the modified u-PA kringle and t-PA kringle 2 (68 %) no fibrin dependent plasminogen activation of this hybrid molecule was observed.
The above-mentioned results are not in agreement with the notion of the functional autonomy of kringle domains within hybrids of the plasminogen activators t-PA and u-PA, and suggest that interactions between kringle and protease domain in hybrid molecules strongly influence their functional features.

Introduction

Tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA) play an important role in the prevention and dissolution of fibrin-containing blood clots (Thorsen et al., 1972; Collen et al., 1980; Carmeliet et al., 1994). Both t-PA and u-PA convert the pro-enzyme plasminogen into plasmin by hydrolysis of a single peptide bond (R^{561}-V^{562}) (Wallén, P., 1978). The thus formed plasmin is held responsible for the proteolytic degradation of fibrin, which constitutes the scaffold of a blood clot. Prolonged plasmin activity eventually results in the lysis of the fibrin network and subsequently the collapse of the blood clot. However, plasmin is not a fastidious enzyme and besides fibrin also other blood proteins may serve as a substrate. This could cause undesirable side effects. Therefore the activity of plasmin under physiological conditions is well controlled and guided. Firstly, fibrin bound plasmin is protected from the plasmin inhibitor α2 plasmin inhibitor (Wiman and Collen, 1978; Aoki et al.,1993) Whereas fluid-phase plasmin is rapidly inhibited. Secondly, plasminogen activation is limited towards the fibrin surface (Thorsen et al., 1972). Localisation of plasminogen activation on the fibrin surface is accomplished in different ways: t-PA which is secreted as a single chain glycoprotein has a high affinity for fibrin. Fibrin not only protects t-PA from rapid inactivation by plasminogen activator inhibitor type 1 (PAI-1) (Kruithof et al., 1984, Robbie et al., 1993) but also acts as a cofactor in plasminogen activation. In the presence of fibrin the conversion of plasminogen to plasmin by t-PA increases 2-3 orders of magnitude (Binder et al.,1980; Hoylaerts et al., 1982; Rånby, 1982; Nieuwenhuizen et al., 1985).
The plasminogen activator u-PA is secreted as an single chain

glycoprotein with low plasminogen activating potential (Lijnen et al., 1986; Pannell and Gurewich, 1987). In contrast to t-PA, single chain u-PA (scu-PA) does not form stable complexes with PAI-1 (Colucci et al.,1993). In the presence of plasmin scu-PA, is rapidly converted to two chain u-PA (tcu-PA) which is a potent plasminogen activator thus creating a positive feedback on the further activation of plasminogen (Lijnen et al.,1986; Pannell and Gurewich ., 1987). Much research has been focused on improving natural plasminogen activators by combining positive features of t-PA (such as fibrin dependent plasminogen activation, affinity for fibrin) and of u-PA (such as no interaction with PAI and low intrinsic plasminogen activation activity (Pannekoek et al.,1988; Higgins and Bennet, 1990; Lijnen and Collen, 1991)). In principle this seemed straightforward since both plasminogen activators consist of highly conserved homologous domain structures. From the aminoterminus, t-PA consists of a finger domain, a growth factor domain, two kringle domains and a protease domain (Ny et al.,1985; Pennica et al.,1983), while scu-PA consists of a growth factor domain, one kringle domain and a protease domain (Günzler et al., 1982; Steffens et al., 1982; Holmes et al., 1985).
Based on the idea that structurally autonomous domains are also functionally autonomous, the domains responsible for fibrin binding in t-PA, the finger domain and kringle 2 domain were transferred to scu-PA creating a variety of chimeric molecules in which parts of t-PA responsible for fibrin specific functions were introduced in the u-PA molecule (Nelles et al.,1987; Gheysen et al., 1987; Lee et al.,1988; Vries et al.,1988; Piérard et al.,1989; Devlin et al.,1989; Heim et al.,1989 ; Lubin et al., 1992; Asselbergs et al., 1993).
The fibrin specific properties such as fibrin binding and fibrin dependent plasminogen activation of these molecules varied depending on the strategy used to construct them but were always less than expected (Heim et al.,1989).
Domain deletion mutants of t-PA consisting of only the kringle 2 domain and the protease domain still possess fibrin binding and fibrin dependent plasminogen activation (Zonneveld et al., 1986a , Verheijen et al., 1986). All these features seem to be associated with the presence of a lysyl binding site in the kringle 2 domain (Zonneveld et al., 1986b; Munk et al., 1989). Indeed, introduction of a lysyl binding site in the non lysyl binding kringle 1 domain of a t-PA mutant consisting only of the kringle 1 domain and the

protease domain resulted in fibrin binding and fibrin dependent plasminogen activation (Bakker et al., 1993). In the present study we attempted to introduce a lysyl binding site in the kringle domain of u-PA, thus creating fibrin dependent features in u-PA with as minimal a change in the molecule as possible. We learned that introduction of a functional lysyl binding domain in u-PA does not result in the expected properties, probably because of a lysyl-binding site mediated domain-domain interaction. Furthermore, the modified u-PA kringle domain containing the consensus residues for a lysyl binding site when placed N-terminal of the protease domain of t-PA does not confer fibrin dependent properties to this hybrid, indicating that more amino acid residue substitutions are required for these properties.

Experimental procedures

proteins used in this report

Nomenclature and numbering of t-PA and u-PA mutant proteins is according to Pannekoek (Pannekoek et al., 1983). The numbering of kringles is according to Tulinsky (Tulinsky et al., 1988). The following proteins were used: recombinant u-PA is referred to as $G_uK_uP_u$; u-PA del(D45-D133)ins(t-PA: G176-C261) referred to as $G_uK2_tP_u$; u-PA del(A77-Q82)ins(t-PA: M207-K212), del(D106-R108)ins(t-PA:D236-D238),del(G118-V123)ins(t-PA:N248-W253) referred to as $G_uK_{u(m)}P_u$; t-PA del(S1-E175) referred to as $K2_tP_t$; t-PA del(S1-S262)ins($G_uK_{u(m)}P_u$: D45-D133) referred to as $K_{u(m)}P_t$; t-PA del(S1-S262)ins(u-PA:D45-G133) referred to as K_uP_t and t-PA del (S1-D262)ins($G_uK2_tP_u$:G45-C133) referred to as $K2_uP_t$ (see figure 2).

construction of mutant proteins

isolation of the u-PA coding frame:
Standard cloning procedures were (unless stated otherwise) performed according to Sambrook et al., 1992. Primers used in this paper were purchased from Isogen (Amsterdam, The Netherlands) The u-PA reading frame was obtained from HT 1080 cells. Total RNA from HT 1080 cells was isolated according to Chomczynski (Chomczynski and Sacchi 1987). Poly A^+ RNA was isolated using oligo dT Sepharose. The synthesis of cDNA was performed as follows: 1 μg of poly A^+ RNA was mixed with a solution consisting of the following components: RNAsin (final concentration 400 U/ml, Promega), DTT (final concentration 5mM), dNTP (final concentration 0.4 mM, Promega), oligo-dT (final concentration 15 μg/ml, Promega) and Taq polymerase buffer (final concentration 0.5x, Amersham). Next this mixture was incubated for 10 minutes at 70 °C, and slowly cooled towards 37 °C. After adding reverse transcriptase (final concentration 200 U/ml M-MLV (Promega) the mixture was incubated for 2 hours at 37 °C.
Primers used to amplify the u-PA cDNA were: forward primer UK7: 5' TAG CGC CCC GGG CTC GCC ACC AT 3', annealing position -21 to +2, backward primer UK4: 5' ACG GGT CTG GGG AGA CCG GT 3' annealing position 1710 to 1692. Amplification condition used were: 5 μl of cDNA solution was added to 90 μL of a solution consisting of Taq polymerase buffer (final concentration 1x, Amersham) primer UK4 and UK7 (final concentration 20 - 40 nM). This mixture was incubated for 7 minutes at 94 °C following the addition of 10 μl of a solution containing Taq polymerase (final concentration 50 u/ml, Amersham) and dNTP (final concentration 250 nM, Promega). After addition of a drop of Nujol oil (Perkin Elmer) the c-DNA amplification was accomplished with a Bioexcellence thermocycler: 1 cycle consisted sequentially of a 1 minute denaturation at 94 °C, a 0.5 minute reannealing at 54 °C, and 5 minutes of amplification at 70 °C. After 30 cycles a last 5 minutes amplification step was performed at 70 °C.

construction of the u-PA expression vector:
The SV-40 based expression plasmid peV_2 t-PA (Verheijen et al., 1986) was digested with Hind III and Bgl II

to remove the complete coding sequence of t-PA. Within this Hind III/ Bgl II site a sequence was cloned in which the amplified u-PA gene could be cloned. This sequence is formed by annealing the following primers 5' : AGC TTC CCG GGA GGC TTG TCG AC 3' and 5' : GAT CTG TCG ACA AGC CTC CCG GGA 3' creating 5' Hind III site and 3' Bgl II site. The resulting plasmid is called peV_2 pre u-PA.

cloning of the amplified u-PA into pre u-PA:

The amplified u-PA c-DNA was digested with Stu I and Sma I respectively creating a c-DNA with 2 blunt ends containing the u-PA reading frame -21 to 1620. This u-PA reading frame was cloned in the stu I site of pre u-PA. The resulting plasmid is called PeV_2 u-PA.

Construction of mutant DNAs with the recombinant circle polymerase chain reaction was performed as described by Jones (Jones et al., 1990) with minor modifications (Bakker et al., 1993).

Modification of the u-PA kringle domain:

construction of $G_xK_{u(m)}P_u$: first construction of u-PA del(A^{77} - Q^{82}) ins (t-PA : M^{207} - K^{212}) referred to as u-PA 1: first PCR product forward mutagenesis primer

UK12: 5' TGG AAC TCT ATG ATC CTG ATA GGC AAG ACG TAC CAT GCC CAC AGA TCT 3'

backward amplification primer

UK11: 5' GTG ACC ATT CCC CTC ATA GCA G 3'

second PCR product

backward mutagenesis primer

UK13: 5' ATG GTA CGT CTT GCC TAT CAG GAT CAT AGA GTT CCA GGG CAG GCA GGG 3'

forward amplification primer

UK10: 5' TGG CCA AAA GAC TCT GAG GCC C 3'

construction of the second step : u-PA 1 del (D^{105} - R^{108}) ins (t-PA : D^{256} - D^{258}),referred to as u-PA 2, first PCR product

forward mutagenesis primer:

UK16: 5' TGC AGG AAC CCA GAT GGG GAT AGG CGA CCC TGG TGC TAT 3',

backward amplification primer UK 11

second PCR product, backward mutagenesis primer

UK17: 5' CCA GGG TCG CCT ATC CCC ATC TGG GTT CCT GCA GTA ATT 3'

the forward amplification primer UK10.

construction of the u-PA 2 del (G^{116} - V^{125}) ins (t-PA : N^{248} - W^{255}), referred to as $G_xK_{u(m)}P_u$, first PCR product, forward mutagenesis primer

UK14: 5' GTG CAG GTG AAC CGC AGG CTG ACG TGG CAA GAG TGC ATG GTG CAT GAC 3'

backward amplification primer UK 11

forward mutagenesis primer

UK15: 5' GCA CTC TTG CCA CGT CAG CCT GCG GTT CAC CTG CAC ATA GCA CCA GGG 3'

the forward amplification primer UK10.

Replacement of the u-PA kringle by the K2 domain of t-PA:

Construction of G_xK2P_u :

To replace the u-PA kringle by the kringle 2 domain of t-PA the same principle as described for the RCPCR was used. Primers used to amplify the Kringle 2 domain from peV_2t-PA:

first PCR product, forward amplification primer

TPA17: 5' TCT TAC CAA GGA AAC AGT GAC TGC TAC TTT 3'

backward amplification primer

TPA19: 5' TAC TGT GAT GTG CCC TCC TGC 3'

second PCR product, forward amplification primer

TPA18: 5' GGA AAC AGT GAC TGC TAC TT 3'

backward amplification primer

TPA31: 5' TGT GAT GTG CCC TCC TGC 3'

primers used to amplify the u-PA, lacking the u-PA kringle domain:

first PCR product, forward primer

UK29: 5' GGA AAA AAG CCC TCC TCT 3'

backward amplification primer

UK27: 5' GAG GGC AGC ACT GTG AGA TCG GAA ACA GT 3'

second PCR product forward amplification primer

UK30: 5' CCC TCC TGC GGA AAA AAG CCC TCC TCT 3'

backward amplification primer

UK28: 5' GAG GGC AGC ACT GTG AGA TC 3'

To transfer the coding sequences of the various kringles (wild type u-PA kringle, the modified u-PA kringle and the kringle 2 domain) Amino terminal to the protease domain of t-PA a different strategy was chosen. Primers were developed to amplify the coding sequences of the various kringle domains and the coding sequences of the protease domain of t-PA. Because a unique Bsa I (a type I restriction site) is present in all primers, unique sticky ends are generated after amplification and Bsa I digestion.
Primers used to amplify the wild type u-PA and the modified u-PA kringle :
forward amplification primer UK42 : 5' AGA AAT TCG GAG GGC AGC GGT CTC ACC AAG ATA AGT CAA AAA CCT GCT AT 3'
backward amplification primer UK43 : 5' TCT TCT GGA GGA GAG GAG GGT CTC ATG GAA TCT GCG CAG TCA TGC ACC A 3'
Primers used to amplify the kringle 2 domain of from the u-PA hybrid (i.e. the kringle 2 domain of t-PA is substituting the kringle domain in u-PA)
forward amplification primer: 5' UK41: 5' AGA AAT TCG GAG GGC AGC GGT CTC CCC AAG GAA ACA GTG ACT GCT ACT TTG G 3'
the backward amplification primer UK46: 5' TCT TCT GGA GGA GAG GAG GGT CTC TTG GAG CAG GAG GGC ACA TCA CAG TCA T 3'
primers used to amplify the Protease domain of t-PA :
forward amplification primer UK44 : 5' TGG GAG TAC TGT GAT GTG CCG GTC TCC TCC ACC TGC GGC CTG AGA CAG TAC 3'
backward amplification primer UK45 : 5' CAG AAG AGG AGC CAG ATC TTA CCA AGG AGA CCG CAG AGA TGA AAA AAC GCA G 3'

Recombinants were screened with restriction enzyme digestion. Nucleotide sequences of the mutant plasmids were checked by plasmid dideoxy sequencing (Sanger and Coulson,) using the T7 sequenase kit (Promega) or the Circumvent kit (Biolabs).

CHO cell transfections

Plasmids containing the coding sequences of the various plasminogen activators and the plasmid pSV_2/Neo were tranfected in CHO cells as described before (Bakker et al.,1993). Recombinant t-PA mutants were purified by immunoaffinity chromatography using a monoclonal antibody against the protease domain of t-PA (ESP-2, Campro Scientific, The Netherlands). Recombinant u-PA mutants were purified by immunoaffinity chromatography using a monoclonal antibody (α-u-PA, American Diagnostica) or a monoclonal antibody against the K2, domain (Pam-2, Biopool) coupled to agarose. Column fractions containing plasminogen activator activity were pooled for further characterization.

Gel electrophoresis and zymography

Polyacrylamide gel electrophoresis in the presence of SDS was performed under non-reducing conditions on 10 % acrylamide gels with 5 % stacking gels (Laemmli, 1970). After electrophoresis, gels were washed in 2.5 % (v/v) Triton X-100 to remove SDS and placed on plasminogen-containing fibrin agarose layers (Granelli-Piperno & Reich., 1978). Upon incubation the position of plasminogen activators appear as clear lysis zones on an opaque background.

Conversion of the single chain to two chain form of PA analogues

Conversion of the single chain form of plasminogen activator analogues to the two chain form was performed as described previously (Wallén et al., 1981) with minor modifications as described previously (Bakker et al., 1993).

Binding of t-PA or u-PA analogues to lysyl Sepharose.

Binding to a lysyl Sepharose column was performed as described before with some minor modifications (Munk et al., 1989; Bakker et al.,1993). Binding was performed in 0.1 M Tris-HCl, 0.01 % (v/v) Tween 80, 0.4 M NaCl, pH 7.5 at room temperature. The amount of plasminogen activator activity was assessed in run-through, washing fluid and eluate with the plasminogen activator assay described below with the following modification that all assay buffers contained 10 mM ε-amino caproic acid (EACA). The fraction of the total activity applied is given.
Instead of washing in batch like fashion, in some experiments the wash fraction was divided over 3 wash samples of 1 ml and 3 elution samples of 1 ml.

Determination of the plasminogen activator analogue concentration.

Concentration of plasminogen activators containing the protease domain of u-PA were determined with Elisa (Binnema et al., 1986) and with the amidolytic substrate L- pyroGlu-L-Gly-L-Arg-p-nitroanilide dihydrochloride (S-2444) (Chromogenix). The concentration of Plasminogen activators containing the protease domain of t-PA was determined with Elisa (Imulyse-Kit, Biopool) and with the amidolytic substrate H-D-Ile-L-Pro-L-Arg-p-nitroanilide (S-2288) (Chromogenix).

Determination of the stimulation factor.

Plasminogen activator activity in the presence and absence of soluble fibrin mimic (CNBr fragments of fibrinogen) was determined as described before (Bakker et al., 1993).
The ratio of the plasminogen activator activity in the prensence and in the absence of fibrin mimics (CNBr-digest of fibrinogen) is the stimulation factor.

Determination of the interaction of isolated aminoterminal fragment from the u-PA hybrid containing the Kringle 2 domain of t-PA with lysyl Sepharose using Western blotting.

Aminoterminal fragments (ATF) consisting of the growth factor and kringle 2 domain were isolated from $G_pK_2P_u$ using either mild reduction and reoxidation (ATF_r), (Rijken et al.,1986) or plasmin digestion (ATF_p),(Barlow et al., 1981). Half of the solution containing ATF_r or ATF_p in 0.1 M Tris-HCl pH 7.4 0.01 % (v/v) Tween - 80 was applied on a 1 ml lysyl Sepharose column, while the other half was applied to a 1 ml Sepharose column. The wash and the elution fraction were dialysed against water, concentrated and analysed by non reduced SDS-PAGE and Western blotting (Towbin et al., 1979) using antibodies against t-PA.

Separation of the kringle 2 domain and the protease domain of t-PA.

The kringle 2 domain and the protease domain of t-PA were prepared from the two-chain form of mutant K_2P_t after mild reduction with 2-mercapto-ethanol (Rijken et al., 1986). Refolded Kringle 2 domain and Protease domain were isolated using lysyl Sepharose and Zn^{2+} Sepharose respectively. The protease domain, showed one band with a molecular weight of approximately 32 kD. The Kringle 2 domain showed one diffuse band with molecular weight between 10 and 16 kD. Only the fractions of the Zn^{2+} Sepharose column contained plasminogen activating activity, which was not enhanced in the presence of fibrin mimic (CNBr digest of fibrinogen).

Labelling of the $K2_t$ domain with ^{125}I

Labelling was performed with the iodogen procedure according to the manufacturers instruction (Pierce Chemical Co). Specific activity of ^{125}[I]$K2_t$ was found to be 6.5 x 10^6 cpm/pmol, corresponding with approximately 1 pmol ^{125}I per pmol of $K2_t$

Binding of the kringle 2 domain to high molecular weight urokinase and low molecular weight urokinase.

Wells of microtiter plates (polystyrene, Nunc) were filled with 100 μl of a solution either containing 20 μg/ml BSA, Casein, Protease domain of t-PA (see above), High Molecular Weight urokinase (Ukidan, Serono, Switzerland) or Low Molecular Weight urokinase (Abbokinase, Abbot, USA) in 0.1 M Na phosphate buffer pH 7.4 containing 0.01 % (w/v) NaN_3 for 16 hours at room temperature. Subsequently the plates were washed three times with 0.1 M Na-phosphate buffer pH 7.4 containing 2 % (v / v) Tween 80 . Binding experiments were performed with ^{125}[I]Kringle 2 domain in 100 μl Phosphate Buffered Saline containing 0.2 % (v/v) Tween-80, pH 7.4. (PBS-Tween) in the absence or presence of 50 mM EACA. After 3 hours incubation at room temperature the plates were washed four times with PBS-Tween. The amount of radioactivity bound to the plate was determined. All experiments were performed in triplicate.

Results

Fibrin dependent plasminogen activation in t-PA is associated with the presence of a lysyl binding site in the kringle 2 domain. The consensus structure for the lysyl binding site of the kringle 2 domain has been described (Byeon and Llinás, 1991; Vos et al.,

1992; Serrano et al., 1993). It consists of a hydrophobic core formed by two tryptophan residues W^{62} and W^{72} which forms a hydrophobic bed on which the hexyl backbone of the lysine analogue ϵ-amino caproic acid (EACA) is positioned (figure 1, panel A). The negative core of this lysyl binding pocket which is

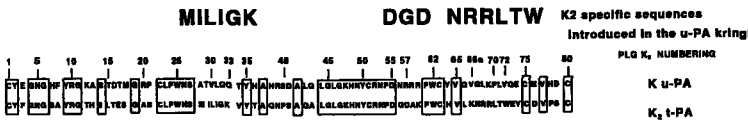

Figure 1. Introduction of a lysyl binding site in the kringle domain of u-PA.

A: Primary amino acid structure of the kringle 2 domain of t-PA and the kringle domain of t-PA. Conserved amino acid residues are boxed (numbering of the aminoacid residues in the kringle are based on the kringle 5 of plasminogen (Tulinsky et al., 1988).

in close contact with the positively charged amino group of EACA is formed by two aspartic acid residues D^{55} and D^{57}. At the other side of the binding pocket there is a positively charged core, which consists of a lysyl residue K^{33} thought to be involved in stabilizing the negatively charged carboxylic group of EACA. In the kringle domain of u-PA only the D^{55} and the W^{62} residues of this consensus structure are present (figure 1, panel B). In the u-PA kringle domain a Q^{33}, R^{57} and V^{72} are found on the positions of the consensus sequence. Introduction of the three stretches of aminoacids M^{28}-K^{33} , D^{55}-D^{57} and N^{67}-W^{72} introduced the three lacking consensus residues K^{33} , D^{57} and W^{72}, creating a theoretical lysyl binding site in the u-PA kringle domain.
Two sets of recombinant plasminogen activators were used to study whether the introduction of the consensus sequence for the lysyl binding site into the u-PA kringle would endow u-PA with fibrin dependent features. One set of molecules consists of the full length u-PA ($G_uK_uP_u$) with the u-PA kringle replaced by either a modified u-PA kringle ($G_uK_{u(m)}P_u$) or a t-PA kringle 2 ($G_uK_{2t}P_u$). The other set of molecules consists of a t-PA protease domain connected to either the wild-type u-PA kringle domain

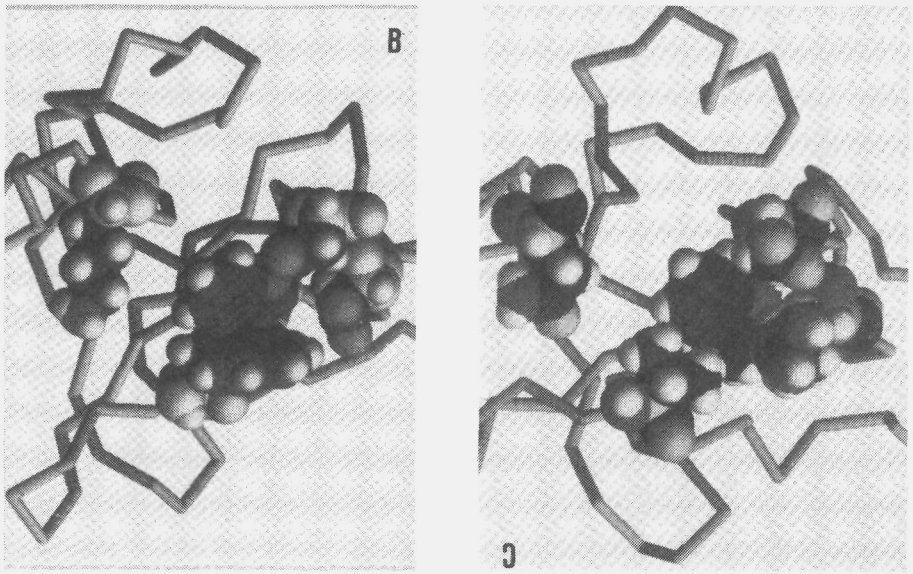

Figure 1 B and C: Comparison of the 3-D structures of the kringle 2 domain of t-PA (panel B) and the u-PA kringle domain (panel C)

Coordinates for the three dimensional solution structures of the two kringles were obtained from the Brookhaven data bank (kringle 2 domain of t-PA (Beyon et al., 1991) and kringle domain of u-PA (Li et al., 1992) and these structures were visualised with the program Rasmol (R. Sayle, Glaxo Research and Development, UK). Residues thought to be responsible for the lysyl binding feature of the kringle 2 domain W^{62}, W^{72}, D^{55}, D^{57} and K^{33} are shown in panel B. In panel C the structure of the u-PA kringle domain is shown. The residues in positions homologous to those involved in lysyl binding of the kringle 2 domain have been indicated (W^{62}, V^{72}, D^{55}, R^{57} and Q^{33}). To create a potential lysyl binding site in the kringle domain of u-PA three regions were changed A^{28}-Q^{33} into M^{28}-K^{33}, G^{67}-V^{72} into N^{67}-W^{72}, and N^{56}-R^{57} into G^{56}-D^{57}.

(K_uP_t) , modified u-PA kringle domain ($K_{u(m)}P_t$) or the t-PA kringle 2 domains originating from t-PA ($K_{2t}P_t$) or from the hybrid $G_uK2_tP_u$ ($K_{2u}P_t$, figure 2).

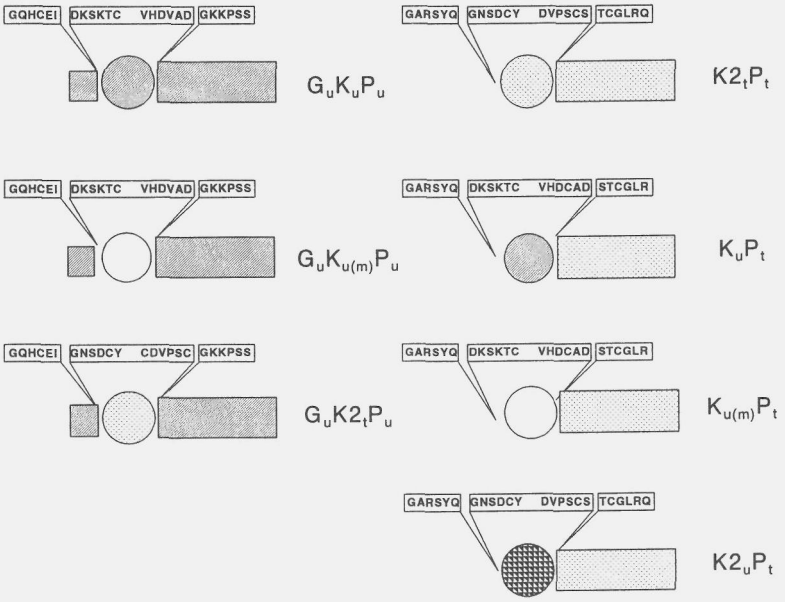

Figure 2. Mutant proteins used in this study.

The following molecules were used: one set of molecules consists of full length u-PA ($G_uK_uP_u$) and two molecules with the u-PA kringle replaced by either a modified u-PA kringle ($G_uK_{u(m)}P_u$) or a t-PA kringle 2 ($G_uK_{2t}P_u$). The other set of molecules consists of a t-PA protease domain connected to either wild type u-PA kringle (K_uP_t), modified u-PA kringle ($K_{u(m)}P_t$) or t-PA kringle 2 domain originating from t-PA ($K_{2t}P_t$) or from the hybrid $G_uK_{2t}P_u$ ($K_{2u}P_t$). Mutants were constructed according to exon-intron boundaries, the amino acid sequences of these boundaries are shown.

The recombinant DNA molecules were expressed in CHO cells and the proteins were purified using an antibody against u-PA, against t-PA or against the kringle 2 domain of t-PA respectively. All molecules showed the expected molecular weight on a zymogram (figure 3). The two molecular forms of K_2P_t and $G_uK_{2t}P_u$ most likely are the result of differences in glycosylation. The 33 kD form

in the $G_uK_{2t}P_u$ preparation most likely corresponds with the protease domain.

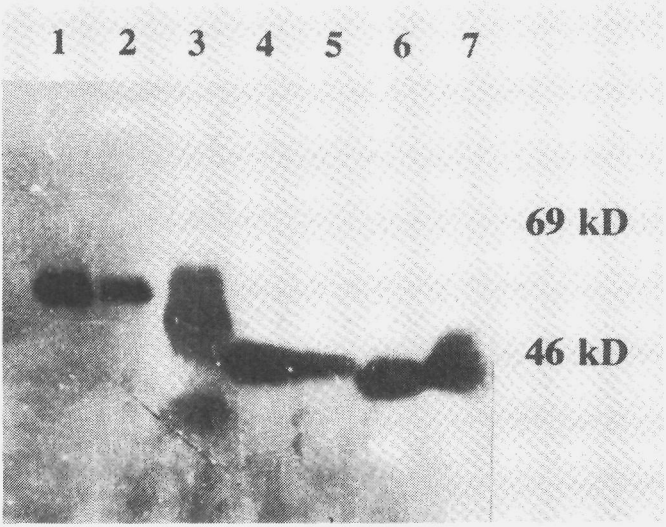

Figure 3. Fibrin zymography of the plasminogen activators.

Plasminogen activators were isolated from culture medium by affinity chromatography. Gel electrophoresis and zymography were performed as described under " Experimental Procedures ". High molecular weight standards were run in a separate lane (not shown).lane 1: $G_uK_uP_u$, lane 2: $G_uK_{u(m)}P_u$, lane 3: $G_uK2_tP_u$, lane 4: K_uP_t , lane 5: $K_{u(m)}P_t$ lane 6: $K2_uP_t$, and lane 7: $K2_tP_t$.

To test whether or not a lysyl binding site is present in the u-PA molecule with the modified u-PA kringle we studied binding to a lysyl Sepharose column (figure 4). The wild type u-PA protein ($G_uK_uP_u$) showed no affinity for lysyl Sepharose as expected (figure 4 C). Also the u-PA protein with the modified kringle domain ($G_uK_{u(m)}P_u$) shows no affinity for lysyl Sepharose. We were surprised to find that when the u-PA kringle domain was replaced by the kringle 2 domain of t-PA ($G_uK_{2t}P_u$) only a very weak interaction with lysyl Sepharose is observed.
Similar experiments were performed with a second set of mutants consisting of different kringle domains connected with the t-PA protease domain (figure 4 D). As expected the t-PA mutant consisting of the kringle 2 domain and the protease domain of t-PA

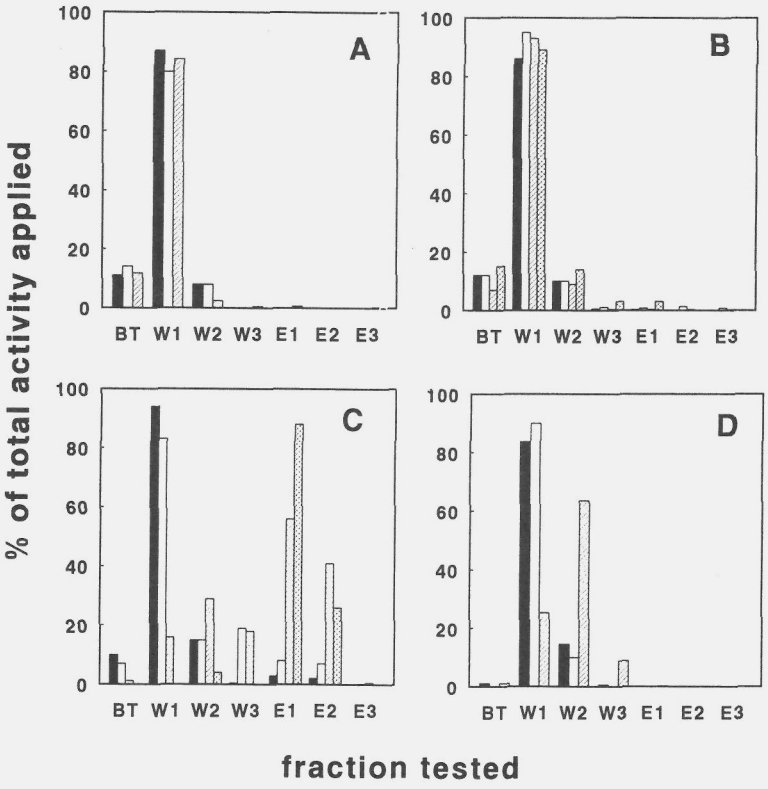

Figure 4. Binding of the plasminogen activators to a Sepharose and lysyl Sepharose.

Approximately 50 fmol of the various plasminogen activators in 250 μl of buffer was applied to a 1 ml Sepharose (panel A and B) or 1 ml lysyl Sepharose column (panel C and D). The columns were washed with 3 ml buffer and eluted with 3 ml buffer containing 10 mM EACA. The plasminogen activating activity was determined in break through (BT), wash (W) and eluate (E). On the Y-axis the percentage of the total applied activity is shown. On the X-axis the fractions are indicated. Panel A and D : $G_uK_uP_u$ (■), $G_uK_{u(m)}P_u$ (□) and $G_uK2_tP_u$ (▨).
For panel B and C: K_uP_t (■), $K_{u(m)}P_t$ (□) $K2_uP_t$ (▨) and $K2_tP_t$ (⊠). For further details see " Experimental Procedures ".

($K_{2t}P_t$) does bind to lysyl Sepharose. The mutant consisting of the kringle 2 domain from the u-PA hybrid placed before the protease domain of t-PA ($K_{2u}P_t$) also binds to lysyl Sepharose. The hybrid molecule containing the wild-type u-PA kringle (K_uP_t), however, did not bind to lysyl Sepharose. Interestingly, the hybrid consisting of the modified u-PA kringle and the t-PA protease domain ($K_{u(m)}P_t$) shows a weak but clearly observable interaction with lysyl Sepharose. None of these molecules interact with a control Sepharose column (figure 4 A and B).
Because the lysyl binding site is present in the hybrid u-PA molecule containing the kringle 2 domain of t-PA ($G_uK_{2t}P_u$) the above-mentioned results suggest that this site does not function properly either by incorrect folding or by restricted accessibility of

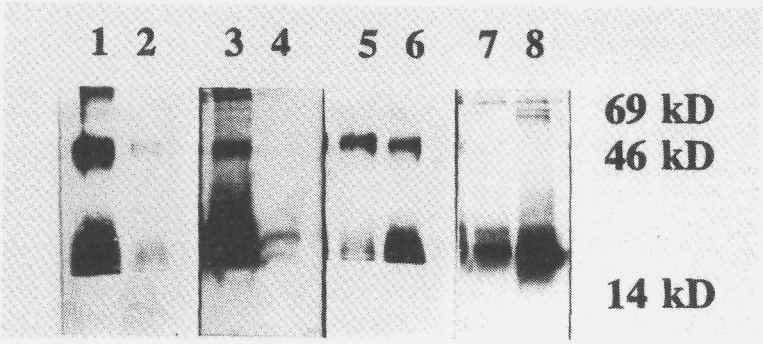

Figure 5. Binding of the aminoterminal fragment of the kringle 2 domain containing u-PA hybrid to lysyl Sepharose and Sepharose.

Reduced (r) or plasmin digested (p) $G_uK_{2t}P_u$ preparations containing ATF_r (S^1-K^{158}) or ATF_p (S^1-K^{135}) respectively, were applied to a 1 ml Sepharose or a 1 ml lysyl Sepharose column. The wash fraction and the elution fraction were dialysed and concentrated and analysed using polyacrylamide gel electrophoresis and Western blotting. ATF_r and ATF_p are present as bands of approximately 22 kD, non-reduced or undigested material was still present as a band of approximately 54 kD. lane 1: ATF_r , wash of the Sepharose column lane 2: ATF_r, eluate of the Sepharose column, lane 3: ATF_p, wash of the Sepharose column, lane 4: ATF_p, eluate of the Sepharose column, lane 5: ATF_r, wash of the lysyl Sepharose column, lane 6: ATF_r, eluate of the lysyl Sepharose column, lane 7: ATF_p, wash of the lysyl Sepharose column, lane 8: ATF_p, eluate of the lysyl Sepharose column.

the lysyl binding site. This may be caused by the presence of the protease domain, since the isolated aminoterminal fragment of this u-PA hybrid molecule, containing the growth factor and the kringle 2 domain of t-PA (G_uK_{2t}) was found to bind to lysyl Sepharose (figure 5).
To detect a possible interaction between the kringle 2 domain and the protease domain, direct binding experiments of isolated t-PA kringle 2 domains with various urokinase forms and control proteins were performed (figure 6). It was found that both High Molecular Weight u-PA (HMW u-PA, $G_uK_uP_u$) as well as Low Molecular Weight u-PA (LMW u-PA, P_u) interact with the kringle 2 domain of t-PA, whereas hardly any binding was observed with control proteins (figure 6) .

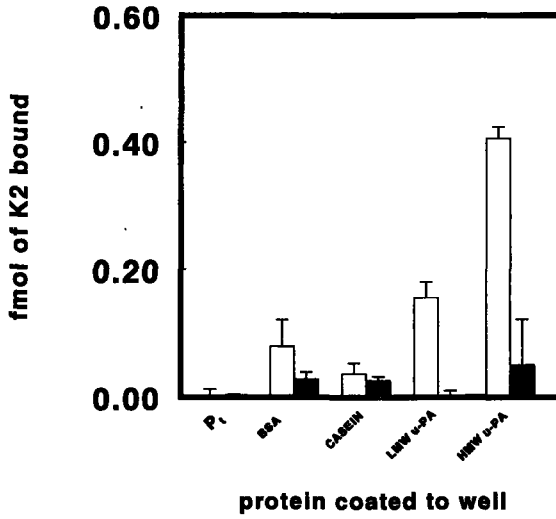

Figure 6. Binding of isolated kringle 2 domain to High Molecular Weight u-PA and Low Molecular Weight u-PA.

A: Binding of kringle 2 domain to different proteins. On the Y-axis amount of kringle 2 domain bound, on the X-axis the different proteins used in this study Binding was done in triplicate and the mean and the standard error is given. In the absence of EACA (□), in the presence of 50 mM EACA (■). For details see "Experimental Procedures".

Binding of the kringle 2 domain with both u-PA forms could be inhibited by EACA, indicating that a lysyl binding site is involved. Introduction of a modified u-PA kringle or a t-PA kringle 2 into urokinase ($G_uK_{u(m)}P_u$, $G_uK_{2t}P_u$) did not result in fibrin induced enhancement of plasminogen activation. Furthermore the modified u-PA kringle did not endow the hybrids consisting of only kringle and t-PA protease domain ($K_{u(m)}P_t$) with fibrin enhancement of plasminogen activation. This is only observed for the hybrids containing the kringle 2 domain originating from both t-PA ($K_{2t}P_t$) or $G_uK_{2t}P_u$ ($K_{2u}P_t$) (figure 7).

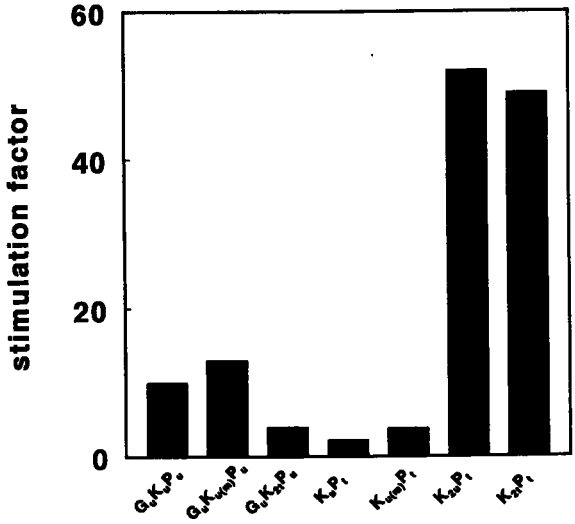

Figure 7: Stimulation factor of the studied plasminogen activators.

The conversion rate of plasminogen to plasmin in the absence and presence of fibrin mimic (CNBr fragments of fibrinogen) was determined as described under the " Experimental procedures ". The ratio of these values (stimulation factor) is plotted on the Y-as. The plasminogen activators tested are plotted on the X-as. For abbreviations of the plasminogen activators see figure 2.

Discussion

In previous studies combinations of structural domains of u-PA and t-PA have not resulted in the desired improvements of the fibrinolytic properties of the resulting hybrid plasminogen

activators. Therefore, instead of substitution of complete domains, we used a more sophisticated approach by substituting only stretches of amino acid residues within the u-PA kringle domain. This strategy has been shown to be successful in t-PA (Bakker et al., 1993).

We set out to create a functional lysyl binding site in the u-PA kringle with a view to introduce fibrin dependent functions in u-PA. Testing of the constructed molecules ($G_uK_{u(m)}P_u$, $G_uK_{2t}P_u$, $K_{2t}P_t$ and $K_{u(m)}P_t$) for lysyl- binding showed that the functioning of the kringle domain is dependent on the protein in which it is placed. The kringle 2 domains of t-PA placed N-terminal of the P_t domain of t-PA ($K_{2t}P_t$) results in a molecule that binds to lysyl Sepharose. However, introduction of the same kringle domain in u-PA result in a molecule ($G_uK_{2t}P_u$) which only very weakly interacts with lysyl Sepharose (Similar results were obtained with the uK2uPA molecule obtained from Asselbergs et al.,1993). The modified u-PA kringle in the u-PA molecule ($G_uK_{u(m)}P_u$) does not bind to lysyl -Sepharose, whereas the same modified kringle placed N-terminal of the protease domain of t-PA ($K_{u(m)}P_t$) does interact weakly with lysyl Sepharose. The difference in functionality of the lysyl binding sites thus seems to depend on the protein framework in which they are present. This is most likely the result of a reduced accessibility (by shielding or by intramolecular occupation) of the lysyl binding site which is stronger in combination with P_u than with P_t. This is corrobarated by the observation that the isolated aminoterminal fragment from the $G_uK_{2t}P_u$ molecule containing the growth factor domain and the kringle domain (G_uK_{2t}) does bind to lysyl Sepharose. Furthermore, the EACA dependent binding of isolated kringle 2 domain to u-PA suggests a direct involvement of the lysyl binding site in an intra-molecular interaction in the hybrids containing P_u.

Placing the modified u-PA kringle domain N-terminal of the protease domain of t-PA results in a weak interaction with lysyl Sepharose. We do not know if this is a consequence of a reduced accessibility of the lysyl binding site or due to the fact that the modified u-PA kringle does not constitute a strong lysyl binding site. Despite the high degree of similarity between the kringle 2 domain and the modified u-PA kringle, remaining differences in structure could be responsible for the relatively weak interaction.

Introduction of the t-PA kringle 2 domain, containing a functional

lysyl binding site, into the u-PA molecule ($G_uK_{2t}P_u$) does not result in an increase in fibrin dependent plasminogen activation. Recently, it was reported that fibrin dependent plasminogen activation of such molecules depends on the nature of the linker peptide between the newly introduced kringle 2 domain and the u-PA protease domain. With the linker peptide originating from t-PA (T^{235}-R^{275}), fibrin dependent plasminogen activation could be demonstrated, whereas when the linker peptide originates from u-PA (G^{144}-K^{158}) no fibrin dependent plasminogen activation was observed (Colucci et al., 1993). Part of this u-PA linker peptide (F^{146}-K^{158}) inhibits the fibrin dependent plasminogen activation of t-PA (Liu et al., 1989) and competes with t-PA for fibrin binding. The presence of R^{154} and R^{156} appears to be essential for the inhibition of binding (Song et al., 1992). Whether this peptide interacts directly with the lysyl binding site of the kringle 2 domain of t-PA has not yet been established.

When the modified u-PA kringle domain is placed N-terminal of the protease domain of t-PA ($K_{u(m)}P_t$) no enhanced fibrin dependent plasminogen activation is observed. This could be due to the limited ability to bind lysyl residues. Recently it was reported that replacement of the u-PA kringle domain by the kringle 1 domain of plasminogen ($G_uK_{1p}P_u$) results in a lysyl Sepharose binding hybrid u-PA molecule (Boutaud and Castellino, 1993). However, no fibrin dependent plasminogen activation was observed indicating that the presence of a functional lysyl binding site is not sufficient for this phenomenon.

All of the above-mentioned observations in hybrid plasminogen activators suggest that the function of an autonomous folding unit is influenced by other domains. This result questions the concept of functional autonomy of structurally autonomous domains (Zonneveld et al., 1986b) and could help explain why the fibrinolytic properties of hybrid plasminogen activators have always been less than expected on the basis of the theory of autonomous domains.

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References

Aoki, N., Sumi, Y., Miura, O., and Hirosawa, S. (1993) Methods in Enzymology 223, 185 - 196
Asselbergs, F. A. M., Bürgi, R., Hamerman, J., Heim, J., van Oostrom, J., and Agnelli, G. (1993) Thrombos. and Haemostas. 69, 50 - 55
Bakker, A. H. F., van de Greef, W., Rehberg, E. F., Marotti, K.R. & Verheijen, J. H. (1993) J. Biol. Chem. 268, 18496 - 18501
Barlow, G.H., Francis, C.W., and Marder, V. J. (1981) Thromb. Res. 23, 541 - 547
Binder, B. C., and Spragg, J. (1980) Protides of the Biological Fluid 28, 391 - 394
Byeon, I. J. L. & Llinás, M. (1991) Journal of Molecular Biology 222, 1035 - 1051
Boutaud, A. and Castellino F. J. (1993) Archives of Biochemistry and Biophysics 303, 222 - 230
Carmeliet, P., Schoonjans, L., Kieckens, L., Ream, B., Degen, J., Bronson, R., Vos, R., Oord, van den J., Collen, D., and Mulligan, R.C. Nature 368, 419 - 424
Chomczynski, P., and Sacchi, P. (1987) Anal. Biochem. 161, 4156 - 159
Christensen, U., and Molgaard, L. (1992) Biochem. J. 285, 419 - 425
Collen, D. (1980) J. Int. Soc. Thrombos. and Haemostas. 43, 77 - 89
Colucci, M., Cavallo, L., G., Agnelli, G., Mele, A., Bürgi, R., Heim, J., and Semeraro, N. (1993) Thrombos. and Haemostas. 69, 446 - 472
Devlin, J. J., Devlin, P. E., Clark, R., O'Rourke, E. C., Levenson, and C., Mark, D. F. Biotechnology 7, 286 - 292
Gheysen, D., Lijnen, H. R., Piérard, L., de Foresta, F., Demarsin, E., Jacobs, P., De Wilde, M., Bollen, A., and Collen, D. (1987) J. Biol. Chem. 262, 11779 - 11784
Granelli-Piperno, A. & Reich, E. (1978) J. Exp. Med. 148, 223 - 234
Günzler, W. A., Steffens, G.J., Otting, F., Kim S. M.A., Frankus E., and Flohe, L. (1982) Hoppe-Seyler's Z. Physiol. Chem. 363, 1155 - 1165.
Heim, H., Asselbergs, F., Bürgi, R., Chaudhuri, B., Küenzi, M., Meyhack, B., Rajput, B., van Oostrom, J. (1989) Thromb. Haemostas. 62, 337
Hoylaerts, M., Rijken, D. C., Lijnen, H. R. & Collen, D. (1982) J. Biol. Chem. 257, Holmes, W. E., Pennica, D., Blaber, M., Rey, M. W., Günzler, W. A., Steffens, G. J., and Heyneker, H. L. (1985) Biotechnology 3, 923 - 929
Higgins, D. L., and Bennet, B. W. (1990) Annual Review of Pharmacology and Toxicology 30, 392 - 397
Jones, D.H., and Howard, B.H. (1990) Biotechniques 8, 178 - 183
Kruithof, E. K. O., Tran-Thang, C., Ransijn, A., and Bachmann, F. (1984) Blood 64, 907 - 913
Laemmli, U. K. (1970) Nature 15, 680 - 685
Lee, S. G., Kaylan, N., Wilhelm, J., Hum, W-T., Rappaport R., Cheng, S-M., Dheer, S., Urbano, C., Hartzell, R. W., Ronchetti - Blume, M., Levner, M., and Hung, P. P. (1988) J. Biol. Chem. 263, 2917 - 2924
Lijnen, R. H., Van Hoef, B., and Collen, D. (1986) Biochimica et Biophysica Acta 884, 402 - 408
Lijnen, R. H., and Collen, D. (1991) Thrombos. and Haemostas. 66, 88 - 110
Lubin, I. M., Caban, R., and Runge, S. M. (1992) J. Biol. Chem. 268, 5550 - 5556 Liu, J., Song, A., Zhu, D., and Gurewich, V. (1992) in Peptides, Chemistry and Bilology. (Smith, J., and Rivier, J.E. eds.) pp. 810 - 811, ESCOM, Leiden
Munk, de G. A. W., Caspers, M. P. M., Chang, G. T. G., Pouwels, P. H., Enger-Valk, B. E. & Verheijen, J. H. (1989) Biochemistry 28, 7318 - 7325
Nelles, L., Lijnen, R. H., Collen, D., and Holmes, W. E. (1987) J. Biol. Chem. 262, 10855 - 10862
Nieuwenhuizen, W., Voskuilen, M., Traas.,D.W., Hoegee - Nobel, B.D. and Verheijen, J.H. (1985) in Fibrinogen-structural variants and interactions (Henschen, A., Hessel, B., McDonagh, J., and Saldeen T. eds.) pp. 331-342, Walter de Gruyter & co, Berlin pp 331-342.
Ny, T., Elgh, F. & Lund, B. (1984) Proc. Natl. Acad. Sci. USA. 81, 5355 - 5359
Onundarson, P. T., Francis, C. W., Marder, V. J. (1992) J. Lab. Clin. Med. 120, 120 -127
Pannell, R., and Gurewich, V. (1987) Blood. 1, 22 - 26
Pannekoek, H., Lijnen, H. R. & Loskutoff, D. J. (1983) Thromb. Haemostas. 64, 600 - 603
Pannekoek, H., Vries, de C., and Zonneveld, van A. J. (1988) Fibrinolysis 2, 123 - 132
Pennica, D., Holmes, W. E., Kohr, W. J., Harkins, R. N., Vehar, G. A., Ward, C. A., Bennett, W. F., Yelverton, E., Seeburg, P. H., Heyneker, H. L. & Goeddel, D. V. (1983) Nature 301, 214-221
Piérard, L., Quintana, L. G., Reff, M. E., and Bpllen, A. (1989) DNA 8, 321 - 328

Rånby, M. (1982) Biochim. Biophys. Acta. 704, 461 - 469
Rijken, D. C. & Groeneveld, E. (1986) J. Biol. Chem. 261, 3098 - 3102
Robbie, A., Booth, N., Croll, A. M., and Bennett, B. (1993) Thrombos. and Haemostas. 70, 301 - 305
Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Song, A., Liu, J., Yu, R., Cui, D., Zhou, T., Cui, H., and Zhu, D. (1992) Science in China. 35, 975 - 973
Serrano, de V. S., and Castellino, F.J. (1993) Biochemistry. 32, 3540 - 3548
Steffens, G.J., Gunzler, W. A., Otting, Frankus E., and Flohe, L. (1982) Hoppe-Seyler's Z. Physiol. Chem. 363, 1043 - 1058
Thorsen, S., Glas-Greenwalt, P. & Astrup, T. (1972) Thrombos. Diathes. heamorrh. 28, 65 - 74
Thorsen, S., Glas-Greenwalt, P. & Astrup, T. (1972) Thrombos. Diathes. heamorrh. 28, 65 - 74
Tulinsky, A., Park, H. P., and Skrzypczak-Jankun, E. (1988) J. Biol. Chem. 28, 885 - 901 Towbin, H., Staehelin, T., and Gordon, J. (1979) Proc. Natl. Acad. Sci. USA 76, 4350 -4354
Verheijen, J. H., Caspers, M. P. M., Chang, G. T. G., Munk, de G. A. W. d , Pouwels, P. H. & Enger-Valk, B. E. (1986) EMBO Journal 5, 3525 - 3530.
Vos, de A. M., Ultsch, M. H., Kelley, R. F., Padmanabhan, K., Tulinsky, A., Westbrook, M. L. & Kossiakoff, A. A. (1992) Biochemistry 31, 270 - 279
Vries, de C. (1990) Structure and Function of Tissue-type Plasminogen Activator. A molecular Biological Approach. Thesis, University of Amsterdam.
Wallén, P., (1978) Progress in Fibrinolysis 3, 167 - 181
Wiman, B., and Collen, D. (1978) Nature 272, 549-550
Zonneveld, van A. J. v., Veerman, H. & Pannekoek, H. (1986a) Proc. Natl. Acad. Sci. USA. 83, 4670 - 4674
Zonneveld, van A. J., Veerman, H. & Pannekoek, H. (1986b) J. Biol. Chem. 261, 14214 - 14218

Chapter 4

The Position of the Structurally Autonomous Kringle 2 Domain Influences the Functional Features of Tissue-type plasminogen activator.

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Summary

Tissue-type plasminogen activator (t-PA) is composed of structurally autonomous domains. From the Aminoterminus of t-PA, a finger domain (F), an epidermal growth factor domain (G), two kringle domains (K1 and K2) and a serine protease domain (P) can be discerned. The K2 domain of t-PA is known to be involved in lysyl binding, fibrin binding and fibrin dependent plasminogen activation. To study the functional autonomy of the K2 domain in t-PA we constructed with the aid of a cassette t-PA gene (Rehberg et al.,(1989) Prot. Engng. 2, 371-377) mutant t-PA genes coding for four molecules (FGK1K2P, FGK2K1P, GK1K2P and GK2K1P) in which the K2 domain was placed in two different positions in t-PA . The DNAs of wildtype t-PA and the t-PA variants were expressed in CHO cells and the recombinant proteins were purified by affinity chromatography. All molecules were expressed in their single chain form and could be converted to their two chain form. With these molecules lysyl binding, fibrin binding and fibrin dependent plasminogen activation were studied. All variants showed affinity for lysyl Sepharose and aminohexyl Sepharose. Reversal of the K domains (FGK2K1P versus FGK2K1P and GK1K2P versus GK2K1P) resulted in a 23 % - 47 % weaker interaction to both lysyl Sepharose and aminohexyl Sepharose. Deleting the F domain (FGK1K2P versus GK1K2P and FGK2K1P versus GK2K1P) resulted in a 20 % - 70 % improvement of the interaction to lysyl Sepharose and aminohexyl-Sepharose. All variants bound to a forming fibrin clot. Reversal of the K domains (FGK1K2P versus FGK2K1P) reduced fibrin binding. In the presence of the lysine analogue ϵ- amino caproic acid only FGK1K2P bound to fibrin. All variants activated plasminogen. In the absence of fibrinogen CNBr fragments (mimic of fibrin) the reversal of the K domain (FGK2K1P) resulted in a 2 times improved plasminogen activation. In the presence of a fibrin mimic the plasminogen activation of the F domain deletion analogues GK1K2P and GK2K1P were found to be 2 - 4 times decreased. From these results we conclude that the functioning of t-PA in lysyl binding, fibrin binding and fibrin dependent plasminogen activation is dependent on the correct spatial orientation of the K2 domain within the t-PA molecule.

Introduction

Tissue-type plasminogen activator (t-PA) is a physiological fibrin selective thrombolytic agent (Thorsen et al., 1972, Collen, 1980). In the presence of polymerised fibrin, the component which gives a bloodclot its rigid structure, t-PA efficiently converts the proenzyme plasminogen to plasmin (Hoylaerts et al., 1982; Rånby, M., 1982a; Binder et al., 1979, Niewenhuizen et al.,1985). Plasmin subsequently degrades the fibrin network resulting in destruction of the blood clot.

Elucidation of the gene structure of t-PA showed the presence of exons coding for domains which also are similar in other proteins (Patthy et al., 1990). From the N-terminus of the protein, t-PA consists of a finger domain (F) analogous to that in fibronectin, an epidermal growth factor domain (G) analogous to that in the epidermal growth factor, two kringle domains (K1, K2) first described in prothrombin and a serine protease (P) first found in trypsin (Pennica et al.,1983; Ny et al., 1984; Friezner-Degen et al., 1986).

Structural studies on isolated K domains of plasminogen, prothrombin, urokinase-type plasminogen activator and t-PA showed an overall similar folding pattern (Mulichak et al., 1991; Wu et al., 1991; Tulinsky et al., 1988; Li et al., 1994; Byeon and Llinás, 1991 ; Vos et al., 1992). These observations support the view that kringle domains are structurally autonomous folding units.

Studies with isolated and in vitro refolded parts of t-PA revealed that functional features of the intact molecule are localized in certain domains. The plasminogen activating property of t-PA and the activity towards synthetic substrates are localized in the P domain (Rijken et al., 1986). Experiments with the isolated and in vitro refolded FGK1K2 part of t-PA showed that this part binds to fibrin (Rijken et al., 1986). Studies with domain deletion variants of t-PA extended this observation. Deletion of the F domain, results in a reduced fibrin binding of the t-PA variant (Verheijen et al., 1986, Zonneveld et al., 1986a). A second fibrin interaction site was located in the K2 domain. This interaction can be disturbed with the anti fibrinolytic ϵ amino caproic acid (EACA) (Zonneveld et al., 1986b). Amino acid residue substitution variants of t-PA showed that only the K2 domain in t-PA is responsible for the interaction with EACA (Weening - Verhoeff et al., 1990,

Bennet et al., 1991). The K_d's for the interaction between EACA and isolated K2 and between EACA and t-PA do not differ significantly, indicating that the surrounding of the K2 domain has little influence on the lysyl binding feature (Munk et al., 1989; Byeon et al., 1989). Therefore this binding site, referred to as lysyl/fibrin binding site is thought to function independently of the F domain. Determination of the stoichiometry and the K_d's of the fibrin binding sites in t-PA supported the existence of two non identical independent fibrin binding sites, one located in the F domain and one located in the K2 domain (Nesheim et al., 1990). The fibrin dependent plasminogen activation was localized in the K2P part of t-PA (Verheijen et al., 1986; Zonneveld et al., 1986a). Since enzymatic activities of both isolated (and in vitro refolded) and recombinant P domain show no fibrin dependency, it was concluded that the K2 domain is involved in fibrin dependent plasminogen activation (Verheijen et al., 1986). Recently it was shown, that the t-PA analogue K1P does not bind fibrin, lysyl residues and that plasminogen activation is not fibrin dependent (Bakker et al., 1993).

The aforementioned observations are in line with the idea that the structurally autonomous domains behave like functionally autonomous domains independent of their surroundings in t-PA. Since the K2 domain is involved in lysyl binding, fibrin binding, and fibrin dependent plasminogen activation, it was possible to study the relation between the position of the K2 domain and the functional performance of the resulting t-PA molecule. To this end we made use of a cassette t-PA molecule (Rehberg et al., 1989). This model gene enabled the construction of mutant molecules in which the K2 and K1 are reversed in the full length molecule (FGK2K1P versus FGK1K2P) and in a F domain deleted variant (GK2K1P versus GK1K2P). Here we report that the functioning of the t-PA molecule in lysyl binding, fibrin binding and fibrin dependent plasminogen activation is dependent on the position of the K2 domain challenging the concept of fully functionally autonomous domains.

Material and Methods

construction of mutant proteins

The following mutants were constructed (Nomenclature according to Pannekoek et al., 1990): 1. UG3 referred to as FGK1K2P : t-PA ins (R2), K49N, S50A, E85D, del (S178), S262A, ins(A264), L265R, Q268Y , 2. UG7 referred to as GK1K2P: UG3 del (S1-A50), 3. UG9 referred to as FGK2K1P: UG3del (N177-C261) ins (UG3:N177-C261) insertion took place after D85, 4. UG31 referred to as GK2K1P: UG9del (S1-A50).
Construction of the mutant DNAs was carried out as described (Rehberg et al.,1989). Briefly, oligonucleotides were synthesized in four synthetic blocks, block 1 (157 bp Bgl 11- Sph1 fragment) coding for the finger domain, block 2 (105 bp Sph1-Cla 1 fragment) coding for the growth factor domain, block 3 (269 bp Cla1 - Bamh1 fragment) coding for kringle one domain and block 4 (561 bp BamH1 - EcoR1 fragment) coding for the kringle 2 domain . After additon of a polylinker EcoR1- BglII (with a unique HindIII restriction site) to block 1, all the blocks were ligated sequentially to yield full length analogue t-PA gene (synthetic heavy chain till Sca1 site and wildtype from there on). This complete t-PA sequence was subcloned in puc 19 as an HindIII - Aat I fragment. GK1K2P was constructed by removal of the sequence, coding for the F domain at the SphI site. FK1K2P was constructed by removing the sequence coding for the G domain between the HpaI and EcoRV sites. FGK1P was constructed by removing the sequence coding for the K2 domain between the HpaI and the MstI site. FGK1K2P was constructed by cutting FGK1P with EcoRV and inserting a K2 domain coding sequence as a HpaI-Mst1 fragment. Finally the GK2K1P was constructed by removal of the of the coding sequence for the F domain at the Sph1 site. For the expression in CHO cells each constructed analogue was cloned in an expression vector containing the gene for dihydrofolate reductase, the immediate early promotor of the cytomegalovirus as well as a polyadenylation signal from the bovine growth hormone gene (Rehberg et al. 1989).

CHO cell transfection

Tissue-type PA expression plasmids were used to transfect dihydrofolate-reductase-deficient Chinese hamster Ovary (CHO^{dhfr-} cells) by calcium phosphate co-precipitation (Rehberg et al., 1989). All transfections were done in duplicate. Cells that incorporated the plasmids and were $dhfr^+$ were selected in Dulbecco's MEM supplemented with 10% (v/v) fetal bovine serum, 0.1 % (w/v) non-essential amino acids, 10 mM Hepes, pH 7.3, 100 U/ml penicillin and 100 ug/ml of streptomycin.
Recombinant t-PA and t-PA analogues were purified by immunoaffinity chromatography using a monoclonal antibody against P domain (ESP-2) of t-PA coupled to agarose (Campro Scientific, the Netherlands). A 6.0 cm x 2.0 cm column packed with 10 ml of sephadex G-25 was overlaid with 1.0 ml of the immuno resin. The column was equilibrated with a buffer containing 0.1 M Tris-HCl, pH 7.5 and 0.01 % (v/v) Triton X-100 . Conditioned media from CHO cells producing recombinant t-PA were loaded onto the column at a flow rate of 50 ml/h at 4 °C. The column was washed with five volumes of a buffer containing 0.1 M Tris-HCl, pH 7.5, 1.0 M NaCl and 0.01 % (v/v) Triton X-100. Subsequently, the column was re-equilibrated with five column volumes of the same buffer without NaCl. The t-PA was eluted from the column with a buffer containing 0.1 M Tris-HCl, pH 7.5, 0.01 % (v/v) Triton X-100 and 3.0 M KSCN. Column fractions containing active enzyme were pooled for further characterization. With these tandem columns t-PA is immediately separated from the KSCN after elution. Typically the t-PA activity peak is around fraction 10, whereas KSCN only appears in fraction 16 or later (Rehberg et
al.,1989).

Gel electrophoresis and zymography

Polyacrylamide gel electrophoresis in the presence of SDS was performed under non-reducing conditions on 10 % acrylamide gels with 5 % stacking gels using the Laemmli system
(Laemmli, 1970). After electrophoresis, gels were washed in 2.5% (v/v) Triton X-100 to remove SDS and placed on plasminogen-containing fibrin agarose layers (Granelli-Piperno and Reich, 1978). Upon incubation the positions of plasminogen activators appear as clear lysis zones on an opaque background.
Gels used to examine active-site inhibited t-PA analogue ([^{125} I]-PAB-CMK treated t-PA analogue, see active site labeling on page 86) were run under reducing conditions. Lanes were subsequently sliced (slice width 2.5 mm) and the amount of radioactivity per slice determined by γ -counting.

Conversion of single chain t-PA to two chain t-PA

Conversion of t-PA and analogues from the single chain form to the two chain form was performed as described previously (Wallén et al., 1981). In short, 5 ml plasmin-Sepharose slurry (200 μg plasmin/ g Sepharose-4B (

wet weight, Pharmacia, Uppsala, Sweden)) or 5 ml Sepharose-4B were washed twice with 50 ml 10 mM Tris-HCl, pH 7.5 and 0.01% (v/v) Tween-80 . 50 µl plasmin-Sepharose slurry (consists of 1 volume of plasmin-Sepharose + 1 volume of 10 mM Tris-HCl, pH 7.5 and 0.01% (v/v) Tween-80) or Sepharose-4B (consists of 1 volume of Sepharose-4B + 1 volume of 10 mM Tris-HCl, pH 7.5 and 0.01% (v/v) Tween-80) was added to 450 µl t-PA analogue (60 nM) in the same buffer. The reaction was carried out with constant mixing at 37 °C. samples (20 µl) were removed from the incubation mixture at time intervals (t=0, t=10, t=20, t=30, t=60, t=90 minutes) spun down and transferred to wells of a microtiterplate containing 130 µl of 100 mM Tris-HCl, pH 7.4, 0.1 % (v/v) Tween-80 and 20 KIU/ml Trasylol (Bayer, Leverkusen, Germany). Conversion of t-PA and analogues from the single-chain to the two-chain form was confirmed by spectrophotometric activity determination with S-2366 (L-Pyro-Glu-L-Pro-L-Arg-p-nitroanilide hydrochloride, KabiVitrum, Stockholm, Sweden) and S-2288 (H-D-Ile-L-Pro-L-Arg-p-nitroanilide dihydrochloride, KabiVitrum, Stockholm, Sweden). Spectrophotometric assays were performed as previously described (Verheijen et al., 1985). Briefly, the reaction mixture (250 µl total volume) contained plasmin Sepharose or Sepharose treated t-PA analogues, 100 mM Tris-HCl, pH 7.4 , 0.1 % (v/v) Tween-80 trasylol 20 KIU/ml and 1.0 mM S-2366 or S-2288. The absorbance of the reaction mixtures was measured at 405 nm in an eight-channel microtiter plate reader against suitable blanks without termination of the reaction. The absorbance at 405 nm was plotted against time ($\Delta A/\Delta h$) for each of the six time points. The activity ratio (plasmin-Sepharose treated analogue/Sepharose treated analogue) is plotted on the Y-as against incubation time which is plotted on the X-as. The mean and standard error of the mean from three independent experiments are shown.

Labeling of t-PA analogues

For labeling of the two-chain t-PA and two-chain analogues an active site directed inhibitor of t-PA was used. The inhibitor 4-aminobenzoyl-Gly-Arg-CH_2Cl (a kind gift of Dr. E. Shaw) was iodinated with [^{125}I] and purified as described (Rauber et al., 1988). t-PA analogues (5nM) in 0.1 M Tris-HCl, pH 7.5, and 0.1% (v/v) Tween 80 were incubated with 2 µM iodinated inhibitor for 4 h at room temperature. Radiolabeled t-PA and t-PA analogues were bound on a 1 ml column of Zn chelate-Sepharose, extensively washed with 0.02 M Tris HCl, pH 7.4, 1 M NaCl, and 0.01% v/v Tween 80, and eluted with the same buffer containing 100 mM imidazole (Merck, Darmstadt, Germany). Specific activity of the labeled t-PA analogues was approximately $2.6x10^4$ cpm/pmol.

Binding of two chain t-PA and analogues to lysyl Sepharose and aminohexyl Sepharose.

Binding to a lysyl Sepharose and aminohexyl Sepharose column was performed as described before (Munk et al., 1989). In short; radiolabeled two chain molecules (approximately 100 fmol in 500 µl) in buffer (0.1 M Tris-HCl, pH 7.4 ,0.4 M NaCL, 0.01 % (v/v) Tween-80) were applied to 1 ml lysyl Sepharose, aminohexyl Sepharose or Sepharose columns (Pharmacia, Uppsala, Sweden) equilibrated in the same buffer (flow rate 50 ml/h at room temperature). These columns were washed with 2.5 ml (0.1 M Tris-HCl, pH 7.4, 0.4 M NaCl, 0.01 % (v/v) Tween-80. Specifically bound analogue was eluted with 2.5 ml buffer containing 50 mM EACA. Radioactivity was assessed in run-through, washing fluid, eluate and in the column . The mean and standard error of the mean of three independent experiments are shown. Subsequently the specifically bound fraction of t-PA analogues was expressed as fraction of the specifically bound FGK1K2P within each of the three experiments, and the mean and standard error of the mean for three independent experiments were determined.

Binding to a forming fibrin clot.

Fibrin binding was performed as described previously (Munk et al., 1989). Radiolabeled two-chain molecules (approximately 0.1 nM, final concentration) were mixed with fibrinogen (Kabi Diagnostica AB, Mölndal, Sweden), which was first made plasminogen-free, in the presence of 500 KIU/ml trasylol. Clotting was performed with 2 NIH/ml thrombin (Leo, Ballerup, Denmark). After 1 hour incubation at 37 °C, clots were centrifuged and radioactivity in the supernatant was determined, t-PA analogue bound was expressed as the fraction of the total amount of t-PA analogue added to the fibrinogen solution.

Determination of the t-PA analogue concentration.
Active site titration was performed as decribed before (Rehberg et al., 1989).

In short: 500 µl plasmin Sepharose treated t-PA analogue diluted in 10 mM Tris-HCl, pH 7.5, 0.01 % Triton X-100 were incubated with 150 µCi (4.4 Ci/mmol) 1,3[^{3}H]DFP for 16 h at 22 °C. For each assay a standard curve was constructed by treating various amounts of known t-PA concentration (American Diagnostica, New York, NY) with 150 µCi (4.4 Ci/mmol) 1,3[^{3}H]DFP under the experimental condition described above. At the end of the incubation period the proteins were treated with 500 µl of a 10 % TCA solution for 2 h at 22 °C.

Precipitates were collected on glass-fiber filters and washed extensively with 5 % TCA and then with isopropanol. The TCA precipitate on each filter was counted in Liquifluor-toluene scintilation coctail. The number of moles of active enzyme in a sample was determined by extrapolation from the t-PA standard curve.

Determination of the plasminogen activating activity of t-PA analogues in the absence and presence of fibrin mimic.

Spectrophotometric assays of the plasminogen activation activity of t-PA were performed as described previously (Verheijen et al., 1982). Briefly, the reaction mixture (250 μl total volume) contained various amounts of plasmin-Sepharose treated t-PA analogues, 100 mM Tris-HCl, pH 7.4, 0.1% (v/v) Tween 80, 0.12 μM Glu-plasminogen and 0.7 mM S-2251 (H-D-Val-L-Leu-L-Lys-p-nitroanilide, KabiVitrum, Stockholm, Sweden). In some cases, 120 ug/ml of CNBr-digested fibrinogen as a soluble fibrin mimic were included. The absorbance of the reaction mixtures was measured at 405 nm in an eight-channel microtiter plate reader against suitable blanks without termination of the reaction. 0, 5, 10 and 20 μl of a 60 nM t-PA solution were tested in an assay volume of 250 μl in the absence of fibrin mimic When CNBr fragments of fibrinogen were present 6 nM of t-PA or t-PA analogue were used. Plasminogen activating activities were determined as follows: the change in absorbance was monitored over time for each t-PA analogue in the presence and in the absence of a CNBr digest of fibrinogen. For each volume of t-PA or t-PA analogue used a slope of the the absorbance change over time-squared ($\Delta mA/\Delta h^2$) was calculated. These slopes, in turn, were plotted as a function of the different volumes of t-PA solution (0, 5, 10 and 20 μl) used, which yields the absorbance change per time-squared per volume of t-PA solution and finally expressed as $\Delta A/\Delta h^2 pmol^{-1}$ t-PA or t-PA analogue. The data of three independent

experiments were averaged and the standard error of the mean calculated.

Results

To study the effect of different positions of the K2 domain on the function of t-PA in lysyl binding, fibrin binding and fibrin-dependent plasminogen activation, we constructed two different sets of mutant molecules. One set consisted of FGK1K2P and FGK2K1P. The K1 domain was reversed with the K2 domain in the full length t-PA molecule leaving the overall structure intact. A second set t-PA analogues consisted of the F domain deletion variants GK1K2P and GK2K1P. Normal wildtype t-PA was included as a control.

After construction of the t-PA variants DNAs, they were expressed in CHO cells. The secreted proteins were purified from tissue culture medium using affinity chromatography. All proteins were expressed and showed the expected molecular weight on a zymogram (fig. 1).

After treatment of these molecules with plasmin Sepharose, an increase in activity ratio was observed towards the synthetic substrate molecules S-2288 and S-2366, indicating that all molecules were converted to their two chain form (fig. 2a, b). To our surprise this increase in activity ratio was exceptionally high for the GK1K2P molecule; 60 fold versus a 10 - 15 fold increase for the single chain preparations, including wildtype single chain t-PA. No increase in activity was observed for the two chain t-PA

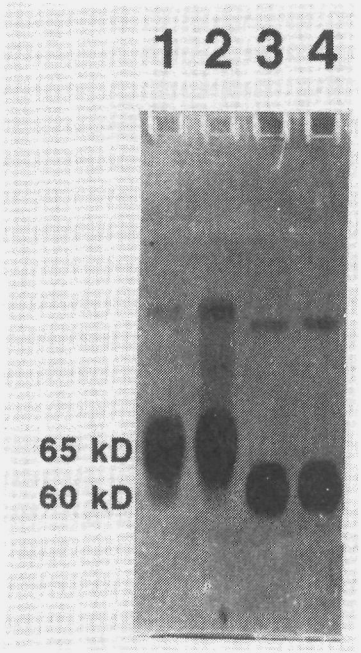

Figure 1: Fibrin zymography of t-PA analogues.

FGK1K2P (lane 1), FGK2K1P (lane 2), GK1K2P (lane 3) and GK2K1P (lane 4) were isolated from culture media by affinity chromatography. Gel electrophoresis under non reducing conditions and zymography were performed as described under Material and Methods. High molecular weight standards were run in a separate lane (not shown).

preparation.
Reversal of the K domain (FGK2K1P versus FGK1K2P and GK2K1P versus GK1K2P) resulted in 3 - 4 fold decrease of the activity ratio upon plasmin Sepharose treatment.
The interaction with lysyl Sepharose and aminohexyl Sepharose and Sepharose. None of the t-PA analogues were retained on a Sepharose column. All t-PA analogues showed affinity for aminohexyl Sepharose and lysyl Sepharose. When the EACA competable fraction was normalized within each experiment assigning to FGK1K2P a value of 1 (table 1), it was found that reversal of the K2 domain (FGK2K1P versus FGK1K2P and GK2K1P versus GK1K2P) resulted in a reduction of the interaction to lysyl Sepharose (40 % and 30 %, respectively) and aminohexyl Sepharose (30 % and 50 %, respectively). Deleting the F domain (GK1K2P versus FGK1K2P and GK2K1P versus FGK2K1P), resulted in an increase in the binding to lysyl Sepharose (20 % and 30 %, respectively) and aminohexyl-Sepharose (70 % and 20 % , respectively). However, it should be noted that the cassette t-PA (FGK1K2P) has a consistendly lower affinity for lysyl and aminohexyl Sepharose than wildtype t-PA.

Both the FGK1K2P, and the wildtype t-PA bound with high affinity to a forming fibrin clot (fig. 3a and fig. 3e). In the presence of the anti-fibrinolytic agent EACA a decreased binding of both molecules to a forming fibrin clot was observed. Deleting the F domain (GK1K2P) resulted in a reduced fibrin binding (fig. 3c) which could be completely inhibited with EACA. The kringle reversal mutant (FGK2K1P) showed a reduced binding to a forming fibrin clot and despite the presence of the F domain, no binding of this molecule was observed in the presence of EACA (fig. 3b). The F domain deletion variant (GK2K1P) showed comparable binding characteristics to full-length FGK2K1P (fig. 3d).

All t-PA analogues converted plasminogen to plasmin (fig. 4a).

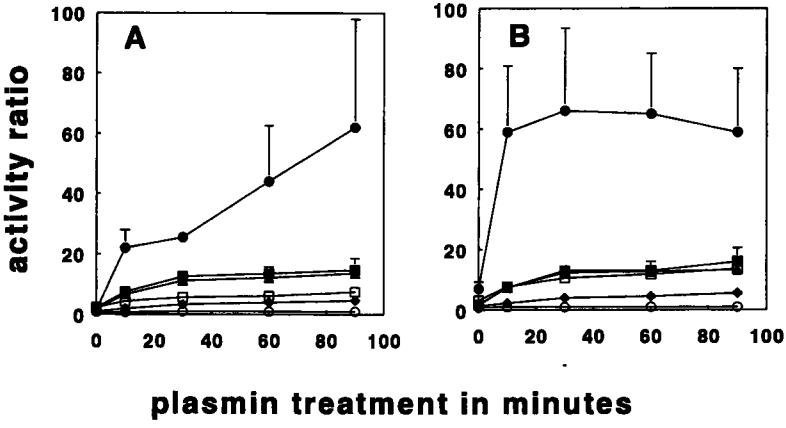

Figure 2: Plasmin Sepharose treatment of t-PA and t-PA analogues.

Single chain forms of t-PA or t-PA analogues were treated with plasmin Sepharose or Sepharose. Samples were taken from the incubation mixture at t= 0, 10, 20, 30, 60 and 90 minutes. The amidolytic activity towards the substrates S-2288 (panel a) and S-2366 (panel b) was determined. The activity ratio (activity ratio = amidolytic activity of plasmin-Sepharose treated t-PA analogue at $t=t_1$ / amidolytic activity of Sepharose treated t-PA analogue at $t=t_0$) is plotted on the Y-as (n=3), the incubation time with plasmin Sepharose or Sepharose in minutes is plotted on the X-as (▲ = FGK1K2P ◆ = FGK2K1P ● = GK1K2P ■ = GK2K1P ○ = t-PA two chain and □ = t-PA one chain , for details see Material and Methods)

t-PA analogue	fraction bound to lysyl - sepharose	fraction bound to aminohexyl - sepharose
FGK1K2P	1	1
FGK2K1P	0.62 ± 0.02	0.7 ± 0.1
GK1K2P	1.2 ± 0.1	1.7 ± 0.2
GK2K1P	0.9 ± 0.1	0.9 ± 0.2
t-PA	1.7 ± 0.1	1.7 ± 0.1

TABLE 1: Interaction of t-PA analogue with Sepharose, lysyl Sepharose and aminohexyl Sepharose.

Radiolabeled t-PA and t-PA analogues were applied to 1 ml columns of lysyl Sepharose or aminohexyl Sepharose.(See fig. 3a, b, c). Flow-through was collected and the columns were washed with 2.5 ml buffer and subsequently eluted with 2.5 ml buffer containing 50 mM of the lysine analogue ϵ amino caproic acid. The radioactivity present in the flow through, the washing fluid , the elution and remaining in the columns was determined. The radioactivity present in the elution fraction is expressed as fraction of the total radioactivity. After normalization (FGK1K2P = 1) within each experiment (n=3) the mean and the standard error of the mean were calculated (for details, see Experimental procedures).

The presence of fibrin mimic resulted in an inceased plasminogen activation for all analogues (fig. 4b, note the different scale). The determination of the stimulation factor revealed that reversal of the K domains or deletion of the F domain resulted in a marked reduction of the stimulation factor (fig. 4c).

Discussion

The K2 domain of t-PA plays an important role in several functional aspects of t-PA. It is important in lysyl binding, fibrin binding and fibrin-dependent plasminogen activation (Zonneveld et al.,1986; Verheijen et al.,1986). Isolated K2 domains interact with lysyl residues indicating that the K2 domain at least in lysyl binding is a functionally autonomous domain (Byeon et al., 1989). To study the functional autonomy of the K2 domain this domain was

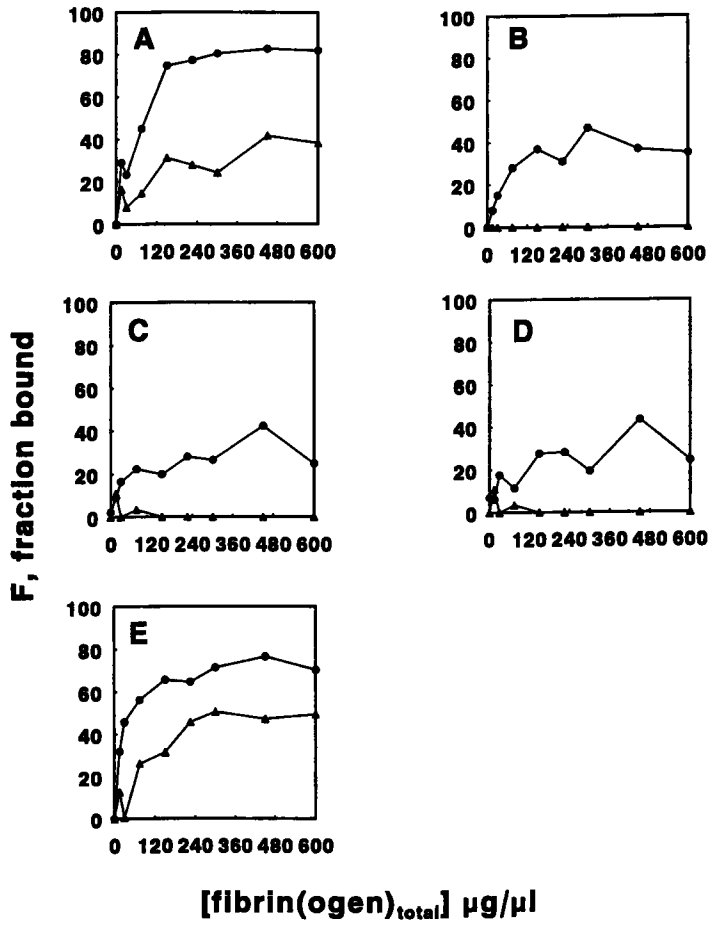

Figure 3: Binding of t-PA analogues to a forming fibrin clot in the presence and absence of the lysine analogue ϵ amino caproic acid.

Radiolabeled t-PA analogues FGK1K2P (panel A), FGK2K1P (panel B), GK1K2P (panel C) and GK2K1P (panel D) or t-PA (panel E) were mixed with varying amounts of fibrinogen and clotted with thrombin in the presence (-▲-▲-) or absence (-●-●-) of ϵ-amino caproic acid. Clots were centrifuged and the amount of label in the supernatant was determined using a γ-counter. Bound t-PA was then calculated on the basis of control experiments without fibrinogen or thrombin present. The fraction of bound t-PA is plotted on the Y-axis, the concentration of fibrinogen is plotted on the X-axis expressed as µg/ml (for details see Material and Methods).

placed in different positions within a t-PA analogue molecule. We constructed FGK2K1P, reversing only the kringle domains but leaving the overall spacing intact. We also constructed GK1K2P and GK2K1P in which the overall spacing was changed. If domains are functionally autonomous, independent of their surroundings, the FGK2K1P molecule should behave in a way comparable with the FGK1K2P and the GK2K1P should behave in a manner comparable with the GK1K2P in lysyl binding, fibrin binding and fibrin-dependent plasminogen activation.

Surprisingly, it was found that the activity ratio upon treatment with plasmin Sepharose differed for the variants. The activity ratio for the FGK2K1P and GK2K1P compared with the FGK1K2P and GK1K2P, differed by a factor 3 to 4. In contrast the activity ratios for FGK1K2P and GK2K1P were between 8 and 12 respectively, similar to the activity ratio of a pure single chain and two chain t-PA preparation. This is in agreement with previously reported activity ratios determined for pure single chain t-PA preparation converted to pure two chain t-PA preparations (Wallén et al. 1981; Rånby et al. 1982b; Verheijen et al.,1985). It has been shown previously that the t-PA analogues FGK1K2P, FGK2K1P and GK1K2P are in their single chain form. When completely converted to their two chain form these two chain t-PA analogues all show comparable amidolytic activities (Rehberg et al.,1989). Therefore, the observed difference in activity ratio is most likely caused by a difference of amidolytic activity of the single chain variants. Although we did not determine the K_M and the k_{cat} of these one chain variants one possible explanation for the observed differences in activity ratio could be the accessibility of the active site in the P domain of the various t-PA analogues. Absence of the F domain results in a less accessible P domain, while changing the position of the K2 domain results in a more accessible P domain.

Also, the interaction of t-PA with its natural substrate plasminogen depends on the position of the K2. Reversal of the kringle domains (FGK2K1P) results in a increased plasminogen activation in the absence of fibrin mimic. In the presence of fibrin mimic the position of the K2 domain influences the fibrin-dependent plasminogen activation only in the absence of the F domain.

Lysyl binding of the K2 domain has always been considered an autonomous functioning entity. Isolated K2 domain has been shown before to bind to EACA with comparable affinity to K2 domain

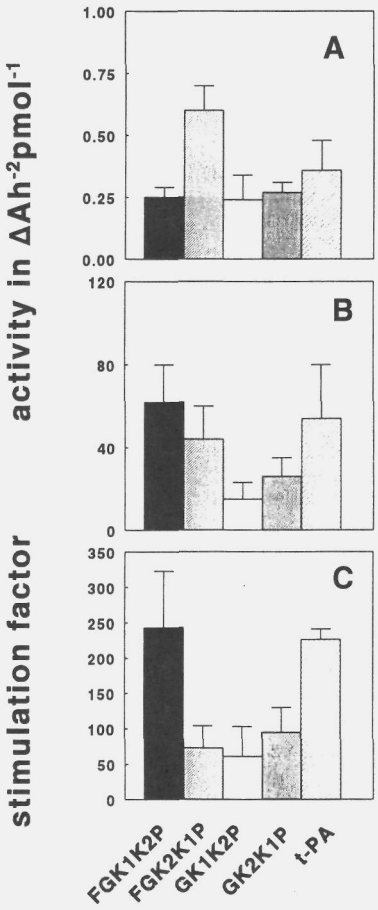

Figure 4: Plasminogen activating activity of t-PA analogues in the absence and presence of fibrin mimic.

Plasminogen activator activity of t-PA and t-PA analogues in the absence (panel A) or in the presence (panel B) of CNBr fragments of fibrinogen were determined as described in the Experimental procedures and expressed as $Ah^{-2}pmol^{-1}$ of t-PA or t-PA analogue. The standard error of the mean of three experiments are presented (for details, see Material and methods). The stimulation factor (panel C) is the ratio of the plasminogen activation activity in the presence of fibrin mimic and the plasminogen activator activity in the absence of fibrin mimic.

within the t-PA molecule, indicating that the surrounding did not significantly influence the lysyl binding properties of this domain (Munk et al.,1989; Byeon and Llinás, 1990). We found that the enviroment of the K2 domain had an effect upon lysyl binding. Changing the position of the K2 domain lowered the EACA competable binding while deleting the F domain increases the EACA competable binding to lysyl Sepharose and to aminohexyl Sepharose. Recently, it was shown that in the presence of Cl^-, isolated K2 domains within a K2 crystal interact via the Lys^{47} of one K2 domain and the lysyl binding site of another K2 domain (Vos et al., 1992). This observation indicates that the K_d for the interaction between K2 and lysine analogues in the presence of Cl^- must be considered with caution; the ligand probably competes with a lysyl residue of another K2 domain for the lysyl binding site in the K2 domain. The influence of the position of the K2 domain on the interaction with lysyl Sepharose and aminohexyl Sepharose might be explained by suggesting an intramolecular interaction between lysyl residues of the other domain in t-PA and the K2 domain. Structural studies on t-PA suggest that t-PA is an ellipsoid molecule, the F domain being in close proximity of the P domain (Margossian et al., 1993 ; Novokahatny et al., 1991). Whether this interaction is stabilised via an intramolecular interaction between lysyl residue and the lysyl binding site in the K2 domain is not yet known.

Another explanation for the difference in interaction with lysyl Sepharose and aminohexyl Sepharose might be a different glycosylation of the K2 domain in the kringle reversal mutant as compared with the normal FGK1K2P. It has been reported that glycosylation of Asn^{184} influences the interaction of t-PA with lysyl Sepharose (Parekh et al., 1989). However, at this moment we do not know if the observed results are caused by a difference in glycosylation.

Fibrin binding sites in t-PA have been localised in the F domain and the K2 domain. Recently the stoichiometry and the K_d for the two binding sites were determined. They justify a model of two independent, non-equivalent fibrin binding sites in t-PA (Nesheim et al., 1990). The kringle reversal mutant (FGK2K1P) which still contains the two potential interaction sites, shows reduced fibrin binding which could be completely abolished with EACA. This result shows that the two binding sites are not independent of one

another. The functioning of the F domain depends on the correct position of the K2 domain in the molecule.

In conlusion, we have shown that some properties of t-PA depend on the position of the K2 domain within the molecule. Furthermore the fibrin binding of the F-domain is dependent on the position of the K2 domain. It seems that the presumed structurally autonomous domains in t-PA do not completely function as autonomous domains but are dependent on their positions within the molecule. Recently it has been shown that the F and G domains of t-PA do not behave as structurally independent units, but more or less as a single domain (Smith et al., 1994).

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References

Bakker, A. H. F., van de Greef, W., Rehberg, E. F., Marotti, K.R. and Verheijen, J. H. (1993) J. Biol. Chem. 268, 18496 - 18501
Bennett, W. F., Paoni, N. F., Keyt, B. A., Botstein, D., Jones, A. J. S., Presta, L., Wurm, F. M. and Zoller, M. J. (1991) J. Biol. Chem., 266, 5191 - 5201
Binder, B. R. and Spragg, J. (1980) In Peeters, H. (ed), Protides of the Biological Fluids Pergamon Press, Oxford, 28, pp. 391 - 394.
Byeon, I. J. L., Kelley, R. F. and Llinás, M. (1989) Biochemistry, 28, 9350 - 9360
Byeon, I. J. L. and Llinás, M. (1991) J. Mol. Biol., 222, 1035 - 1051
Cleary, S., Mulkerrin, M. G. and Kelley, R. F. (1989) Biochemistry, 28, 1884 - 1891
Collen, D. (1980) J. Int. Soc. Thrombos. and Haemostas., 43, 77 - 89
Friezner-Degen, S. J., Rajput, B. and Reich, E. (1986) J. Biol. Chem., 261, 6972 - 6985
Graham F. L. and van der Eb A.J. (1973) Virology, 52, 456 - 467
Granelli-Piperno, A. and Reich, E. (1978) J. Exp. Med., 148, 223 - 234
Hoylaerts, M., Rijken, D. C., Lijnen, H. R. and Collen, D. (1982) J. Biol. Chem. 257, 2912 - 2919
Ichinose, A., Takio, K. and Fujikawa, K. (1986) J. Clin. Invest., 78, 163 - 169
Jay, D. G. (1984) J. Biol. Chem., 259, 15572 - 15578 Laemmli, U. K. (1970) Nature, 15, 680 - 685
Li, X., Smith, R. A. G. and Dobson, C. M. (1992) Biochemistry, 31, 9562 - 9571
Margossian, S. S., Slayter, H. S., Kaczmarek, E. and Mcdonagh, J. (1993) Biochim. Biophys. Acta, 1163, 250 - 256
Mulichak, A. M., Tulinsky, A. and Ravichandran, K. G. (1991) Biochemistry, 30, 10576 - 10588
Munk, de G. A. W., Caspers, M. P. M., Chang, G. T. G., Pouwels, P. H., Engervalk, B. E. and Verheijen, J. H. (1989) Biochemistry, 28, 7318 - 7325
Nesheim, M., Fredenburgh, J. C. and Larsen, G. R. (1990) J. Biol. Chem., 265, 21541 - 21548

Niewenhuizen, W., Voskuilen, M., Traas.,D.W., Hoegee-Nobel, B.D. and Verheijen, J.H. (1985) In Henschen, A., Hessel, B., McDonagh, J., and Saldeen T. (eds) Fibrinogen-structural variants and interactions. Walter de Gruyter and co, Berlin, pp 331-342.
Novokhatny, V. V., Ingham, K. C. and Medved, L. V. (1991) J. Biol. Chem., 266, 12994 - 13002
Ny, T., Elgh, F. and Lund, B. (1984) Proc. Natl. Acad. Sci. USA, 81, 5355 - 5359
Pannekoek, H., Lijnen, H. R. and Loskutoff, D. J. (1990) Thromb. Haemostas., 64, 600 - 603
Parekh, R. B., Raymond, A. D., Thomas, J. R., Opdenakker, G., and Rademacher, T. W. (1989) Biochemistry, 28, 7644 - 7662
Patthy, L. (1990) Blood coagulation and fibrinolysis, 1, 153 - 166
Pennica, D., Holmes, W. E., Kohr, W. J., Harkins, R. N., Vehar, G. A., Ward, C. A., Bennett, W. F., Yelverton, E., Seeburg, P. H., Heyneker, H. L. and Goeddel, D. V. (1983) Nature, 301, 214 - 221
Rånby, M., Bersdorf, N. and Nilsson, T. (1982b) Thromb. Res., 27, 175 - 183
Rånby, M. (1982a) Biochim. Biophys. Acta., 704, 461 - 469
Rauber, P., Wikstrom, P. and Shaw, E. (1988) Anal. Biochem., 168, 259 - 264
Rehberg, E. F., Theriault, N. Y., Carter, J. B., Palermo, D. P., Hubert, E. V., Bergum, P. W., Wunderlich, C. J., Erickson, L. A. and Marotti, K. R. (1989) Protein Engng., 2, 371 - 377
Rijken, D. C. and Groeneveld, E. (1986) J. Biol. Chem., 261, 3098 - 3102
Smith, B. O., Downing, A. K., Dudgeon, T. J., Cunningham, M., Driscol, P. C. and Campbell, I. D. (1994) Biochemistry, 33, 2422 - 2429
Tulinsky, A., Park, C. H. and Skrzypczak-Jankun, E. (1988) J. Mol. Biol., 202, 885 - 901
Thorsen, S., Glas-Greenwalt, P. and Astrup, T. (1972) Thrombos. Diathes. heamorrh., 28, 65 - 74
Tulinsky, A. (1991) Thrombos. and Haemostas., 66, 16 - 31
Verheijen, J. H., Caspers, M. P. M., Chang, G. T. G., Munk, de G. A. W. d., Pouwels, P. H. and Enger-Valk, B. E. (1986) EMBO J., 5, 3525 - 3530.
Verheijen, J. H., Jong, Y. F. d. and Chang, G. T. G. (1985) Thromb. Res., 39, 281 - 288.
Verheijen, J. H., Nieuwenhuizen, W. and Wijngaards, G. (1982) Thromb. Res., 27, 377 - 385.
Vos, de A. M., Ultsch, M. H., Kelley, R. F., Padmanabhan, K., Tulinsky, A., Westbrook, M. L. and Kossiakoff, A. A. (1992) Biochemistry 31, 270 - 279
Wallén, P., Rånby, M., Bergsdorf, N. and Kok, P. (1981) Progress in Fibrinolysis, 5, 16 - 23
Weening - Verhoeff, E. J. D., Quax, P. H. A., van Leeuwen, R. T. J., Rehberg, E. F., Marotti, K. R. and Verheijen, J. H. (1990) Protein Engng., 4, 191 - 198
Wu, T. P., Padmanabhan, K., Tulinsky, A. and Mulichak, A. M. (1991) Biochemistry, 30, 10589 - 10594
Zonneveld, van A. J. v., Veerman, H. and Pannekoek, H. (1986a) Proc. Natl. Acad. Sci. USA, 83, 4670 - 4674
Zonneveld, van A. J. v., Veerman, H. and Pannekoek, H. (1986b) J. Biol. Chem., 261, 14214 - 14218

The Role of the Lysyl Binding Site of Tissue-type Plasminogen Activator in the Interaction with a Forming Fibrin Clot.

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Summary

To describe the role of the lysyl binding site in the interaction of tissue-type plasminogen activator (t-PA, FGK1K2P) with a forming fibrin clot, we performed binding experiments with domain deletion mutants GK1K2P, K2P and the corresponding point mutants lacking the lysyl binding site in the absence and the presence of EACA. Occupation of the lysyl binding site in the K2 domain with EACA has a pronounced effect on the binding of FGK1K2P to a fibrin clot (C_{50} = 77 ± 11 nM versus 376 ± 45 nM with EACA). Deleting the lysyl binding site in the K2 domain (substitution D236N) also impairs fibrin binding but to a lesser extent (C_{50} = 169 ± 20 nM). Although the binding of K2P to a fibrin clot is weak (C_{50} = 1163 ± 490 nM), it still is two orders of magnitude stronger than the binding of EACA to K2P. Therefore it was surprising to find, that deletion of the lysyl binding site in K2P completely abolishes fibrin binding. Even when both the F domain and the lysyl binding site were deleted considerable fibrin binding is still observed (C_{50} = 557 ± 126 nM), suggesting other than F and K2 mediated interactions. The binding of FGK1K2P, FGK1K2P (D236N), GK1K2P and GK1K2P (D236N) to fibrin could be competitively inhibited by FGK1K2P and K2P, indicating that all molecules recognize the same interaction sites on a fibrin clot. Based on these results a new model for the interaction of t-PA with a forming fibrin clot is proposed. The fibrin binding sites in t-PA are not confined to the F and K2 domain. The main role of the lysyl binding site in the K2 domain of t-PA appears indirect rather than direct, most likely stabilizing a conformation favourable for fibrin binding.

Introduction

Only in the presence of fibrin does t-PA efficiently convert its substrate plasminogen into the fibrin degrading enzyme plasmin. The enzyme t-PA appears to play an essential role in dissolving fibrin rich clots in the blood stream (Thorsen et al., 1972; Collen., 1980; Carmeliet et al., 1994). Fibrin binding of t-PA is thought to be a prerequisite for this enhanced plasminogen activation (Hoylaerts et al., 1982; Rånby., 1982; Nieuwenhuizen et al., 1985). This fibrin binding is localized in the heavy chain of t-PA (

Rijken et al., 1986). After the elucidation of the c-DNA structure it became apparent that t-PA is composed of several domains (Pennica et al., 1983; Ny et al., 1984). From the N-terminus, t-PA consists of a finger domain (F), an epidermal growth factor domain (G), two kringle domains (K1, K2) and a serine protease domain (P). Both the F and K2 domain were found to be involved in fibrin binding to a forming fibrin clot (Verheijen et al., 1986; Zonneveld et al., 1986a). It was further shown that t-PA interacts with lysyl Sepharose and arginyl Sepharose (Radcliffe & Heinze, 1978 ; Wallén et al., 1981). The interaction with lysyl - Sepharose can be disturbed by L-lysine, L-arginine or the lysine analogue ϵ - amino caproic acid (EACA) (Radcliffe & Heinze, 1978; Allen & Pepper, 1981 ; Munk et al., 1989). The binding of t-PA with fibrin can be partially blocked with the lysine analogue EACA (Zonneveld et al., 1986b, Munk et al., 1989). Subsequently, isolated kringle 2 domains were shown to interact with EACA and lysyl Sepharose (Byeon et al., 1989; Serrano et al., 1993; Vos et al., 1992). This led to the view that t-PA binds to a forming fibrin clot via two modes: a lysyl binding site and a non-lysyl dependent interaction which requires the presence of the F domain (Zonneveld et al., 1986b).

Based on binding isotherms of t-PA to a forming fibrin clot, two independent non identical binding sites on the fibrin are proposed (Nesheim et al., 1990). The high affinity interaction is F domain mediated while a lower affinity interaction is K2 domain mediated. However, this model is questioned by the observation of one class of t-PA binding sites on a forming fibrin clot. Furthermore, the high affinity of t-PA for a forming fibrin clot could not be fully accounted for by the F mediated and the K2 mediated interaction (Horrevoets et al., 1994).

The role of the lysyl binding site of t-PA in fibrin binding is intricate. The K2 domain is thought to interact via an intra chain lysyl residue of the fibrin network and therefore the binding site was called aminohexyl binding site. It was shown that increasing the amount of carboxy terminal lysyl residues in the fibrin network by partial degradation with plasmin, results in new binding sites for t-PA (Vries et al., 1989). Although, the affinity of the K2 domain for aminohexyl Sepharose differs from the affinity for lysyl Sepharose, both interactions can be inhibited with EACA (Munk et al., 1989) Furthermore, deletion of the lysyl binding site in the K2

domain by the substitution of one aminoacid residue (Asp236 → Asn236) abolishes binding to aminohexyl and lysyl Sepharose (Weening-Verhoeff et al., 1990). Therefore, the structures in the K2 domain mediating lysyl binding and aminohexyl binding must be considered equivalent.
Substitution of one amino acid residue in the K2 domain (t-PA (D236N), t-PA (D236A) results in a t-PA analogue which no longer interacts with lysyl or aminohexyl-Sepharose but still possesses high affinity for fibrin (Weening-Verhoeff et al., 1990; Bennet et al., 1991). This observation stands in clear contrast to the large effect of EACA on the fibrin binding of t-PA (Zonneveld et al., 1986b, Munk et al., 1989).
We studied the role of the lysyl binding site of t-PA in fibrin binding by performing fibrin binding experiments with domain deletion mutants lacking a functional lysyl binding site in the absence and presence of EACA. To describe the interaction site of t-PA and t-PA variants on a forming fibrin clot we performed competition experiments with FGK1K2P and K2P.
We have found that for fibrin binding of t-PA other interactions than the F and the lysyl binding site mediated binding exists. Furthermore, the binding sites of FGK1K2P (D236N) mediated and the K2P mediated t-PA binding sites on a forming fibrin clot are in close proximity to each other. The lysyl binding site in the K2 domain appears not to interact directly with an aminohexyl group of the fibrin network, but is probably involved in stabilizing a favourable conformation of t-PA needed for fibrin binding.

Experimental procedures

proteins used in this report

Nomenclature and numbering of t-PA mutant proteins is according to Pannekoek (Pannekoek et al., 1983). Recombinant t-PA referred to as FGK1K2P, t-PA del (I5-H44) referred to as GK1K2P and t-PA del (R7-C168) referred to as K2P and the point mutant t-PA D236N referred to as FGK1K2P (D236N) have been described before (Verheijen et al., 1986; Weening-Verhoeff et al., 1990). The construction of the corresponding domain deletion mutants t-PA D236N del (I5-H44) referred to as GK1K2P (D236N) and t-PA D236N del (R7- C168) referred to as K2P (D236N) are described below.

construction of mutant proteins

The construction of the t-PA D236N del (I5-H44) was performed as follows: from the plasmids containing the reading frame for GK1K2P (peV2t-PA4, Verheijen et al., 1986) A 3835 bp NarI - SacI restriction fragment lacking the K1K2 and part of the P domain was isolated. From the plasmid containing the sequence coding for FGK1K2P (D236N) (Weening Verhoeff et al., 1990) a 900 pb NarI - SacI restriction fragment containing K1K2 (with the D236N substitution) and part of the P domain was isolated. This fragment was ligated into the above mentioned 3835 bp fragment according to Sambrook (Sambrook et al.,1989).

Construction of t-PA D236N del (R7-C168) was performed as follows: from the plasmid containing the sequence coding for FGK1K2P (D236N) (Weening-Verhoeff et al., 1990) a Pst 1 partial fragment of 4249 bp, missing the FGK1 fragment of 486 bp was isolated, and ligated with itself according to Sambrook (Sambrook et al.,1989).

LB6 cell transfections

t-PA expression plasmids were used to transfect mouse L cells (LB6) by calcium phosphate co-precipitation with peV2/Neo which contains the gene for Aminoglycoside Phoshotransferase 3' (Graham and van der Eb., 1973). Cells that incorporated the plasmids and thus were Neo-resistant were selected in Dulbecco's MEM supplemented with 10 % (v/v) fetal calf serum (Boehringer / Mannheim, Germany), L-glutamine (Gibco)), 100 U penicillin/ml and 100 ug/ml streptomycin (Gibco) and 1.2 mg/ml of the neomycin analogue geneticin (Gibco). For purification of the recombinant proteins, cells were cultured in Dulbecco's MEM supplemented with 100 KIU/ml Trasylol (Bayer, Leverkusen, Germany) and 10 mM ϵ- amino caproic acid (Merck, Darmstadt, Germany) to prevent plasmin activity, 0.3 g/l human serum albumin (CLB, Amsterdam, The Netherlands) L-glutamine , 100 U penicillin/ml and 100 ug/ml streptomycin and 1.2 mg/ml geneticin. Recombinant t-PA mutants were purified by immunoaffinity chromatography using a monoclonal antibody ESP-2 (ESP-2, Campro Scientific, The Netherlands) against the protease domain of t-PA coupled to agarose. A 0.5 ml aliquot of anti t-PA -Sepharose suspension was placed on a disposable PD-10 gel filtration column (Pharmacia). The tandem column was equilibrated with 0.1 M Tris-HCl, 0.01 % (v/v) Tween 80, pH 7.5. Approximately 50 ml of conditioned medium was loaded onto the column followed by washing with the buffer mentioned above. The column was then washed with 2 column volumes of a buffer containing 0.1 M Tris-HCl, pH 7.5, 1.0 M NaCl and 0.01 % (v/v) Tween 80. Subsequently the column was reequilibrated with the same buffer without NaCl. The t-PA mutant was eluted from the column with a buffer containing 0.1 M Tris-HCl, 0.01 % (v/v) Tween 80 and 3.0 M KSCN, pH 7.5 (Merck). Column fractions containing plasminogen activator activity were pooled for further characterization.

Gel electrophoresis and zymography

Polyacrylamide gel electrophoresis in the presence of SDS was performed under non-reducing conditions on 10 % acrylamide gels with 5 % stacking gels using the Laemmli system (Laemmli, 1970). After electrophoresis, gels were washed in 2.5 % (v/v) Triton X-100 to remove SDS and placed on plasminogen-containing fibrin agarose layers (Granelli-Piperno & Reich., 1978). Upon incubation the positions of plasminogen activators appear as clear lysis zones on an opaque background.

Conversion of the single chain to the two chain form of t-PA and t-PA analogues

Conversion of the single chain form t-PA analogues to the two chain form was performed as described previously (Wallén et al., 1981). In short, plasmin-Sepharose slurry (200 μg plasmin/ g wet Sepharose-4B or Sepharose-4B (Pharmacia) was washed with 10 mM Tris-HCl,pH 7.5 and 0.01 % (v/v) Tween-80. 50 μl of a 50 % plasmin-Sepharose suspension or 50 % Sepharose-4B suspension was added to 450 μl t-PA analogue (10 pmol) in the same buffer. The reaction was carried out with constant mixing at 37 °C. Samples were removed from the incubation mixture at time intervals (t=0, t=10, t=20, t=30, t=60, t=90 minutes) spun down and 20 μl was transferred to wells of a microtiterplate containing 130 μl of 100 mM Tris-HCl, pH 7.4, 0.1 % (v/v) Tween-80 and 20 KIU/ml trasylol . Conversion of t-PA analogues from the single-chain to the two-chain form was confirmed by spectrophotometric activity determination with S-2288 (H-D- Ile-L-Pro-L-Arg-p-nitroanilide dihydrochloride, Chromogenix, Mölndal, Sweden). Spectrophotometric assays were performed as previously described (Verheijen et al., 1985). Briefly, the reaction mixture (250 μl total volume) contained plasmin-Sepharose or Sepharose treated t-PA analogues, 100 mM Tris-HCl, pH 7.4 , 0.1 % (v/v) Tween-80 trasylol 20 KIU/ml and 1 mM S-2288. The absorbance of the reaction mixtures was measured at 405 nm in an eight-channel microtiter plate reader against suitable blanks without termination of the reaction. The absorbance at 405 nm was plotted against time ($\Delta A/\Delta t$) for six time points.

Labelling of t-PA and t-PA analogues

For labelling of two chain t-PA and t-PA analogues an active site directed inhibitor of t-PA was used. The inhibitor 4-aminobenzoyl-Gly-Arg-CH2Cl (a kind gift of Dr. E. Shaw) was iodinated with [^{125}I] and purified as described before (Rauber et al., 1988). 1.5 pmol of t-PA analogues in 100 μl 0.1 M Tris-HCl, pH 7.5, and 0.1 % (v/v) Tween 80 were incubated with 2 μM iodinated inhibitor for 4 h at room temperature. Radiolabelled t-PA and t-PA analogues were bound on a 1 ml column of Zn chelate-Sepharose and extensively washed with 0.02

M Tris-HCl, pH 7.4, 1 M NaCl, and 0.01 % (v/v) Tween 80, and then eluted with the same buffer containing 100 mM imidazole (Merck, Darmstadt, Germany). Specific activities of the labelled t-PA or t-PA analogues (final concentration 5 nM) were approximately 2.6 10^4 cpm/pmol.

Binding of two chain t-PA or t-PA analogues to lysyl Sepharose.

Binding to a lysyl Sepharose column was performed as described before (Munk et al., 1989). In short radiolabelled two-chain t-PA analogues (approximately 100 fmol in 500 μl) in buffer (0.1 M Tris-HCl, pH 7.4 ,0.4 M NaCl, 0.01 % (v/v) Tween-80) were applied to 1 ml lysyl Sepharose columns (Pharmacia) equilibrated in the same buffer
(flow rate 50 ml/h at room temperature). These columns were washed with 2.5 ml buffer. Specifically bound analogues (see below) was eluted with 2.5 ml buffer containing 50 mM EACA. Radioactivity was assessed in run-through, washing fluid, eluate and in the column. The fraction of the total cpm is given.

Binding of t-PA or t-PA analogues to a forming fibrin clot.

Fibrin binding was performed as described before (Munk et al., 1989). Radiolabelled t-PA analogues (0.06 nM, final concentration) were mixed with fibrinogen (Chromogenix, Mölndal, Sweden), which was depleted of plasminogen and plasmin as described before (Munk et al., (1989) in a buffer containing 15 mM Veronal, 140 mM NaCl, 0.5 mM $CaCl_2$, 0.2 mM $MgCl_2$, 5 mM Tris HCl and 0.005 % Tween and 500 Trasylol KIE/ml pH 7.75. After 1 hour incubation at 37°C, clots were centrifuged and radio-activity in the supernatant was determined. t-PA bound was expressed as the fraction of the total amount of t-PA analogue added to the fibrinogen solution (F). The data were fitted to :

$$F = [\text{ t-PA}_{\text{bound}}] / [\text{ t-PA}_{\text{total}}] = [\text{ fibrino(gen)}_{\text{total}}]/ ([\text{ fibrino(gen)}_{\text{total}}] + C_{50})$$

with the non-linear regression analysis algorithm of slide write 5.0 (Advanced Graphics Software, Inc. Carlsbad U.S.A.). C_{50} stands for the concentration of fibrin(ogen) where 50 % of the total $\text{t-PA}_{\text{bound}}$ is bound to fibrin(ogen). For n = 1 the standard error of the nonlinear regression estimate of the 10 data points is given. When n > 1 the mean C_{50} (with the standard error of the mean) of different binding experiments is given.

Determination of the t-PA and t-PA analogue concentration.

Spectrophotometric assays were performed as previously described (Verheijen et al.,
1985). Briefly, the reaction mixture (250 μl total volume) contained plasmin-treated t-PA analogues, in 100 mM Tris-HCl, pH 7.4 , 0.1 % (v/v) Tween 80 and 1.0 mM S-2288 (H-D-Ile-L-Pro-L-Arg-p-nitroanilide, Chromogeninx, Mölndal, Sweden). The two chain t-PA or t-PA analogue sample was tested at four different concentrations. The absorbance change at 405 nm ($\Delta A/\Delta t$) for each concentration was determined. These were plotted against the four different concentrations of t-PA or t-PA analogues, representing the absorbance change per concentration t-PA analogue. The absorbance change per concentration for a known amount of t-PA standard was compared with the absorbance change per concentration of the t-PA analogues. Since the amidolytic activity for the P domain of the t-PA standard is similar to the amidolytic activivty of the P domain of the t-PA analogues, the concentration of t-PA was calculated (Bakker et al., 1993).

Determination of the stimulation factor.

Spectrophotometric assays were performed as previously described (Verheijen et al., 1982). Briefly, the reaction mixture (250 μl total volume) contained various amounts of plasmin-treated t-PA analogues, 100 mM Tris-HCl (pH 7.4), 0.1% (v/v) Tween 80, 0.12 μM Glu-plasminogen and 0.7 mM S-2251 (H-D-Val-L-Leu-L-Lys-p-nitroanilide) (Chromogeninx. Mölndal, Sweden). In certain cases, 120 ug/ml of CNBr-digested fibrinogen were included. The absorbance of the reaction mixtures was measured at 405 nm in an eight-channel microtiter plate reader against suitable blanks without termination of the reaction. The t-PA analogue sample was tested at 10, 20 and 40 pM (final concentration) of active enzyme both for reaction mixtures containing fibrinogen fragments and for reactions mixtures without fragments. Fibrinogen fragments were prepared as described (Verheijen et al., 1982). The enhancement factors were determined as follows. The change in absorbance was monitored over time for each t-PA analogue in the presence and in the absence of CNBr digest of fibrinogen. For each enzyme concentration a slope was calculated, representing the absorbance change over time-squared ($\Delta A/\Delta t^2$). These slopes, in turn, were plotted against enzyme concentration, representing the absorbance change per time-squared per molar concentration of enzyme, and finally expressed as $\Delta A\ h^{-2}\ pmol^{-1}$. The ratio of the slope in the presence of fibrinogen fragments to the slope in the absence of fragments is the enhancement factor. This ratio reflects the extent to which fibrinogen fragments enhance the activity of the particular t-PA analogue preparation.

Competition experiments.

t-PA (final concentration approximately 4 μM) and K2P (final concentration approximately 8 μM) were inactivated with 50 μM PPACK in 0.1 M Tris-HCl pH 7.5, 0.01 % (v/v) Tween - 80 and 1 M NaCl for 6 h at room temperature. PPACK treated t-PA and K2P were separated from Phe-Pro-Arg-CH_2Cl with a Sephadex G-50 fine column equilibrated in 0.1 M Tris-HCl pH 7.5, 0.01 % (v/v) Tween 80 and 1 M NaCl. The inhibition of t-PA and K2P was confirmed using a spectrophotometric assay (see Determination of the t-PA analogue concentration.)

880 nM plasminogen-free fibrinogen was incubated with radiolabelled t-PA analogues (final concentration 0.1 nM) and t-PA (final concentration approximately 0.5 μM) or K2P (final concentration approximately 0.5 μM) or human serum albumin (final concentration approximately 0.3 μM) in 15 mM Veronal, 140 mM NaCl, 0.5 mM $CaCl_2$, 0.2 mM $MgCl_2$, 5 mM Tris-HCl and 0.005 % (v/v) Tween pH 7.75.. Clotting was performed with 2 NIH units thrombin/ml. After 1 h of incubation at 37 °C, clots were disrupted by vortexing. After centrifugation radioactivity in the supernatant was determined. t-PA bound is expressed as the fraction of total addd amount of t-PA analogue.

Results

To study the role of the lysyl binding site in the K2 domain of t-PA in the interaction of t-PA with a forming fibrin clot, we constructed t-PA domain deletion analogues in which the lysyl binding site is impaired by a single amino acid substitution, FGK1K2P (D236N), GK1K2P (D236N) and K2P (D236N). The recombinant proteins show the expected molecular weight on a zymogram (figure 1).

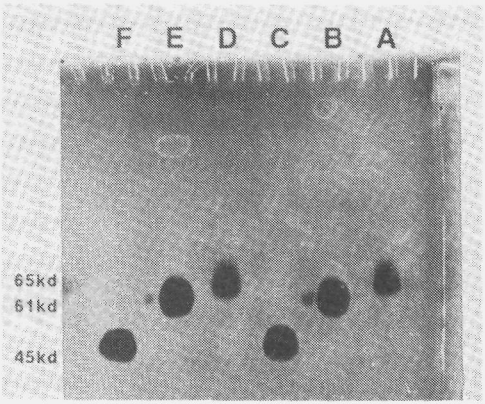

Figure 1: Fibrin zymography of t-PA analogues.

FGK1K2P (lane A), GK1K2P (lane B), K2P (lane C), FGK1K2P(D236N) (lane D), GK1K2P(D236N) (Lane E) and K2P(D236N) (lane F) were isolated from culture media by affinity chromatography. Gel electrophoresis and zymography were performed as described under Experimental procedures. High molecular weight standards were run in a separate lane (not shown).

All the domain deletion variants specifically interact with lysyl Sepharose. The mutation D236N in the different domain deletion

mutants results in a loss of lysyl binding capacity (table 1). All molecules convert plasminogen to plasmin (table 2). In comparison with the t-PA variants which show interaction with lysyl Sepharose, the t-PA variants lacking the lysyl binding site activate plasminogen with lower efficiency (table 2, column 2) In the presence of fibrin mimic the plasminogen activation of all molecules was enhanced (table 2, column 3). The FGK1K2P and the GK1K2P molecules showed a higher enhancement of plasminogen activation than the corresponding lysyl binding site mutants FGK1K2P (D236N) and GK1K2P (D236N). Interestingly K2P (D236N) which shows no interaction with a forming fibrin

t-PA or t-PA analogues studied	fraction of total cpm in			
	run through	wash	eluate	column
FGK1K2P	0	0.08	0.9	0.02
GK1K2P	0.2	0.1	0.7	0
K2P	0.05	0.05	0.9	0
FGK1K2P(D236N)	0.1	0.8	0.1	0
GK1K2P(D236N)	0.3	0.7	0	0
K2P(D236N)	0.2	0.8	0	0

Table 1: Binding to Lysyl - Sepharose.

Radio-labelled t-PA analogues were applied to a 1 ml lysyl Sepharose column. Run-through was collected. The columns were washed with 2.5 ml of buffer and eluted with 2.5 ml buffer containing 50 mM of the lysine analogue EACA. The radioactivity present in the flow through, wash fraction, elution fraction and remaining on the columns was determined and expressed as a fraction of the total radioactivity. For details see " Experimental procedures ".

clot (see below), still activates plasminogen in the presence of fibrin mimic as efficiently as K2P.

t-PA or t-PA analogue studied	Plasminogen activation $\Delta A\ h^{-2}\ pmol^{-1}$		stimulation factor
	no CNBr fragment of fibrinogen	CNBr fragments of fibrinogen	
FGK1K2P	0.24	30	125
GK1K2P	0.17	5.6	33
K2P	0.34	7.6	22
FGK1K2P(D236N)	0.22	11.1	50
GK1K2P(D236N)	0.09	1.1	12
K2P(D236N)	0.11	2.7	25

Table 2: Fibrin dependent plasminogen activation.

The amount of two-chain t-PA variant was determined using the amidolytic substrate S 2288 as described under " Experimental Procedures ". Plasminogen activator activity of t-PA and the t-PA variants in the absence (column 2) and presence of CNBr fragments of fibrinogen (column 3) were determined as described under " Experimental Procedures " and expressed as $\Delta A\ h^{-2}\ pmol^{-1}$.
Stimulation factors (column 4) were calculated as follows: the $\Delta A\ h^{-2}\ pmol^{-1}$ (in the presence of CNBr fragments of fibrinogen) divided by $\Delta A\ h^{-2}\ pmol^{-1}$ (in the absence of CNBr fragments of fibrinogen).

Fibrin binding experiments were performed with low concentrations of t-PA or t-PA analogues (< 0.1 nM) and the fraction of total t-PA or t-PA analogues bound at different fibrin(ogen) concentrations

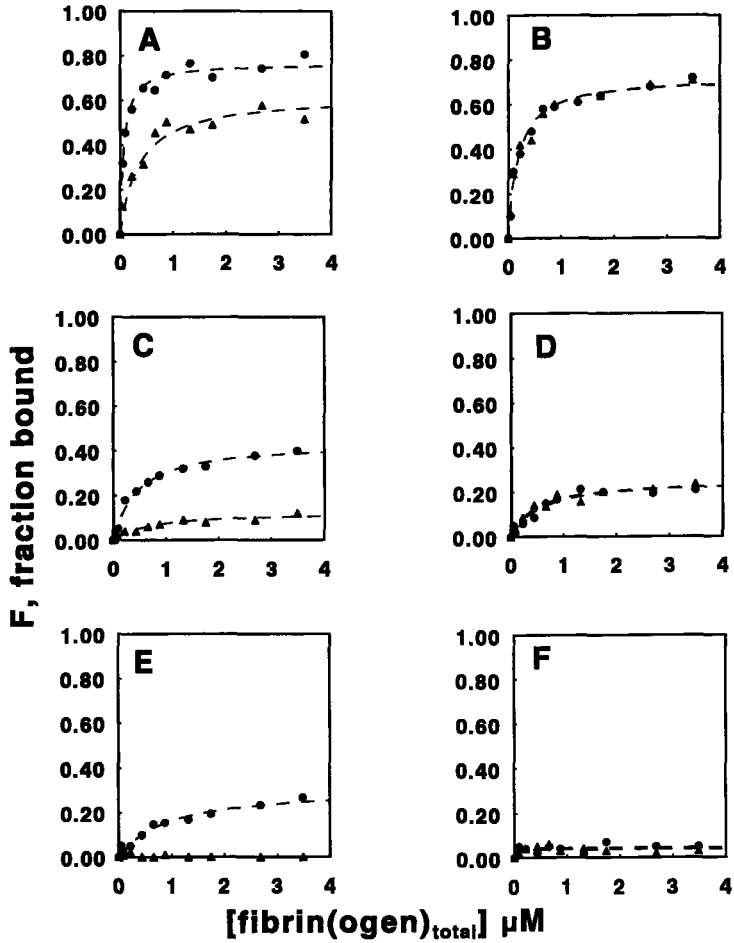

Figure 2: Experimental determination of the fraction of total t-PA bound (F) at various fibrin(ogen) concentrations.

Radiolabelled t-PA was incubated with various amounts of fibrinogen (0 - 3.4 μM) in the absence and presence of EACA. After clotting, the amount of radiolabelled t-PA or t-PA analogue bound to the fibrin clot was determined. On the Y-axis F = the fraction of total t-PA bound to the fibrin clot, on the X-axis the amount of fibrin(ogen) present in the clot: panel A = FGK1K2P; panel B = FGK1K2P (D236N); panel C = GK1K2P; panel D = GK1K2P (D236N); panel E = K2P ; panel F = K2P (D236N); ● = no addition ▲ = in the presence of 5 mM EACA (see Experimental procedures and for further explanation see text).

was assessed (figure 2). For t-PA a high affinity interaction with a forming fibrin clot is found (figure 2 panel A). When the C_{50} of this interaction was determined a value of 77 $\pm$ 11 nM was found (table 3). Fibrin binding of t-PA in the presence of 5 mM EACA is markedly reduced (figure 2, panel A). Determination of the C_{50} for this interaction resulted in a value of 376 $\pm$ 46 nM (table 3). Deletion of the lysyl binding site (FGK1K2P (D236N) also effects the binding to a forming fibrin clot (figure 2, panel B). Determination of the C_{50} resulted in a value of 169 $\pm$ 20 nM (table 3). The presence of 5 mM EACA had no influence on the

t-PA or t-PA analogue studied	C_{50} in nM			
		5 mM EACA	D236N	D236N 5mM EACA
FGK1K2P	77 $\pm$ 11 n = 9	376 $\pm$ 46 n = 6	169 $\pm$ 20 n = 2	186 $\pm$ 32 n = 2
GK1K2P	414 $\pm$ 26 n = 1	710 $\pm$ 189 n = 1	557 $\pm$ 126 n = 2	446 $\pm$ 126 n = 2
K2P	1163 $\pm$ 490 n = 2	NB n = 2	NB n = 2	NB n = 2

Table 3: Determination of C_{50} of t-PA and t-PA analogues to fibrin in the absence and presence of EACA.

Column 1: t-PA analogues studied. Column 2-5 Half maximal t-PA binding were determined from binding experiments of t-PA or t-PA analogues to a forming fibrin clot, comparable to the ones described (see figure 2). Using non-linear regression analysis the C_{50} of t-PA and t-PA domain deletion analogues to a forming fibrin clot in the absence or presence of 5 mM ϵ aminocaproic acid (EACA) and before and after deletion of the lysyl binding site (D236N) were determined. The concentration of fibrin(ogen) where half of the $t\text{-}PA_{bound}$ is bound and the standard deviation of the C_{50} is presented. NB = no binding observed. For details see " Experimental Procedures ".

fibrin binding of FGK1K2P (D236N), confirming the absence of a functional lysyl binding site in this molecule. Deletion of the F domain in t-PA (GK1K2P) reduces fibrin binding (figure 2 panel C). Blocking the lysyl binding site in GK1K2P with 5 mM of EACA resulted in a lowered fibrin binding. Surprisingly even when the F domain and the lysyl binding site in t-PA are lacking (GK1K2P (D236N)), considerable fibrin binding is still observed (figure 2 panel D, GK1K2P (D236N) C_{50}: 557 $\pm$ 126 nM (table 3)). As noticed with t-PA, occupying the lysyl binding site with EACA has a greater effect on fibrin binding than deleting the lysyl binding site. Binding of K2P to fibrin is weak (figure 2 panel E,

labelled t-PA or t-PA analogue	**fibrin binding in the presence of competitor**		
	NONE	**FGK1K2P**	**K2P**
FGK1K2P	0.73 $\pm$ 0.01	0.43 $\pm$ 0.01	0.23 $\pm$ 0.02
GK1K2P	0.29 $\pm$ 0.01	NB.	0.08 $\pm$ 0.02
FG1K2P(D236N)	0.63 $\pm$ 0.01	0.26 $\pm$ 0.01	0.26 $\pm$ 0.01
GK1K2P(D236N)	0.14 $\pm$ 0.01	NB	NB

Table 4: Competition experiments of radiolabelled t-PA and t-PA analogues with t-PA and K2P.

Radiolabelled t-PA or t-PA analogue was incubated with 880 nM fibrin(ogen) in the absence (column 2) or in the presence of 0.5 μM FGK1K2P (column 3) or 0.5 μM K2P (column 4). After clotting the fraction of total t-PA bound to fibrin was determined. The numbers in the table represent the fraction of total t-PA bound to the clot. The standard deviation was calculated from three data points. NB = no binding observed. For details see " Experimental Procedures ".

C_{50}: 1163 $\pm$ 490 nM (table 3)) and is completely inhibited in the presence of 5 mM EACA. Deleting the lysyl binding site in this molecule also abolishes fibrin binding (figure 2 panel F).
To study the t-PA binding site on a forming fibrin clot, we performed competition experiments. Table 4 shows the result of such a competition experiment. The binding of radiolabelled FGK1K2P is partially competed by FGK1K2P and K2P. Fibrin binding of FGK1K2P (D236N) lacking the lysyl binding site was only partially inhibited by FGK1K2P but also by K2P. This result suggests that the t-PA binding site in fibrin is also recognized by K2P. Binding of GK1K2P lacking the F domain is competitively inhibeted by K2P but also by FGK1K2P. Fibrin binding of GK1K2P (D236N) lacking both the F domain and the lysyl binding site in the K2 domain could be completely inhibited by FGK1K2P and K2P, indicating that the GK1K2P (D236N) molecule still recognizes the same t-PA binding sites on fibrin. Competition with human serum albumin at concentrations comparable to K2P and FGK1K2P did not occur (not shown).

Discussion

The first models describing the interaction of t-PA with a forming fibrin clot, were based on the idea that t-PA not only consists of structurally autonomous domains but also of functionally autonomous domains (Zonneveld et al., 1986a). In these first models there is a prominent role for the F and the K2 domain (Zonneveld et al., 1986b, Verheijen et al., 1986, Vries et al., 1990, Nesheim et al., 1990, Horrevoets et al., 1994). However a model in which the K1 domain plays an important role in the interaction of t-PA to preformed fibrin has been described (Kaczmarek et al., 1993). The F/K2 models may be further subdivided into models in which the t-PA interaction sites on fibrin are in juxtaposition (Zonneveld et al., 1986b, Vries et al., 1990 ; Horrevoets et al., 1994) and into models in which the t-PA interaction sites are further apart (Nesheim et al., 1990). All F/K2 models stress the importance of an direct interaction between the lysyl binding site in the K2 domain and a lysyl side chain of the fibrin network. Besides these models in which the functional autonomy of domains is stressed, a model was presented in which the fibrin interaction sites

in t-PA were spread over many domains, except the K2 domain (Bennet et al., 1991).
Our results with GK1K2P (D236N), a molecule which shows considerable interaction with a forming fibrin clot, suggest that besides the F and K2 mediated interaction, other fibrin interaction sites in t-PA exist. Interestingly, this molecule also shows enhanced plasminogen activation in the presence of fibrin, indicating that besides the Finger domain and lysyl binding site in the K2 domain other domains of t-PA are involved in fibrin dependent plasminogen activation. Remarkably, fibrin binding of FGK1K2P (D236N) a molecule which no longer can interact via its lysyl binding site in the K2P part, can be competitively inhibited by K2P. It seems therefore unlikely that the binding sites on fibrin for K2P and FGK1K2P (D236N) are far apart on the fibrin surface. This result questions the model of Nesheim (Nesheim et al., 1990) in which no such competition would be expected.
The role of the lysyl binding site in binding to a forming fibrin clot is more complicated than expected. In the presence of 5 mM EACA fibrin binding is more perturbed than after the deletion of the lysyl binding site. Such a result could be explained by steric hindrance. EACA binding to the lysyl binding site blocks the fibrin binding site and so reduces fibrin binding. Deletion of the lysyl binding site abolishes the interaction with EACA and therefore a possible inhibition of fibrin binding by EACA should no longer be possible. To test this steric hindrance hypothesis we studied the fibrin binding of K2P in more detail. Fibrin binding of this molecule can be completely inhibited by 5 mM EACA. It is known that the dissociation constant of EACA for K2P is approximately 100 μM (Byeon et al., 1991, Munk et al., 1989), two orders of magnitude higher than the dissociation constants for binding of K2P to fibrin (C_{50}: 1163 $\pm$ 490 nM). Therefore it seems reasonable to assume that fibrin binding is not solely aminohexyl mediated and that the fibrin binding site comprises more than the lysyl binding site. However, deletion of the lysyl binding site in the K2P molecule not only abolishes binding to EACA but also to fibrin. Therefore steric hindrance does not seem to be a satisfying explanation for the observed difference in fibrin binding between t-PA in the presence of EACA and t-PA without a lysyl binding site.
A more likely explanation for this effect is to assume additional indirect effects of occupation of the lysyl binding site such as

induction of a conformational change in the t-PA molecule. Conformational changes upon occupation of a lysyl binding site in the closely related and structurally similar molecule plasminogen have been described (Markus et al., 1978; Christensen & Molgaard, 1992). Plasminogen can occur in a closed conformation in the absence of EACA and an open conformation in the presence of EACA. The most likely explanation for this behaviour is the occupancy of the lysyl binding site by an intramolecular lysyl or arginyl residue in the closed conformation (Ponting and Marshall, 1992). Circumstantial evidence suggests that a similar mechanism could be operating in t-PA since the solubility of t-PA increases considerably upon addition of lysine or arginine (Hasegawa, 1985; Ichimura, 1987). Furthermore electronmicroscopic studies suggest that the structure of the molecule is ellipsoidal with the domains folded towards each other (Margossian et al., 1993). Based on differential scanning calorimetry experiments an interaction between the FG domains and the P domain was predicted. Although no involvement of the lysyl binding site could be detected (Novokhatny et al., 1991) a strong interdomain interaction involving lysyl binding sites has been observed in crystals of the kringle 2 domain (Vos et al., 1992). Recently it was shown that replacement of stretches of charged amino acid residues containing lysyl residues or arginyl residues for alanyl residues influences the fibrin binding of the resulting t-PA analogue (Bennet et al., 1991).

In view of these data we propose an alternative model for the high affinity interaction of t-PA with fibrin and the role of the lysyl binding site in this (figure 3). In analogy to plasminogen, t-PA could occur in two conformations, an open conformation and a closed conformation. The interaction between the lysyl binding site in the K2 domain and a lysyl residue stabilizes the closed conformation. Addition of EACA or mutation of the lysyl binding site would free t-PA in a more open conformation. In the closed conformation the affinity for fibrin is higher than in the open conformation. It has not escaped our notice that occupation of the lysyl binding site in t-PA by EACA could reflect a first step in the pathway of fibrin dependent plasminogen activation. Occupation of the lysyl binding site by plasminogen would free the P domain of t-PA from the fibrin surface making it available for the hydrolysis of the Arg^{561} - Val^{562} bond of plasminogen. However, the enhancement

of the plasminogen to plasmin conversion by K2P (D236N), that does not interact with a forming fibrin clot suggests that yet another mechanism of plasminogen activation exists, independent of the t-PA binding to fibrin.

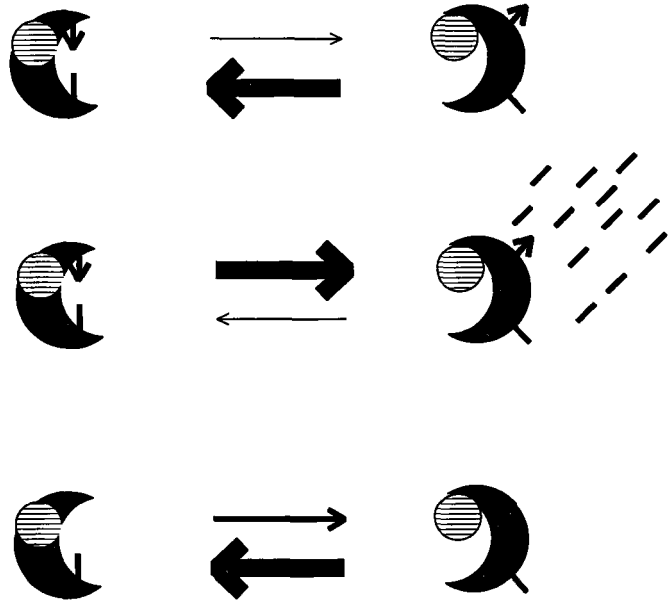

Figure 3: Presentation of a model to explain the effect of EACA and the deletion of the lysyl binding site in t-PA on the fibrin binding to a forming fibrin clot.

Within the t-PA molecule (⊖ = FGK1 part; ℂ = K2P part; ↓ = LBS; | = lysyl/arginyl residue from another t-PA domain or EACA) an intramolecular interaction probably between the lysyl binding site and a lysyl/ arginyl residue of the P domain, results in an equilibrium between a closed and an open conformation. The closed conformation is hypothesized to possess the structural requirements for a high affinity interaction with a forming fibrin clot (upper part). In the presence of EACA the interaction between the lysyl binding site in K2 and the lysyl/arginyl residue is disturbed resulting in a shift in equilibrium toward the open conformation (middle part). The open conformation is hypothesized to possess a fibrin interaction site of lower affinity. Deletion of the lysyl binding site also results, although to a lesser extent, in a shift in equilibrium toward a more open conformation (lower part).

Acknowledgements

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References

Allen, R.A., and Pepper, D.S. (1981) Thrombos. Haemostas. 45, 43 - 50
Bakker, A. H. F., van de Greef, W., Rehberg, E. F., Marotti, K.R., and Verheijen, J. H. (1993) J. Biol. Chem. 268, 18496-18501
Bennett, W. F., Paoni, N. F., Keyt, B. A., Botstein, D., Jones, A. J. S., Presta, L., Wurm, F. M., and Zoller, M. J. (1991) J. Biol. Chem. 266, 5191-5201
Byeon, I. J. L., and Llinás, M. (1991) Journal of Molecular Biology 222, 1035-1051
Carmeliet, P., Schoonjans, L., Kieckens, L., Ream, B., Degen, J., Bronson, R., Vos, de R., Oord, van den J., Collen, D., and Mulligan, R.C. Nature 368, 419 - 424
Christensen, U., and Molgaard, L. (1992) Biochem. J. 285, 419 - 425
Collen, D. (1980) J. Int. Soc. Thrombos. and Haemostas. 43, 77-89
Munk, de G. A. W., Caspers, M. P. M., Chang, G. T. G., Pouwels, P. H., Enger-valk, B. E., and Verheijen, J. H. (1989) Biochemistry 28, 7318-7325
Horrevoets, A.J.G., Smilde, A., Vries, de C., Pannekoek, H. (1994)J. Biol. Chem. 269, 12639 - 12644
Serrano, de V. S., and Castellino, F.J. (1993) Biochemistry. 32, 3540 - 3548
Vos, de A. M., Ultsch, M. H., Kelley, R. F., Padmanabhan, K., Tulinsky, A., Westbrook, M. L., and Kossiakoff, A. A. (1992) Biochemistry 31, 270-279
Vries, de C., Veerman, H., and Pannekoek, H. (1989) J. Biol. Chem. 264, 12604-12610.
Vries, de C. (1990) Structure and Function of Tissue-type Plasminogen Activator. A molecular Biological Approach. Thesis, University of Amsterdam.
Graham, F. L., and van der Eb A.J. (1973) Virology 52, 456-467
Granelli-Piperno, A., and Reich, E. (1978) J. Exp. Med. 148, 223-234
Hasegawa, A., Kondo, S. (1985) European patent application publication number EP0156169.
Hoylaerts, M., Rijken, D. C., Lijnen, H. R., and Collen, D. (1982) J. Biol. Chem. 257, 2912-2919
Ichimura, M., Saki, K., Kunihiro, Y., and Nakashiro, T. (1987) European patent application publication number 0217379
Kaczmarek, E., Lee, M.H., and McDonagh, J. (1993) J. Biol. Chem. 268, 2474-2479
Laemmli, U. K. (1970) Nature 15, 680-685
Margossian, S. S., Slayter, H. S., Kaczmarek, E., and McDonagh, J. (1993) Biochimica et Biophysica Acta 1163, 250-256
Markus, G., Evers, J.L., and Hobika, G.H. (1978). J. Biol. Chem. 253, 733 -739
Nesheim, M., Fredenburgh, J. C., and Larsen, G. R. (1990) J. Biol. Chem. 265, 21541-21548
Nieuwenhuizen, W., Voskuilen, M., Traas.,D.W., Hoegee-Nobel, B.D. and Verheijen, J.H. (1985) In Henschen, A., Hessel, B., McDonagh, J., and Saldeen T. (eds) Fibrinogen-structural variants and interactions. Walter de Gruyter and co, Berlin, pp 331-342.
Novokhatny, V. V., Ingham, K. C., and Medved, L. V. (1991) J. Biol. Chem. 266, 12994-13002
Ny, T., Elgh, F., and Lund, B. (1984) Proc. Natl. Acad. Sci. USA. 81, 5355-5359
Pannekoek, H., Lijnen, H. R., and Loskutoff, D. J. (1983) Thromb. Haemostas. 64, 600-603
Pennica, D., Holmes, W. E., Kohr, W. J., Harkins, R. N., Vehar, G. A., Ward, C. A., Bennett, W. F., Yelverton, E., Seeburg, P. H., Heyneker, H. L., and Goeddel, D. V. (1983) Nature 301, 214-221
Ponting, C.P., Marshall, S.A. (1992) Blood Coagulation and Fibrinolysis 3, 605 - 614
Radcliffe, R., and Heinze, T. (1978) Archives of Biochemistry and Biophysics 189, 185 - 194
Ránby, M. (1982) Biochim. Biophys. Acta. 704, 461-469
Rauber, P., Wikstrom, P. and Shaw, E. (1988) Anal. Biochem. 168, 259-264
Rijken, D. C., and Groeneveld, E. (1986) J. Biol. Chem. 261, 3098-3102
Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
Thorsen, S., Glas-Greenwalt, P., and Astrup, T. (1972) Thrombos. Diathes. heamorrh. 28, 65-74

Verheijen, J. H., Nieuwenhuizen, W., and Wijngaards, G. (1982) Thromb. Res. 27, 175-183.
Verheijen, J. H., de Jong, Y. F., and Chang, G. T. G. (1985) Thromb. Res. 39, 281-288.
Verheijen, J. H., Caspers, M. P. M., Chang, G. T. G., Munk, de G. A. W. d., Pouwels, P. H., and Enger-Valk, B. E. (1986) EMBO Journal 5, 3525-3530.
Wallén, P., Rånby, M., Bergsdorf, N., and Kok, P. (1981) Progress in Fibrinolysis 5, 16-23
Weening - Verhoeff, E. J. D., Quax, P. H. A., van Leeuwen, R. T. J., Rehberg, E. F., Marotti, K. R., and Verheijen, J. H. (1990) Protein Engineering 4, 191-198
Zonneveld, van A. J., Veerman, H., and Pannekoek, H. (1986a) Proc. Natl. Acad. Sci. USA. 83, 4670-4674
Zonneveld van, A. J., Veerman, H., and Pannekoek, H. (1986b) J. Biol. Chem. 261, 14214-14218

Chapter 6

Improving the Thrombolytic Properties of Tissue-type plasminogen Activator and Urokinase-type Plasminogen Activator

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Preface

The interest in plasminogen activators as possible thrombolytic agents has resulted in an enormous information flow (fig.1). Most aspects of these enzymes have been excellently reviewed and a limited list of reviews is given in table 1. For this reason we have confined ourselves to certain aspects of plasminogen activators as therapeutical enzymes. Current thrombolytic agents have a number of disadvantages. Here we will focus our attention on possible improvements of plasminogen activators as thrombolic agents and discuss the difficulties encountered to achieve this.

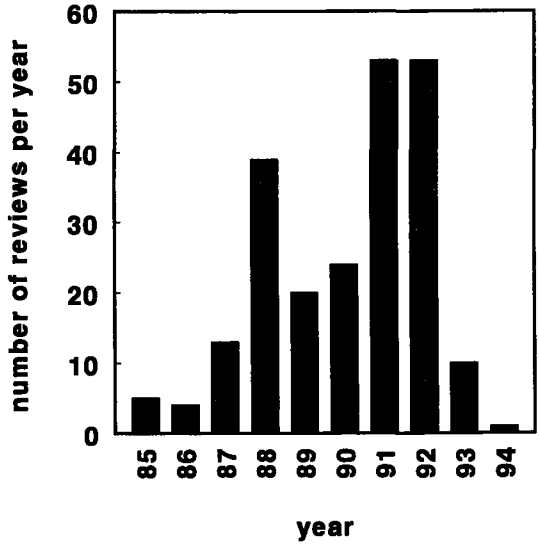

Figure 1: Reviews on plasminogen activation in the last ten years.

1. Introduction

Thrombosis is characterized by the formation of a blood clot within a bloodvessel [1,2]. Such a clot might occlude a bloodvessel and interferes with bloodcirculation. Then oxygen and nutrient deprivation of tissues will lead to tissue damage or death. Depending on the place in the body where the blood clot obstructs the circulation this blockade might result in myocardial infarction,

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Table 1: Reviews on plasminogen activators

	t-PA	u-PA	Plasminogen
chromomal localisation	8p21-8q11.2 (259)	10q24 (260)	6q26-27 (261)
main site of synthesis	endothelium (262)	kidney (263)	liver (264)
molecular weight	68 kD (8)	54 kD (8)	92 kD (8)
plasma concentration	70pM (8)	150pM (8)	2μM (8)

Table 2: Some characteristics of plasminogen and plasminogen activators

stroke, pulmonary embolism or simple thrombosis when the blood vessel supplies oxygen and nutrients to the heart, brain, lung or periferal tissue respectively. In 1994 thrombosis still the most important cause of morbidity and mortality in the western society. A blood clot within a vessel is often formed after damage of the vascular wall [3,4]. This damage frequently is a consequence of the process known as artherosclerosis. Vessel damage triggers a complicated cascade of proteolytic reactions. The final event is the conversion of inactive serine protease prothrombin into active

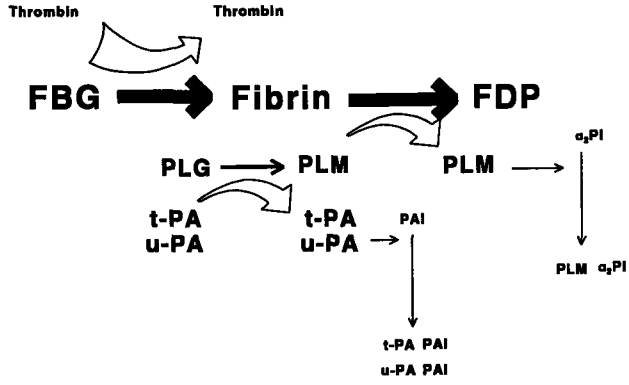


Figure 2: Schematic representation of fibrinolysis.

Upon vesseldamage thrombin (THR) is formed. Thrombin quickly converts the soluble blood protein fibrinogen (FBG) into an insoluble fibrin network. During tissue repair this fibrin network is slowly degraded by plasmin (PLM).
The activity of the non specific enzyme plasmin is localized towards the fibrin surface in two ways: 1 - Fluid phase plasmin is rapidly inhibited by α_2 plasmin inhibitor (α_2 PI). 2 - Plasminogen, (PLG) the inactive form of plasmin is preferentially activated on the fibrin surface by plasminogen activators (PA). When in the fluid phase plasminogen activators also are rapidly inhibited by plasminogen activator inhibitors (PAI).
Fibrin degradation products (FDP). Plasmin and plasminogen activator activity are confined to the fibrin surface by fast acting inhibitors α_2 plasmin inhibitor (PI, hatched triangles) and plasminogen activator inhibitor (PAI, hatched triangles). The inactive complexes between inhibitor and enzym are shown as open triangles and open boxes.

thrombin (THR) [5]. The latter transforms the soluble blood protein fibrinogen into insoluble fibrin. Fibrin polymerizes into a three dimensional network forming the scaffold of a blood clot (fig.2) [6]. Besides fibrin a blood clot also contains cells, like platelets and other bloodproteins.
Normally a blood clot is a temporary structure involved in physiological repair processes in response to vessel damage normally not occurring within a vessel. During this repair the clot is gradully degraded, finally resulting in its disappearance. The decomposition of a blood clot is called thrombolysis. An important event in thrombolysis is the proteolytic demolishing of the scaffold of the blood clot; the insoluble fibrin network is proteolytic degraded to soluble fibrin degradation products. This process is called fibrinolysis [7,8]. The main enzyme involved in fibrinolysis is the serine protease plasmin (PLM) [9]. Plasmin is not a fastidious enzyme, it may degrade many blood proteins. Probably, therefore plasmin occurs in the blood as an inactive precursor plasminogen. An interesting property of plasminogen is its ability to interact to a fibrin clot [10-16]. During fibrinolysis plasminogen is activated by plasminogen activators [17]. Plasminogen activators are very specific serine proteases, they virtually only catalyze the conversion of plasminogen into plasmin involving the hydrolysis of a single Arg-Val peptide bond (fig.3). At present two different plasminogen activators have been characterized in man; tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA). Tissue-type plasminogen activator binds to a fibrinclot [18-24]. Binding of both plasminogen and tissue type plasminogen activator are thought to be necessary for the efficient conversion of plasminogen to plasmin on the fibrin surface [25]. In contrast to t-PA, u-PA occurs in a proenzyme form pro-u-PA and does not bind to fibrin. Despite its lack of fibrin binding pro-u-PA also appears to activate preferentially plasminogen on the fibrin surface. The mechanism although not yet established might involve the activation of only fibrin bound plasminogen by pro-u-PA resulting in formation of plasmin which subsequently converts the pro u-PA into fully active u-PA [26-29]. To limit the proteolytic activity of plasmin to the fibrin surface specific inhibitors for both plasmin (α_2 plasmin inhibitor (α_2 PI)), and plasminogen activators (plasminogen activator inhibitor) (PAI 1) are present [30-37] (fig.2).

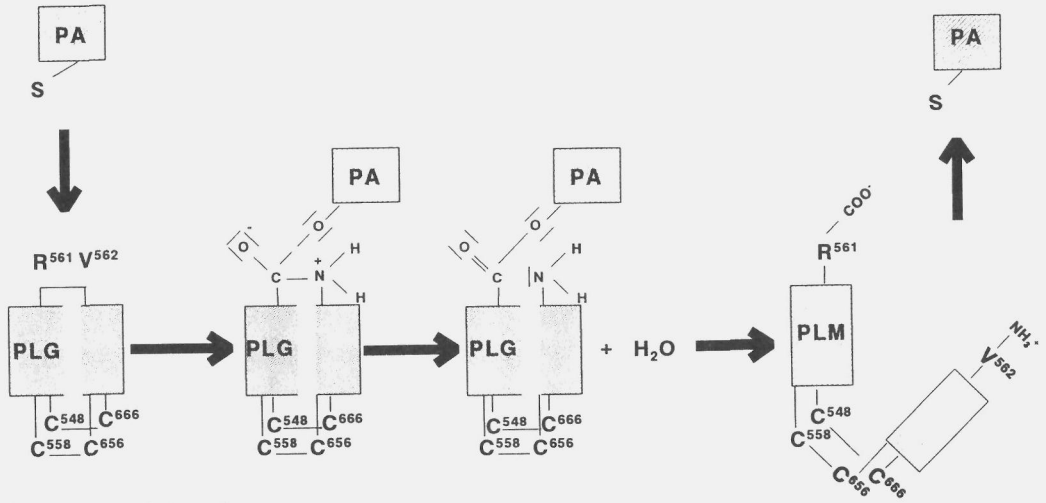


Fig.3. Mechanism of plasminogen activation.

Plasminogen activators (PA) are serine proteases which convert the zymogen plasminogen (PLG) into the active protease plasmin (PLM) by hydrolysis of a single peptide bond Arg561-Val562 (R_{561}-V_{562}). The resulting two chains remain connected via two disulfide bonds: one between cysteine residue 548 and cysteine residue 666 (C^{548}-C^{666}) and one between cysteine residue 558 and cysteine residue 656 (C^{558}-C^{656})and one between . The reaction is thought to follow the general mechanism of serine proteases involving the formation of a tetrahedral complex between the active site serine (S) in the enzyme PA with the substrate plasminogen. The acylcomplex formed is eventually hydrolysed leading to disruption of the R^{561}-V^{562} bond and formation of active two chain plasmin.

Disturbance of the normal regulation of coagulation and fibrinolysis might result in the formation of a blood clot within a vessel [38,39]. To secure the oxygen and nutrient supply route and thus prevent irreversible tissue damage such a clot has to be removed rapidly. One possible approach is thrombolytic therapy: external application of compounds leading to rapid degradation of blood clots. The obvious candidate would be plasmin [40]. Because of its low specificity the use of plasmin would lead to many side effects and therefore is not very successful. After the description of plasminogen activators the most obvious step was the application of plasminogen activators for this purpose. In the beginning of thrombolytic therapy plasminogen activators of bacterial origin (streptokinase) were used. Recently a renewed interest in bacterial plasminogen activator staphylokinase has emerged [41-47]. The antigenicity of these proteins has always been considered a problem in therapy [48]. The use of human urokinase isolated from human urine circumvented this problem of antigenicity [49-50]. However, u-PA is not a fibrin specific agent and the high doses needed to induce thrombolysis limited its use. Application of the fibrin specific agents like t-PA and pro-u-PA only became possible after development of recombinant DNA technology, which could supply the large amount of plasminogen activator needed for thrombolytic therapy. This resulted in a renewed interest in thrombolytic therapy and boosted research on plasminogen activators [51-53].
At the present time many studies with the accent on the treatment of acute myocardial infarction with various plasminogen activators have been reported [54-57,257]. The thrombolytic agents in use today, streptokinase, u-PA and t-PA, differ in their efficiency with which they induce reperfusion. The time of onset of the therapy, the optimal dose, the way of application and, the age of the patient are considered important variables determining the final outcome of treatment. Four main problems of thrombolytic therapy remain:
1- Inability to obtain reperfusion within a limited time span,
2- bleeding complications and 3- reocclusion. A fourth problem is the very short time interval after myocardial infarction in which thrombolytic therapy is effective. These remaining problems have initiated research on possible improvements of thrombolytic therapy. The attention was mainly focussed on the possible improvement of t-PA and u-PA. Especially the structure function relationship and the mechanism of plasminogen activation of

plasminogen activators was studied [58-61].

2. Plasminogen Activation

2.A. Structure and function of plasminogen and its activators

The most important characteristics of u-PA, t-PA and plasminogen have been summarized in table 2. Within the primary structure of plasminogen, u-PA and t-PA four different types of autonomous folding unit, domains, may be discerned (see chapter 1 figure 3, 4 and 5) The finger domain (F) present only in t-PA shares homology with similar domains of fibronectin [62]. The growth factor domain (G) present in both t-PA and u-PA was first described in epidermal growth factor [63]. The kringle domain (K) present in t-PA, u-PA and plasminogen was first described in prothrombin [64-66]. Plasminogen differs from both t-PA and u-PA in the presence of the N-terminal peptide (NTP). Finally and most important is the presence of a trypsin like protease domain (P) in all three proteins. Recent experiments with isolated domains and whole molecules confirmed, that several of these domains indeed behave like autonomous folding units [67-72].
When the gene structure of these proteins became available it was discovered that an autonomous folding unit in the protein corresponded with one or more exons in the gene [73-76] (see chapter 1 figure 3, 4 and 5). These and other observations resulted in the idea that exons can be considered genetic building blocks coding for discrete (parts of) protein domains. Based on the presence of these genetic building blocks a model for evolution of these proteins has been proposed [77-78]. This model based on the exon shuffling theory of Gilbert and the splice junction theory of Craik suggests that nature uses the same basic domains over and over again [79-80].
The description of the autonomous folding units within these proteins has led to the assumption that these autonomous folding units are also autonomous functioning units. Protein chemical and recombinant DNA techniques have shown that at least some of these domains of t-PA indeed appear to behave as autonomous functional units. The protease domain of t-PA is sufficient for

enzymatic activity [19]. Kringle structures are most likely involved in interaction with other macromolecules. K_2 of t-PA (like K_5 of Plg) are thought to interact with fibrin, while the K of u-PA binds to heparins [81,82,16,168,169]. The growth factor domain in urokinase mediates binding to a cellular receptor whereas no definite function for this domain in t-PA has been established [170]. The finger domain of t-PA is involved in fibrin binding [81,82]. Since autonomous folding domains seem to be autonomous functioning domains research focused on the ability to build new thrombolytic agents by adding, deleting and replacing domains thought to be responsible for a certain function.

2.B. Mechanism of plasminogen activation

2.B.1. Tissue-type plasminogen activator.

Tissue-type plasminogen activator is adsorbed to fibrin [18-24]. In the absence of fibrin t-PA is a poor enzyme. Whereas in the presence of fibrin t-PA catalyzed plasminogen activation is increased 2 to 3 orders of magnitude [83]. These two observations formed the basis of the development of t-PA as a thrombolytic agent. A possible mechanism explaining these two observations was presented by Hoylaerts and coworkers [25,84] (fig.5a).
It was proposed that both t-PA and plasminogen bind to fibrin on neighbouring binding sites. In this way a high local concentration of these components is reached responsible for the efficient plasminogen activation. In kinetic terms this results in a lower K_M term of t-PA for plasminogen in the presence of fibrin whereas the k_{cat} does not change. Central in this model is the strict relation between binding and enhancement of activity. Also other models were proposed. The explanations provided in these models are not exclusively based on concentration of t-PA and plasminogen on the fibrin surface. The central issues in those models are conformational changes in either t-PA, plasminogen or both upon binding to the fibrin surface. These conformational changes transform t-PA into a more efficient enzyme and/or plasminogen into a better substrate. In kinetic terms such a mechanism results in an increased k_{cat} for t-PA catalyzed plasminogen activation in the presence of fibrin. Of course both effects can occur simultaneously when a concentration effect and conformational change both are

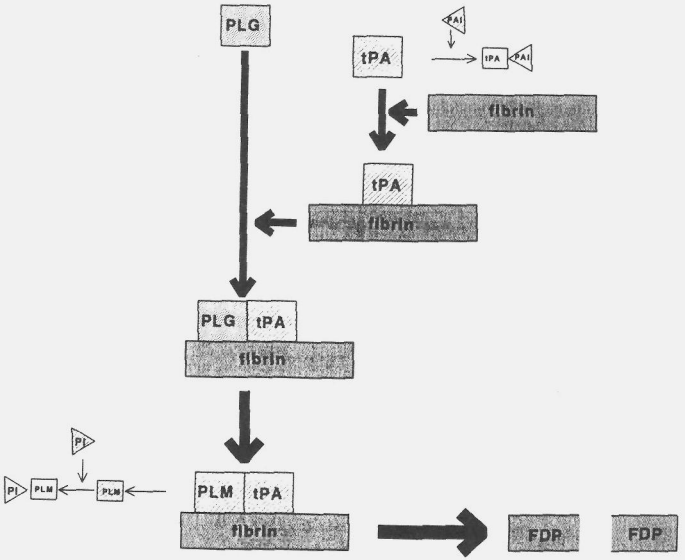


Figure 5.A. Mechanism of fibrin dependent plasminogen activation by t-PA.

To explain the fibrin dependent plasminogen activation of t-PA the following model was proposed [25]. The concentration of plasminogen (PLG, dotted box) in circulation ($\sim 2\mu M$) is low; hardly any complex formation with t-PA will occur. $k_m \approx k_D \approx$ 65mM). However when t-PA binds to fibrin, the affinity of t-PA-fibrin for plasminogen decreases ($k_m \approx k_D = 0.16\mu M$). Then over 90% of t-PA-fibrin will be complexed with plasminogen, and plasmin hatched box, PLM will be generated.The plasmin formed degrades the fibrin network to fibrin degradation products.

involved. In this case both K_M and k_{cat} change in the presence of fibrin [85-88].

2.B.2. Urokinase-type plasminogen activator.

Urokinase occurs as a proenzyme pro-u-PA which has a very low activity towards plasminogen [89-92]. Pro-u-PA can be converted into active u-PA by plasmin. Active u-PA is an efficient plasminogen activator in the absence of fibrin and does not bind to fibrin. However, it was observed that pro-u-PA in the presence of fibrin behaves as a plasminogen activator without conversion into

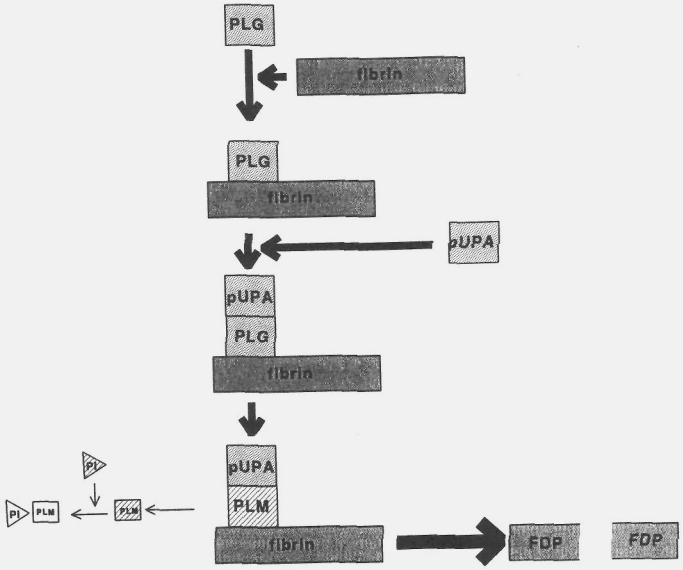


Fig.5.B.1. Fibrin dependent plasminogen activation by pro-u-PA.

To explain the fibrin dependent plasminogen activation of pro-u-PA a model was proposed [26-29,97,98]. Central in this model is the plasminogen (PLG, dotted box) binding to fibrin. Upon binding plasminogen changes its conformation. This conformational change results in an interaction with pro-u-PA (p-u-PA, dotted box). As such pro-u-PA has no affinity for fibrin.
The complex between pro-u-PA and plasminogen leads to plasmin formation (PLM, hatched box).

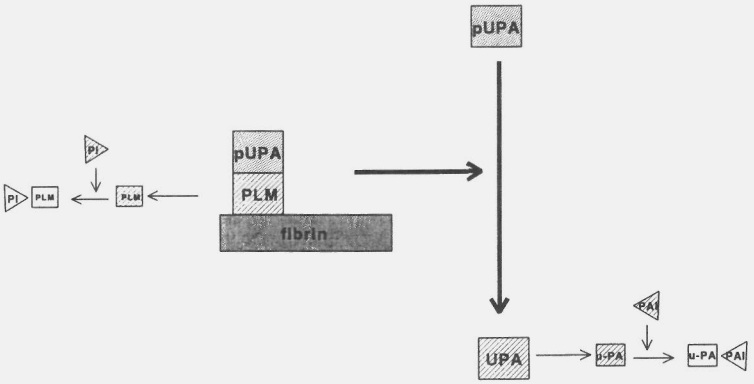


figure 5 B.2: Fibrin dependent plasminogen activation by pro-u-PA.

The plasmin, that is formed, activates pro-u-PA.

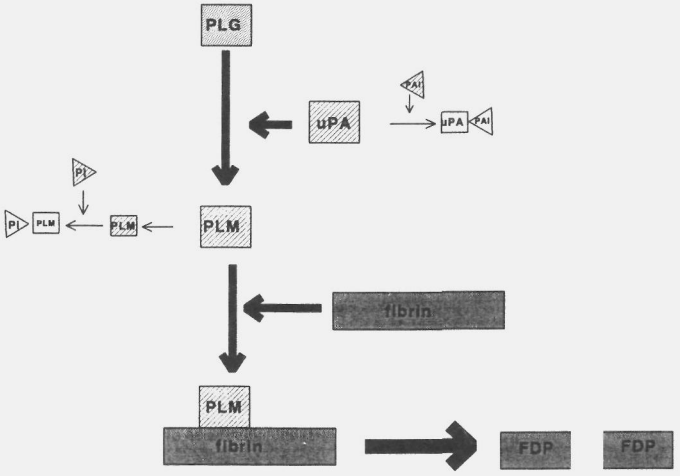


Figure 5 B.3: Fibrin dependent plasminogen activation by pro-u-PA.

This u-PA can activate plasminogen, leading to more plasmin, which again converts more pro-u-PA into active u-PA.
To localize the plasmin activity and the plasminogen activation activity on the fibrin surface, specific fast acting inhibitors are present (see also fig.5A.).

active urokinase [89-92]. Amazingly also pro-u-PA like u-PA does not bind to fibrin. In vivo pro-u-PA acts as a fibrin specific thrombolytic agent [93-96].
Recently a model was suggested to explain these remarkable observations [26-29,97,98], (fig.$5B_1$). Although pro-u-PA does not interact with plasminogen in solution it does convert fibrin bound plasminogen into plasmin at a low but significant rate.

Subsequently, the formed plasmin converts pro-u-PA into active u-PA (fig.$5B_2$). Finally this u-PA quickly converts plasminogen into plasmin (fig.$5B_3$). It is thought that upon binding to fibrin plasminogen adopts another conformation which is recognized as a substrate by pro-u-PA.
Such a mechanism is not without precedent. It has been known for many years that the bacterial protein streptokinase which has no enzymatic activity at all can induce plasminogen activation [41-47]. The mechanism of action of streptokinase appears to be dependent on formation of a complex with plasminogen. This interaction results in a complex with enzymatic activity without actual conversion of plasminogen into plasmin. Remarkably the streptokinase-plasminogen complex unlike plasmin itself has plasminogen activation activity. In this case it has been established that a conformational change induced by complex formation is a critical step in the mechanism.

3. Strategies to improve plasminogen activator as therapeutical enzymes.

3.A. Bleeding complications

All thrombolytic agents, at the therapeutic doses used in therapy today, activate plasminogen in the circulation [54-57]. The plasmin formed is first counteracted by α_2 plasmin inhibitor and α_2 macroglobulin. When this inhibitor pool is exhausted plasmin, begins due to its broad substrate specificity to degrade many plasma proteins including fibrinogen, and the coagulation factors factor V and factor VIII. This depletion of coagulation proteins may induce a long lasting hypocoagulation with increased bleeding risk. Surprisingly, it was observed that also the fibrin specific

thrombolytic agent t-PA has substantial systemic effects leading to bleeding complications in about 20% of the cases. Thus reducing bleeding complications would be an important improvement of thrombolytic therapy. Several concepts to improve this aspect of thrombolytic therapy were initiated.

3.A.1. Improving fibrin binding of t-PA.

Based on the hypothesis that the binding to fibrin is a prerequisite for the enhancement of plasminogen activation of t-PA research in the first place focused on improving the fibrin affinity of thrombolytic agents [51-53]. Fibrin binding features of t-PA are thought to be localized in the finger and kringle 2 domains [81,82]. Since these domains were considered to be independent functional units a logical way for improvement was to increase the number of these domains in the t-PA molecule. Following these lines t-PA mutants with multiple finger domains, extra kringle 2 domains and replacements of kringle 1 by a second copy of a kringle 2 domain were constructed [99-104]. Although fibrin binding properties in vitro were in some cases improved no major improvement of in vivo thrombolysis has been observed. Other more subtle ways to enhance fibrin affinity were tried. Instead of introduction of complete domains relatively small changes within existing domains were proposed. It has been shown that in principle it is possible to introduce fibrin affinity in the none fibrin binding kringle 1 domain of t-PA by substituting 6 amino acid residues [105]. In vivo experiments with such subtly altered molecules did not yet result in better thrombolytic properties [106].
On the contrary deletion of domains in t-PA presumably involved in fibrin binding had in many cases no adverse effects on the thrombolytic properties [107].

3.A.2. Introduction of fibrin binding in urokinase

The systemic effects of urokinase could be due to its lack of fibrin binding properties. A possible improvement would be to endow urokinase with such properties. Both t-PA and u-PA are proteins thought to be composed of similar domains. Homologous growth factor, kringle and protease domains occur in t-PA as well as u-PA. When these domains act as autonomous functional units it should be

possible to introduce new functions in u-PA by introduction of a functional domain from t-PA resulting in hybrid molecules. A large number of such hybrids were made. Molecules with the complete heavy chain of t-PA harboring the finger, growth factor and both kringle domains connected to the protease domain of u-PA or molecules with only part of this heavy chain or just various single domains connected to the protease domain of u-PA were constructed [60,108-112]. Also molecules consisting of complete urokinase with various extra t-PA domains inserted on various different positions have been investigated [60,108-112]. Not only hybrid molecules consisting of part of t-PA and u-PA but also hybrids of t-PA and u-PA with plasminogen were constructed, based on the fibrin binding properties of this latter molecule [113,114]. Indeed some hybrids of t-PA and u-PA acquired fibrin binding properties but never as good as t-PA itself. Furthermore the thrombolytic properties and fibrin binding did not correlate [60].

3.A.3. Using proenzymes.

Apart from reducing the systemic effects by improving the fibrin binding properties of thrombolytic agents it has also been tried to increase their proenzyme character. A further advantage of such molecules would be a reduced inhibition by plasma inhibitors. This problem has been tackled from two different directions: using the existing proenzyme form of urokinase or modified versions of it, which is relatively simple, or making t-PA a proenzyme which is much more difficult. A number of studies have been performed with pro u-PA indicating that this agent has better properties than u-PA [93,96]. Introduction of fibrin binding into pro u-PA did not lead to further improvements [60,108-112]. Recently modified forms of t-PA with proenzyme character similar to pro u-PA have been described using different strategies [166,117]. No data concerning their thrombolytic properties are available at this moment.

3.A.4. Concluding Remarks.

Two surprising conclusions may be drawn from this wealth of data. Firstly improving the fibrin binding ability by transfer or mutation

of domains is much less straight forward than anticipated. Secondly improving fibrin binding is not coupled to a decrease of systemic activity and reducing fibrin binding does not lead to an increased systemic effect.
Reducing the bleeding complications of thrombolytic agents by improving their fibrin affinity was disappointing. This might implicate that the concept behind these approaches is not entirely valid. Fibrin dependent plasminogen activation does not seem to be completely explained by concentration of activator and plasminogen on the fibrin surface.
The observed intracranial haemorrhages with t-PA show that fibrin binding might even lead to increased bleeding tendency [118]. Recent data with a less fibrin binding variant of t-PA show possibly lower intracranial haemorrhages. This might challenge the concept that improving the fibrin binding is desirable: apart from lysis of the pathologic clot also useful physiologic clots will be more rapidly lysed.

3.B. Resistance to lysis.

In about 40% of the cases thrombolytic therapy does not lead to reperfusion [3,59,60]. This might have many causes. Other obstructions than fibrin clots occur, which cannot be attacked by thrombolytic therapy. But even fibrin containing clots could be resistant to lysis. When a clot completely occludes a vessel there is no blood flow and a systemically given thrombolytic agent can only reach the clot by diffusion, a very slow and inefficient process.
A solution is local administration of the agent. Paradoxically, reducing the fibrin affinity of plasminogen activators could improve penetration of the agent into the clot. It is known that plasminogen activators present within the clot are at least hundred fold more effective than administered from the outside [119,120].
Besides this concentration effect another reason for lysis resistance could be the composition of the clot. For instance the amount of plasminogen present in the clot is an important variable [121]. The presence of high concentrations of lp(a) in blood is associated with increased risk for coronary artery disease and has been reported to interfere with plasminogen binding [122,123]. The outcome of thrombolytic therapy is also influenced by the structure of a clot [124,125]. Until now not much attention has been given to these

aspects. One aspect, however, deserves special mention. The presence of inhibitors of the plasminogen activation system like plasminogen activator inhibitor-1 in the clot influences effective lysis. Platelets are a rich source of PAI-1 making platelet rich clots difficult targets for lysis [126,127,172]. Furthermore also the presence of α_2 plasmin inhibitor contributes to lysis resistance of a clot [128,171].

3.B.1. Resistance to inhibitors

The plasminogen activators u-PA and t-PA are rapidly inhibited by PAI 1 by formation of a covalent complex of the inhibitor with the active site serine residue of the activator [129].
Using the known three dimensional structure of trypsin and trypsin inhibitor to model the interaction between t-PA and PAI-1 a secondary site of interaction, apart from the catalytic site was predicted.
With this knowledge a PAI 1 resistant t-PA interacting normally with plasminogen was made by mutation of the secondary interaction site [130-132] (see also fig.4c). A similar change in urokinase also resulted in PAI 1 resistance [133] see fig.4b. In vitro studies with platelet poor and platelet rich clots show that the latter are more rapidly lysed by the mutant t-PA than by wild type t-PA [134]. This confirms the inhibiting effect of platelet derived PAI 1 on clot lysability.

3.C. Reocclusion.

Reocclusion is a frequently occurring complication after thrombolysis [54-57]. Most likely it is a result of reexposure of the endothelial interface or the ruptured atherosclerotic lesion which are considered to be highly thrombogenic surfaces. On the other hand high plasmin concentrations which might occur upon thrombolysis can activate factors of the coagulation pathway. An effect of t-PA on platelet aggregation has been described but there is still no agreement concerning the direction of the effect [56]. The current way to diminish reocclusion is to combine thrombolytic therapy with anti-coagulation using heparin and aspirin [54]. Another

observation is that systemic effects of thrombolysis might be beneficial. This might explain the lower reocclusion frequency observed with streptokinase as compared with t-PA without anticoagulants [54].

3.D. Early administration as a bolus versus late administration by infusion.

An important factor influencing the outcome of thrombolytic therapy is the time of onset of therapy after beginning of the myocardial infarction. Considerable evidence has accumulated that an early reperfusion within four hours after the beginning of myocardial infarction is essential for a successful outcome of the therapy [54]. Current therapy can only be applied in a hospital. The diagnosis of an ongoing myocardial infarction and subsequent transportation of the patient to a hospital is responsible for a considerable delay in starting the therapy. It would be beneficial to apply thrombolytic therapy immediately after onset of the symptoms e.g. by a general practitioner or in an ambulance. Thrombolytic therapy requires a high blood level of the agent for several hours. Continuous infusion is the only possibility to achieve this with the existing agents. The rapid clearance of t-PA and urokinase by the liver results in a very short (approximately 5 min.) half life in the circulation, so continuous administration is a necessity [135-137]. This does not only delay the start of therapy until a hospital is reached but makes the therapy laborious and expensive since a massive dose is needed which is mostly rapidly cleared.

3.D.1. Reducing the rapid clearance.

A major improvement would be to have a less rapidly cleared agent which could be given as a bolus injection with a much higher efficiency. Therefore much effort has been devoted in elucidating the structures involved in rapid clearance. At least two mechanisms of clearance of t-PA exist [138]. One involves the carbohydrate structures of t-PA and the other the finger and or growth factor domains. Deleting certain domains of t-PA or preventing glycosylation by mutation of the critical residues required for

carbohydrate attachment resulted in molecules with significantly prolonged half life in the circulation. It has been shown that many different changes in the t-PA molecule all lead to a prolonged half-life in the circulation [55,56,107]. A number of these molecules have been developed further. Thrombolytic properties in animals have been performed and in some cases even clinical trials are on-going. One of the most thoroughly investigated examples of such a molecule is a t-PA deletion mutant missing the finger, growth factor and kringle-1 domains and missing all glycosylation due to its expression in E.coli. Many of the in vitro data appear less attractive than those of t-PA e.g. the affinity for fibrin is significantly decreased and the stimulation factor in the presence of fibrin is lower [139]. In vivo, however, this molecule also known as K_2P, BM 06.022 or r-PA appears to be very effective [118]. In humans the thrombolytic efficacy appears to be higher than that of t-PA when applied as a bolus [140]. Despite the much longer half-life and lesser affinity for fibrin the systemic effects and bleeding complications were low.
In conclusion increasing the half-life appears to be the most promising improvement made in a thrombolytic agent until now. It is likely that one or more of these molecules will be the first genetically modified proteins for clinical use in the near future.

4. Discussion

Thrombolytic therapy today makes use of the endogenous pathway of plasminogen activation. Administering high doses, two or three orders of magnitude above the physiological concentration of a normally present agent, which are needed to reach therapeutic levels disturb balances between coagulation and fibrinolysis. Plasminogen activator inhibitor pools will be depleted. Depletion of the PAI 3 (PCI) pool will extent the activity of protein C, disturbing the inhibition of the coagulation process [32,36,141]. Extensive systemic activation of plasminogen will occur exceeding the endogenous plasma inhibitors like α_2 plasmin inhibitor leading to degradation of fibrinogen. These are two examples of a disturbed balance leading to a bleeding tendency [56]. Other possible actions of plasmin or plasminogen activators, activation of the coagulation factors VIII and V or effects on platelets could result in a tendency to reocclusion [56]. Two other problems with current thrombolytic

therapy are resistance of clots to lysis and the time between first symptoms and onset of therapy.
The ideal thrombolytic therapy should only dissolve the pathologic blood clot that occludes a blood vessel leaving the useful physiologic clots unharmed. It further should not disturb existing delicate balances between coagulation and fibrinolysis, which as explained before may result in bleeding or rethrombosis. To prevent or limit tissue necrosis, thrombolytic therapy should be very efficient in lysing the pathologic blood clot within a short time span.
A further important point of consideration, not discussed in this chapter, is the problem to get the thrombolytic agent to the clot. This is an even more serious problem in these circumstances when blood flow is seriously hampered.
Much research has been devoted to reduce these side effects by improving plasminogen activators as thrombolytic agent. Most attention was focused on enhancing fibrin binding and decreasing the rapid clearance.

4.A.1. Autonomous domains, autonomous functions?

Based on the idea that autonomous domains posses autonomous functions in plasminogen activators it was tried to combine the desired properties and to remove unwanted properties into new rationally designed molecules. The results were disappointing. The fibrin binding of t-PA could not be significantly improved. And only a very limited fibrin binding could be introduced in urokinase, in comparison with that of normal t-PA. In contrast to fibrin, binding the rapid clearance of t-PA could be easily influenced in many different ways such as deletion of finger, growth factor or kringle 1 domains or interference with glycosylation of t-PA. When tested in vivo, many of these compounds had remarkable thrombolytic properties, despite their reduced fibrin interaction in vitro.
These observations, the failure to significantly improve fibrin binding and the relative ease to interfere with clearance could be explained as follows. Although the domains are autonomous folding units, the assumption that they function autonomously might be presumptive. A first observation was the binding of the isolated heavy chain of t-PA, consisting of the finger, growth factor and

both kringle domains, to fibrin [19]. When a recombinant hybrid between the t-PA heavy chain and the protease domain of urokinase was made, only an extremely weak interaction with fibrin was observed. Similarly an isolated kringle 2 domain of t-PA shows fibrin interaction whereas replacing the urokinase kringle by this kringle 2 domain does not lead to fibrin binding of the resulting hybrid [20,109].
The data presented here show that not merely the presence of a domain determines a function but that interaction between domains in a three dimensional folded structure ultimately determines the properties. Physciochemical techniques, differential scanning calorimetry and fluorescence spectroscopy suggest that in t-PA the serine protease domain strongly interacts with and is stabilized by the finger or growth factor domains in the intact protein [71]. Electronmicroscopy studies of the t-PA molecule support this observation singe chain as well as two chain t-PA show an ellipsoidal and relative compact structure [142]. Another striking observation confirming domain-domain interactions within t-PA is that destroying the lysyl/fibrin binding site in kringle 2 by a single point mutation did not result in a diminished fibrin binding of the whole t-PA molecule [143,117].
These results, obtained with point mutants, further stress that the results obtained with domain deletion mutants have to be interpreted with great care.

4.A.2. Fibrin binding and fibrin dependent plasminogen activation.

The kinetic mechanism proposed by Hoylaerts and coworkers [25] suggested that improving the fibrin binding of plasminogen activators would lead to more efficient plasminogen activation. During the numerous structure function studies it was discovered, however that hardly any correlation between fibrin binding and fibrin dependent plasminogen activation exists [144,59,60]. This might imply that the actual mechanism is not completely based on a concentration of plasminogen and activator on the fibrin surface. Over the years, much experimental data has accumulated suggesting the involvement of conformational changes in the mechanism of enhancement of plasminogen activation.

It has been observed that soluble fragments of fibrin could still enhance plasminogen activation by t-PA [145]. Even small peptides of no more than 12 residues derived from these fragments could enhance this reaction [146]. In these cases complex formation leading to concentration of t-PA and plasminogen is highly unlikely or impossible respectively, pointing to a mechanism based on conformational changes.

The exact three dimensional structure of plasminogen is not known at this time. Low resolution structural studies of plasminogen suggest a helical structure [147,148]. Important in this conformation is the intramolecular interaction between the N-terminal peptide and kringle 5 and between kringle 1-3 and kringle 4. These interactions are hypothesized to be lysyl residue mediated and the Lys^{50} residue in the N-terminal peptide is proposed to interact with a lysyl binding site in kringle 5 [149]. This closed helical conformation may change in a more open conformation in which all the domain-domain interactions are broken. The conformational change between the closed and the open form of plasminogen is the largest thus- far reported for a protein molecule. The open form can be more easily converted into plasmin. There are various ways to influence the balance between the open and the closed conformation. Binding with ligands and fibrin change the balance towards an open conformation [156-157]. Conformational changes upon binding to fibrin have also been described for t-PA although with less dramatic effects [158].

Mechanisms completely driven by conformational changes are not without precedent. The studies with vampire bat saliva plasminogen activator (bat-PA) also show that fibrin binding and fibrin dependent plasminogen activation are separate features. bat-PA resembles the human t-PA molecule in its modular structure although in the vampire bat molecule one kringle domain is missing [159,160]. There exist four naturally occurring forms, two FGKP forms, GKP and KP. Only the full length FGKP has affinity for fibrin. However, all three forms activate plasminogen only in the presence of fibrin. In contrast to human t-PA no activity at all was observed in the absence of fibrin. Surprisingly it was observed that only certain forms of fibrin caused this enhancement, whereas human t-PA has no such preferences [161,162]. These observations imply that enhancement of plasminogen activation is not dependent on fibrin binding of the activator. Introduction of a conformational

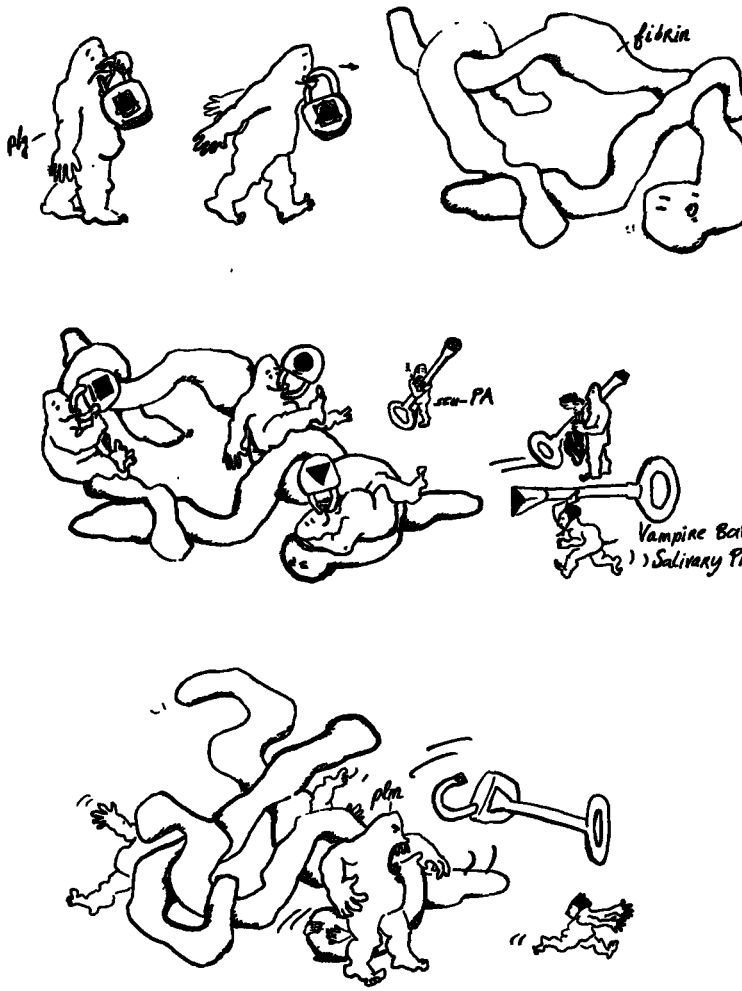
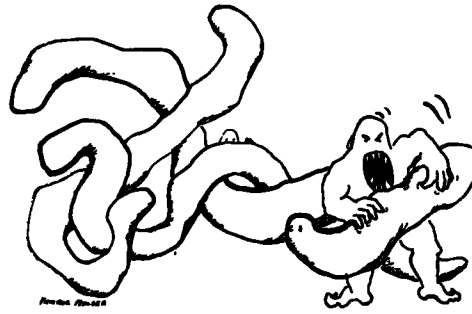


Figure.6: Cartoon proposing a model for fibrin dependent plasminogen activation.

Depending on the binding site on the fibrin surface, plasminogen (PLG) may adopt various conformations. Plasminogen activators (PA) recognize one or more of these conformational changed plasminogen molecules.
Once activated the non fastidious plasmin (PLM) degrades fibrin, until it meets its fast acting inhibitor $\alpha 2$ plasmin inhibitor (α_2 PI).




α_2-PI

change in plasminogen by binding to fibrin and a recognition of this conformational changed plasminogen by the bat-PA appears an attractive model to explain the fibrin dependency of the reaction. A similar mechanism, based on recognition of conformational changed fibrin bound plasminogen was recently proposed to explain the fibrin dependent plasminogen activation by pro urokinase. While pro-urokinase has no affinity for fibrin it does show fibrin dependent plasminogen activation [26-29,89-92].
It is difficult to unequivocally state which model of fibrin dependent plasminogen activation is followed. However, there is ample evidence to suggest that a conformational change of plasminogen upon binding to fibrin plays an essential role.
The fibrin bound forms of plasminogen recognized by t-PA, bat-PA and pro-urokinase respectively appear to be different. The conformational change of plasminogen induced by fibrin depends on the exact site of interaction in fibrin. Pro urokinase has a strong preference for plasminogen bound to the fibrin region E_2; t-PA prefers plasminogen bound to the D regions whereas bat-PA only recognizes plasminogen bound to intact fibrin (fig.6) [29,163-165,161].

5. Final conclusions.

Attempts to improve thrombolytic agents until now were based on two ideas: 1- fibrin binding of the activator is an essential feature in thrombolysis 2- the domains of which plasminogen activators consist are independent functional units. The new insights in the mechanism of the fibrin dependent plasminogen activation and the role of domains in the molecules suggest another strategy.
One approach is to take an existing thrombolytic agent randomly mutate its amino acid sequence and select for the molecules with the desired properties [117]. The success of this method depends on the selection criteria used. For instance the difference in the interaction of the plasminogen activator with fibrin and fibrinogen has been used [166,167]. In this way it was possible to obtain mutants with a significantly larger difference in behaviour in the presence of fibrin compared with fibrinogen than wild type t-PA. Moreover some mutations leading to such behaviour could not be explained nor expected by the current models of plasminogen activation mechanism. A limitation of this approach is the high number of

variants that have to be generated in order to find the desired property. Therefore a simple in vitro selection system predicting the use in vivo is required. The development of in vitro selection criteria relevant for in vivo thrombolytic behaviour is the major bottle neck.

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references

1. C. R. M. Prentice. Haemostasis (1990) 20, 50-59
2. L. T. Clark. American Heart Journal (1992) 123, 1106-1109
3. V. Fuster, L. Badimon, J. J. Badimon and J. H. Chesebro (1992) The New England Journal of Medicine., 326, 242-250
4. V. Fuster, L. Badimon, J. J. Badimon and J. H.Chesebro (1992) The New England Journal of Medicine., 326, 310-316
5. B. Furie and B. C. Furie (1988) Cell 53, 505-518 .
6. R. F. Doolittle Scientif American (1981) 245, 92-97
7. D. Collen, (1990) Thrombosis and Haemostasis 43, 77-89
8. F. Bachman (1989) in Proceedings of the XIIIth international congress on clinical chemistry, edited by den Boer et al. Plenum press, New York
9. K. C. Robbins (1987) in Haemostasis and thrombosis, edited by R. W. Colman, J. Hirsch, V. J. Marder and E. W. Salzman, Lippincott Co., Philadelphia
10. M. Adams-Lucas, L. J. Fretto and P. A. McKee (1983) The Journal of Biological Chemistry 258, 4249-4256
11. A. Váradi and L. Patthy (1983) Biochemistry 22, 2440-2447.
12. M. S. Lewis, F. Carmassi and S. I. Chung (1984) Biochemistry 23, 3874-3879
13. A. Váradi and L. Patthy (1984) Biochemistry 23, 2108-2112
14. R. A. Bok and W. F. Mangel (1985) Biochemistry 24, 3279-3286
15. J. Soria, C. Soria, F. Dunn, K. Deguchi, M. Mirshahi, R. Lijnen, W. Nieuwenhuizen, F. Haverkate and M. Samama (1985) in Fibrinogen-Structural Variants and Interactions, edited by A. Henschen, B. Hessel, J. McDonagh and T. Saldeen, Walter de Gruyter Co, Berlin
16. H. L. Wu, B. I. Chang, D. H. Wu, L. C. Chang, C. C. Gong, K. L. Lou and G. Y. Shi (1990) J. Biol. Chem. 265, 19658-19664.
17. F. Bachmann (1987) in Haemostasis and thrombosis Basic principles and clinical practice second edition, edited by R. W. Colman J. B. Lippincott Co, Philadelphia
18. S. Thorsen, P. Glas-Greenwalt and T. Astrup (1972) Thrombos. Diathes. Haemorrh. 28, 65-74
19. D. C. Rijken and E. Groeneveld (1986) J. Biol. Chem. 261, 3098-3102
20. A. Ichinose, K. Takio and K. Fujikawa (1986) J. Clin. Invest. 78, 163-169
21. D. L. Higgins and G. A. Vehar (1987) Biochemistry 26, 7786-7791
22. P. J. Bosma, D. C. Rijken and W. Nieuwenhuizen (1988) Eur. J. Biochem. 172, 299-404
23. G. A. W. Munk, de M. P. M. Caspers, G. T. G. Chang, P. H. Pouwels, B. E. Engervalk and J. H. Verheijen (1989) Biochemistry 28, 7318-7325
24. M. Nesheim, J. C. Fredenburgh and G. R. Larsen (1990) J. Biol. Chem. 265, 21541-21548
25. M. Hoylaerts, D. C. Rijken, H. R. Lijnen and D. Collen (1982) J. Biol. Chem. 257, 2912-2919
26. R. H. Lijnen, D. C. Stump and D. C. Collen (1987) Sem. Thromb. Hemotas. 13, 152-159
27. V. Gurewich (1988) Sem. Thromb. Haemostas. 14, 110-115

28. V. Gurewich (1988) Sem. Thromb. Hemostas. 15, 123-128
29. J. N. Liu and V. Gurewich (1991) Journal of Clinical Investigation 88, 2012-2017
30. B. Wiman, H. R. Lijnen and D. C. Collen (1979) Biochimica et Biophysica Acta. 579, 142-154
31. N. Aoki, Y. Sumi, O. Miura and S. Hirosawa (1993) Methods in Enzymology 223, 185-195
32. D. C. Stump, M. Thienpont and D. Collen (1986) J. Biol. Chem. 261, 12759-12766
33. E. K. O. Kruithof (1988) in Tissue-type plasminogen activator (t-PA) : Physiological and clinical aspects, edited by C. Kluft, CRC Press Inc, Boca Raton Fla
34. J. Mimuro, M. Kaneko, T. Murakami, M. Matsuda and Y. Sakata (1992) Biochimica et Biophysica Acta 1160, 325-334
35. E. L. Madison, E. J. Goldsmith, M. J. H. Gething, J. F. Sambrook and R. D. Gerard (1990) J. Biol. Chem. 265, 21423-21426
36. M. Geiger, U. Priglinger, J. H. Griffin and B. R. Binder (1991) J. Biol. Chem. 266, 11851-11857
37. J. Mottonen, A. Strand, J. Symersky, R. M. Sweet, D. E. Danley, K. F. Geoghegan, R. D. Gerard and E. J. Goldsmith (1992) Nature, 355, 270-273
38. C. W. Francis and V. J. Marder (1987) in Haemostasis and Thrombosis Basic principles and clinical practice, edited by R. W. Colman, J. Hirsch, V. J. Marder and E. W. Salzman, J.B. Lippincott Co, Philadelphia
39. B. Wiman and A. Hamsten (1988) Sem. Thromb. Haemostas. 16, 207-216
40. O. Storm, P. Ollendorff, E. Drewsen and P. Tang (1974) Thromb. Diath. Haemorrh. 32, 468-482
41. K. W. Jackson, N. Esmon and J. Tang (1981) Progress in Fibrinolysis 5, 49-52
42. K. W. Jackson and J. Tang (1982) Biochemistry 21, 6620-6625
43. H. R. Lijnen, B. Vanhoef, L. Vandenbossche and D. Collen (1992) Fibrinolysis 6, 214-225
44. D. Collen, Z. A. Zhao, P. Holvoet and P. Marynen (1992) Fibrinolysis 6, 226-231
45. D. Collen, M. Demol, E. Demarsin, F. Decock and J. M. Stassen (1993) Fibrinolysis 7, 242-247
46. T. Trieu, D. Behnke, D. Gerlach and J. Tang (1993) Methods in Enzymology 223, 156-167
47. J. T. Radek, D. J. Davidson and F. J. Castellino (1993) Methods in Enzymology 223, 145-155
48. D. Collen, F. Decock, I. Vanlinthout, P. J. Declerck, H. R. Lijnen and J. M. Stassen (1992) Fibrinolysis 6, 232-242
49. J. Ploug and B. O. Kjeldgaard (1957) Biochim. Biophys. Acta. 24, 278-279
50. T. Ueno, N. Kobayashi and T. Maekawa (1979) Thromb. Haemostas. 42, 885-894
51. D. Collen and H. R. Lijnen (1984) Arteriosclerosis 4, 579-585
52. T. J. R. Harris (1987) Protein Engineering 1, 449-458
53. R. Fears (1989) Biochem. J. 261, 313-324
54. M. Grunewald and E. Seifried (1994) Fibrinolysis 8, 67-86
55. J. L. Francis and D. Wood (1988) in Fibrinogen, Fibrin stabilisation and fibrinolysis, edited by J. L. Francis VCH, Weinheim
56. W. R. Bell (1989) in Dose-Response and patency, edited by S. Sherry, Current medical literature Ltd., London
57. A. J. Tiefenbrunn and B. E. Sobel (1989) Fibrinolysis 3, 1-15
58. H. Pannekoek, C. de Vries and A. J. van Zonneveld (1988) Fibrinolysis 2, 123-132
59. D. L. Higgins and W.F. Bennett (1990) Annual Review of Pharmacology and Toxicology 30, 91-121
60. H. R. Lijnen and D. Collen (1991) Thrombosis and Haemostasis 66, 88-110
61. E. L. Madison and J. F. Sambrook (1993) Methods in Enzymology 223, 249-271
62. L. Banyai, V. Varadi and L. Patthy (1983) FEBS letts. 163, 37-41
63. P. A. Handford, M. Mayhew, M. Baron, P. R. Winship, I. D. Campbell and G. G. Brownlee (1991) Nature 351, 164-167
64. L. Patthy, M. Trexler, Z. S. Vali, L. Banyai and A. Varadi (1984) FEBS letts. 171, 131-136
65. F. J. Castellino and J. M. Beals (1987) J. Mol. Evol. 26, 358-369
66. A. Tulinsky (1991) Thrombosis and Haemostasis 66, 16-31
67. M. Trexler and L. Patthy (1983) Proc. Natl. Acad. Sci. USA. 80, 2457-2461
68. V. V. Novokhatny, S. A. Kudinov and P. L. Privalov (1984) J. Mol. Biol. 179, 215-232
69. S. Cleary, M. G. Mulkerrin and R. F. Kelley (1989) Biochemistry 28, 1884-1891
70. M. J. Bogusky, C. M. Dobson and R. A. G. Smith (1989) Biochemistry 28, 6728-6735
71. V. V. Novokhatny, K. C. Ingham and L. V. Medved (1991) J. Biol. Chem. 266, 12994-13002
72. V. Novokhatny, L. Medved, A. Mazar, P. Marcotte, J. Henkin and K. Ingham (1992) J. Biol. Chem. 267, 3878-3885
73. T. E. Petersen, M. R. Martzen, A. I. Ichinose and E. W. Davie (1990) J. Biol. Chem. 265, 6104-6111
74. A. Riccio, G. Grimaldi, P. Verde, G. Sebastio, S. Boast and F. Blasi (1985) N.A.R. 13, 2759-2772
75. S. J. Friezner-Degen, B. Rajput and E. Reich (1986) J. Biol. Chem. 261, 6972-6985
76. T. Ny, F. Elgh and B. Lund (1984) Proc. Natl. Acad. Sci. USA. 81, 5355-5359
77. L. Patthy (1985) Cell 41, 657-663
78. L. Patthy (1990) Blood coagulation and fibrinolysis 1, 153-166

79. W. Gilbert, M. Marchionni and G. McKnight (1986) Cell 46, 151-154
80. C. S. Craick, W. J. Rutter and R. Fletterick (1983) Science 220, 1125-1129
81. J. H. Verheijen, M. P. M. Caspers, G. T. G. Chang, G.A. W. de Munk, P. H. Pouwels and B. E. Enger-Valk (1986) EMBO J. 5, 3525-3530
82. A. J. van Zonneveld, H. Veerman and H. Pannekoek (1986) J. Biol. Chem. 261, 14214-14218
83. P. Wallén (1978) Progress in Chemical Fibrinolysis and Thrombolysis 3, 167-181
84. C. Zamarron, H. R. Lijnen and D. Collen (1984) J. Biol. Chem. 259, 2080-2083
85. B. C. Binder and J. Spragg (1980) Protides of the Biological fluid 28, 391-394
86. M. Rånby (1982) Biochim. Biophys. Acta. 704, 461-469
87. W. Nieuwenhuizen, M. Voskuilen, D. W. Traas, B. d. Hoegee-Nobel and J. H. Verheijen (1985) in Fibrinogen-structural variants and interactions, edited by A. Henschen, B. Hessel, J. McDonagh and T. Saldeen Walter de Gruyter & Co,, Berlin
88. R. Lijnen and D. Collen (1993) Methods in Enzymology 223, 197-206
89. G. A. W. de Munk and D. C. Rijken (1990) Fibrinolysis 4, 1-9
90. L. Nelles, H. G. Lijnen, D. Collen and W. E. Holmes (1987) J. Biol. Chem. 262, 5682-5689
91. H. R. Lijnen, B. van Hoef, L. Nelles, W. E. Holmes and D. Collen (1988) Eur. J. Biochem. 172, 185-188
92. H. R. Lijnen, D. Gheysen, D. E. F. Foresta, L. Pierard, P. Jacobs and D. Collen (1988) Fibrinolysis 2, 85-93
93. C. Zamarron, H. R. Lijnen, B. van Hoef and D. Collen (1984) Thromb. Haemostas. 52, 19-23
94. D. Collen, J. M. Stassen, M. Blaber, M. Winkler and M. Verstraete (1984) Thromb. Haemostas. 52, 27-30
95. H. Kambara, C. Kawai, N. Kajiwara, H. Niitani, S. Sasayama, K. Kanmatsuse, K. Kodama, H. Sato and M. Nobuyoshi (1988) Circulation 78, 899-905
96. D. C. Gulba and K. L. Neuhaus (1989) in Controversies in Coronary Thrombolysis, edited by S. Sherry, R. Schroder, C. Kluft, A. J. Six and K. L. Mettinger, Current Medical Literature Ltd., London
97. V. Gurewich, J. N. Liu and R. Pannell (1992) Annals of the New York Academy of Sciences 667, 224-232
98. V. Fleury, H. R. Lijnen and E. Anglescano (1993) J. Biol. Chem. 268, 18554-18559
99. W. Markland, D. Pollock and D. J. Livingston (1989) Prot. Engng. 3, 111-116
100. A. Stern, R. Mattes, P. Buckel and U. H. Weidle (1989) Gene 79, 333-344
101. M. J. Gething, B. Adler, J. A. Boose, R. D. Gerard, E. L. Madison, D. McGookey, R. S. Meidell, L. M.Roman and J. Sambrook (1988) EMBO J. 7, 2731-2740
102. S. Urano , A. R. Metzger and F. J. Castellino (1989) Proc. Natl. Acad. Sci. USA. 86, 2568-2571
103. I. Dodd, D. L. Mitchell, C. Entwisle, M. J. Browne and J. H. Robinson (1993) Fibrinolysis 7, 237-241
104. E. F. Rehberg, N. Y. Theriault, J. B. Carter, D. P. Palermo, E. V. Hubert, P. W. Bergum, C. J.Wunderlich, L. A. Erickson and K. R. Marotti (1989) Prot. Engng. 2, 371-377
105. A. H. F. Bakker, W. van de Greef, E. F. Rehberg, K. R. Marotti and J. H. Verheijen (1993) J. Biol. Chem. 268, 18496-18501
106. D. Collen, H. R. Lijnen, F. Bulens, A. M. Vandamme, A. Tulinsky and L. Nelles (1990) J. Biol. Chem. 265, 12184-12191
107. K. Wikstrom, C. Mattsson, C. Sterky and G. Pohl (1991) Fibrinolysis 5, 31-41
108. C. de Vries, H. Veerman, F. Blasi and H. Pannekoek (1988) Biochemistry 27, 2565-2572
109. L. Pierard, L. G. Quintana, M. E. Reff and A. Bollen (1989) Journal of Molecular & Cellular Biology 8,321-328
110. F. A. M. Asselbergs, R. Burgi, J. Hamerman, J. Heim, J. van Oostrum and G. Agnelli (1993) Thrombosis and Haemostasis 69, 50-55.
111. M. Colucci, L. G. Cavallo, G. Agnelli, A. Mele, R. Burgi, J. Heim and N. Semeraro (1993) Thrombosis and Haemostasis 69, 466-472
112. I. M. Lubin, R. Caban and M. S. Runge (1993) J. Biol. Chem. 268, 5550-5556
113. S. Wilson, P. Chamberlain, I. Dodd, A. Esmail and J. H. Robinson (1990) Thromb. Haemostas. 63, 459-463
114. A. Boutaud and F. J. Castellino (1993) Archives of Biochemistry and Biophysics 303, 222-230
115. J. Madhani, H. Movsowitz and M. N. Kotler (1993) Therapeutic Drug Monitoring 15, 546-551
116. E. L. Madison, A. Kobe, M. J. Gething, J. F. Sambrook and E. J. Goldsmith (1993) Science 262, 419-421
117. W. F. Bennett, N. F. Paoni, B. A. Keyt, D. Botstein, A. J. S. Jones, L. Presta, F. M. Wurm and M. J. Zoller (1991) J. Biol. Chem. 266, 5191-5201
118. U. Martin, G. Sponer and K. Strein (1993) Blood Coagulation & Fibrinolysis 4, 235-242
119. E. J. P. Brömmer, B. J. Potter van loon, D. C. Rijken and J. H. Vanbockel (1992) Annals of the New York Academy of Sciences 667, 283-285
120. S. Thorsen (1992) Annals of the New York Academy of Sciences 667, 52-63
121. P. T. Onundarson, C. W. Francis and V. J. Marder (1992) J.Lab.Clin.Med. 120, 120-127
122. L. Hervio, M. J. Chapman, J. Thillet, S. Loyau and E. Angles-Cano (1993) Blood 82, 392-397

123. P. C. Harpel and W. Borth (1992) Annals of the New York Academy of Sciences 667, 233-238
124. C. Soria, J. Soria, M. McMirshahi, O. Bertrand and J. P. Caen (1987) in Fundamental and Clinical Fibrinolysis, edited by F. Castellino, P. Gaffney, M. Samama and A. Takada, Exerpta Medica, Amsterdam
125. J. P. Collet, J. Soria, M. Mirshahi, M. Hirsch, F. B. Dagonnet, J. Caen and C. Soria (1993) Blood 82, 2462-2469
126. I. K. Jang, H. K. Gold, A. A. Ziskind, J. T. Fallon, R. E. Holt, R. C. Leinbach, J. W. May and D. Collen (1989) Circulation 79, 920-928
127. E. J. P. Brommer, A. L. Boks, J. Koopman and F. Haverkate (1985) Thromb. Res. 39, 271-280
128. J. A. Paramo, P. S. Gascoine, J. B. Pring and P. J. Gaffney (1990) Fibrinolysis 4, 169-175
129. H. R. Lijnen, B. Vanhoef and D. Collen (1991) J. Biol. Chem. 266, 4041-4044
130. E. L. Madison, E. J. Goldsmith, R. D. Gerard, M. J. H. Gething and J. F. Sambrook (1989) Nature., 339: 721-724
131. E. L. Madison, E. J. Goldsmith, R. D. Gerard, M. J. H. Gething, J. F. Sambrook and R. S. Basselduby (1990) Proc. Natl. Acad. Sci. USA. 87, 3530-3533
132. X. K. Li, H. R. Lijnen, L. Nelles, B. Vanhoef, J. M. Stassen and D. Collen (1992) Blood 79, 417-429
133. D. S. Adams, L. A. Griffin, W. R. Nachajko, V. B. Reddy and C. M. Wei (1991) J. Biol..Chem. 266, 8476-8482
134. R. V. Shohet, S. Spitzer, E. L. Madison, R. Basselduby, M. J. Gething and J. F. Sambrook (1994) Thrombosis and Haemostasis 71, 124-128
135. J. Krause, W. Seydel, G. Heinzel and P. Tanswell (1990) Biochem. J. 267, 647-652.
136. R. Hiramatsu, S. Kasai, Y. Amatsuji, T. Kawai, M. Hirose, M. Morita, T. Tanabe, H. Kawabe and H.Arimura (1989) Fibrinolysis 3, 147-151
137. M. Johannessen, V. Diness, K. Pingel, L. C. Petersen, D. Rao, P. Lioubin and P. O'Hara (1990) Thromb Haemostas. 63, 54-59
138. M. Otter, J. Kuiper, T. J. C. van Berkel and D. C. Rijken (1992) Annals of the New York Academy of Sciences 667, 431-442
139. U. Kohnert,R. Rudolph, J. H. Verheijen, E. J. D. Weening-verhoeff, A. Stern, U. Opitz, U. Martin, H. Lill and H. Prinz (1992) Prot. Engng. 5, 93-100
140. U. Tebbe, R. Vonessen, A. Smolarz, P. Limbourg, J. Rox, J. Rustige, A. Vogt, J. Wagner and W. Meyersabellek (1993) American Journal of Cardiology 72, 518-524
141. L. A. Erickson, G. J. Fici, J. E. Lund, T. P. Boyle, H. G. Polites and K. R. Marotti (1990) Nature 346, 74-76
142. S. S. Margossian, H. S. Slayter, E. Kaczmarek and J. Mcdonagh (1993) Biochimica et Biophysica Acta 1163, 250-256
143. E. J. D. Weening-verhoeff, P. H. A. Quax, R. T. J. van Leeuwen, E. F. Rehberg, K. R. Marotti and J. H. Verheijen (1990) Prot. Engng. 4, 191-198
144. G. R. Larsen, K. Henson and Y. Blue (1988) J. Biol. Chem. 263, 1023-1029
145. J. H. Verheijen, W.Nieuwenhuizen, D. W. Traas, G. T. G. Chang and E. Hoegee (1983) Thromb. Res. 32, 87-92
146. W. J. G. Schielen, H. P. H. M. Adams, M. Voskuilen, G. J. Tesser and W. Nieuwenhuizen (1991) Biochem. J. 276, 655-659
147. C. P. Ponting, S. K. Holland, S. A. Cederholm-Williams, J. M. Marshall, A. J. Brown, G. Spraggon and C. C. F. Blake (1992) Biochimica et Biophysica Acta 1159, 155-161
148. C. P. Ponting, J. M. Marshall and S. A. Cederholm-Williams (1992) Blood Coagulation & Fibrinolysis 3, 605-614
149. T. Urano, Y. Takada and A. Takada (1991) Thromb. Res. 61, 349-359
150. G. Markus, J. L. Evers and G. H. Hobika (1978) J. Biol. Chem. 253, 733-739
151. L. Banyai and L. Patthy (1984) J. Biol. Chem. 259, 6466-6471
152. B. N. Violand, R. Byrne and F. J. Castellino (1978) J. Biol. Chem. 253, 5395-5401
153. A. Takada and Y. Takada (1981) Thromb. Res. 22, 437-443
154. M. Adams-Lucas, D. L. Straight, L. J. Fretto and P. A. McKee (1983) J. Biol. Chem. 258, 12171-12177
155. B. A. K. Chibber and F. J. Castellino (1986) J. Biol. Chem. 261, 5289-5295
156. F. J. Castellino (1988) in Tissue-type plasminogen activator (t-PA) : Physiological and clinical aspects Vol 1, edited by C. Kluft, CRC Press Inc, Boca Raton Fla
157. T. Urano, V. S. de Serrano, P. J. Gaffney and F. J. Castellino (1988) Biochemistry 27, 6522-6528
158. A. Takada and Y. Takada (1991) in Recent Advances in Thrombolysis and Fibrinolysis, edited by K. Tanaka, Academic Press, Tokyo
159. S. J. Gardell, L. T. Duong, R. E. Diehl, J. D. York, T. R. Hare, R. B. Register, J. W. Jacobs, R. A. F. Dixon and P. A. Friedman (1989) J. Biol. Chem. 264, 17947-17952
160. J. Kratzschmar, B. Haendler, G. Langer, W. Boidol, P. Bringmann, A. Alagon, P. Donner and W. D. Schleuning (1991) Gene 105, 229-237

207. M. L. Dyken, T. Brott, L. Caplan, T. R. Price, J. Moossy, J. Zivin, E. Flamm, J. S. Meyer and M. Ginsberg (1990) Stroke 21, 10-11 .
208. E. S. Monrad (1991) Journal of the American College of Cardiology 18, 1573-1578
209. B. E. Sobel and D. Collen (1992) American Journal of Cardiology 70, 385-389
210. E. Braunwald, C. P. Cannon and C. H. Mccabe (1992) Circulation 86, 683-687
211. A. J. Doorey, E. L. Michelson and E. J. Topol (1992) Journal of the American Medical Association 268, 3108-3114
212. D. Collen (1993) Zeitschrift Fur Kardiologie 82, 119-123
213. S. Sherry and V. J. Marder (1991) Journal of the American College of Cardiology 18, 1579-1582
214. A. Terashi, Y. Kobayashi, Y. Katayama, K. Inamura, M. Kazama and T. Abe (1990) Sem. Thromb. Haemostas. 16, 236-241
215. S. Z. Goldhaber (1991) Progress in Cardiovascular Diseases 34, 113-134
216. S. Z. Goldhaber (1992) Chest 101, S183-S185
217. G. Agnelli and P. Parise (1992) Chest 101, S172-S182
218. A. A. Sasahara, C. C. Stmartin, J. Henkin and W. M. Barker (1992) Hematology - Oncology Clinics of North America 6, 1141-1159
219. D. Collen (1991) Plasminogen Activators from Cloning to Therapy 77, 151-159 (1991).
220. L. A. Miles and E. F. Plow (1987) In: Castellino FJ, Gaffney PJ, Samama MM, Takada A, eds.Fundamental an Clinical Fibrinolysis. Exerpta Medica, Amsterdam: 111-124
221. M. Delrosso, G. Fibbi, G. Dini, C. Grappone and M. Pucci (1991) Sem. in Thrombosis and Hemostasis 17, 262-267
222. G. C. Shih and K. A. Hajjar (1993) Proceedings of the Society for Experimental Biology and Medicine., 202, 258-264
223. V. W. M. van Hinsbergh, T. Kooistra, J. J. Emeis and P. Koolwijk (1991) International Journal of Radiation Biology 60, 261-272
224. E. Levin and D. J. Loskutoff (1982) Annals of the New York Academy of Sciences 401, 185-194
225. P. Wallén (1987) in: Fundamental and Clinical Fibrinolysis, edited by F. Castellino, P. Gaffney, M. Samama and A. Takada, Exerpta Medica, Amsterdam
226. K. Robbins, G. H. Barlow, G. Nguyen and M. M. Samama (1987) Sem. Thromb. Hemostas. 13, 131-138
227. P. Wallén (1988) in: Fundamental and Clinical Fibrinolysis, edited by F. J. Castellino, Exerpta Medica, Amsterdam
228. S. Sherry (1985) New England Journal of Medicine 313, 1014-1017
229. A. Takada and Y. Takada (1987) In:Castellino FJ, Gaffney PJ, Samama MM, Takada A, eds.Fundamental an Clinical Fibrinolysis. Exerpta Medica, Amsterdam : 33-44
230. H. R. Lijnen and D. Collen (1988) Enzyme 40, 90-96
231. V. Gurewich (1989) Fibrinolysis 3, 59-66
232. F. J. Castellino, T. Urano, V. de Serrano, J. P. Morris and B. A. K. Chibber (1988) Haemostas. 18, 15-23
233. W. F. Mangel (1991) Plasminogen Activators: from Cloning to Therapy. 77, 43-53
234. W. Nieuwenhuizen, M. Voskuilen, A. Vermond, G. H. Veeneman, J. H. van Boom, E. A. Klasen and N. D. Zegers (1987) in: Fundamental and Clinical Fibrinolysis, edited by F. J. Castellino, P. J. Gaffney, M. Samama and A. Takada, Exerpta Medica, Amsterdam
235. H. R. Lijnen and D. Collen (1982) Sem. Thrombos. Hemostas. 8, 2-10
236. R. D. Gerard, K. R. Chien and R. S. Meidell (1986) Mol, Biol, Med. 3, 449-457
237. D. A. Hart and A. Rehemtulla (1988) Comp. Biochem. Physiol. 90, 691-708 .
238. J. Krause (1988) Fibrinolysis 2, 133-142
239. L. Patthy (1987) FEBS letts 214, 1-7
240. M. Verstraete (1992) Cardiovascular Drugs and Therapy 6, 111-124
241. A. Klausner (1986) Bio/Technology 4, 706-711
242. J. Loscalzo (1990) Circulation 82, 1062-1063
243. H. R. Lijnen and D. Collen (1991) Thrombosis and Haemostasis 66, 88-110
244. H. R. Lijnen and D. Collen (1991) Biotechnology of Plasma Proteins 58, 69-72
245. M. S. Runge, C. Bode, E. Haber and T. Quertermous (1991) Molecular Biology & Medicine 8, 245-255
246. N. U. Bang (1989) Circulation 79, 1391-1392
247. J. H. Robinson and M. J. Browne (1991) TIBTECH. 9, 86-90
248. J. Krause and P. Tanswell (1989) Drug Res. 39, 632-637
249. M. Dewerchin and D. Collen (1991) Bioconjugate Chemistry 2, 293-300
250. D. J. Hayzer, I. M. Lubin and M. S. Runge (1991) Bioconjugate Chemistry 2, 301-308
251. D. E. Vaughan and J. Loscalzo (1991) Trends in Cardiovascular Medicine 1, 36-39
252. E. Haber, T. Quertermous, G. R. Matsueda and M. S. Runge (1989) Science 243, 51-56
253. E. J. Topol (1989) Seminars in Hematology 26, 25-31
254. T. Astrup (1966) Fed. Proc. 25, 42-51

207. M. L. Dyken, T. Brott, L. Caplan, T. R. Price, J. Moossy, J. Zivin, E. Flamm, J. S. Meyer and M. Ginsberg (1990) Stroke 21, 10-11 .
208. E. S. Monrad (1991) Journal of the American College of Cardiology 18, 1573-1578
209. B. E. Sobel and D. Collen (1992) American Journal of Cardiology 70, 385-389
210. E. Braunwald, C. P. Cannon and C. H. Mccabe (1992) Circulation 86, 683-687
211. A. J. Doorey, E. L. Michelson and E. J. Topol (1992) Journal of the American Medical Association 268, 3108-3114
212. D. Collen (1993) Zeitschrift Fur Kardiologie 82, 119-123
213. S. Sherry and V. J. Marder (1991) Journal of the American College of Cardiology 18, 1579-1582
214. A. Terashi, Y. Kobayashi, Y. Katayama, K. Inamura, M. Kazama and T. Abe (1990) Sem. Thromb. Haemostas. 16, 236-241
215. S. Z. Goldhaber (1991) Progress in Cardiovascular Diseases 34, 113-134
216. S. Z. Goldhaber (1992) Chest 101, S183-S185
217. G. Agnelli and P. Parise (1992) Chest 101, S172-S182
218. A. A. Sasahara, C. C. Stmartin, J. Henkin and W. M. Barker (1992) Hematology - Oncology Clinics of North America 6, 1141-1159
219. D. Collen (1991) Plasminogen Activators from Cloning to Therapy 77, 151-159 (1991).
220. L. A. Miles and E. F. Plow (1987) In: Castellino FJ, Gaffney PJ, Samama MM, Takada A, eds.Fundamental an Clinical Fibrinolysis. Exerpta Medica, Amsterdam: 111-124
221. M. Delrosso, G. Fibbi, G. Dini, C. Grappone and M. Pucci (1991) Sem. in Thrombosis and Hemostasis 17, 262-267
222. G. C. Shih and K. A. Hajjar (1993) Proceedings of the Society for Experimental Biology and Medicine., 202, 258-264
223. V. W. M. van Hinsbergh, T. Kooistra, J. J. Emeis and P. Koolwijk (1991) International Journal of Radiation Biology 60, 261-272
224. E. Levin and D. J. Loskutoff (1982) Annals of the New York Academy of Sciences 401, 185-194
225. P. Wallén (1987) in: Fundamental and Clinical Fibrinolysis, edited by F. Castellino, P. Gaffney, M. Samama and A. Takada, Exerpta Medica, Amsterdam
226. K. Robbins, G. H. Barlow, G. Nguyen and M. M. Samama (1987) Sem. Thromb. Hemostas. 13, 131-138
227. P. Wallén (1988) in: Fundamental and Clinical Fibrinolysis, edited by F. J. Castellino, Exerpta Medica, Amsterdam
228. S. Sherry (1985) New England Journal of Medicine 313, 1014-1017
229. A. Takada and Y. Takada (1987) In:Castellino FJ, Gaffney PJ, Samama MM, Takada A, eds.Fundamental an Clinical Fibrinolysis. Exerpta Medica, Amsterdam : 33-44
230. H. R. Lijnen and D. Collen (1988) Enzyme 40, 90-96
231. V. Gurewich (1989) Fibrinolysis 3, 59-66
232. F. J. Castellino, T. Urano, V. de Serrano, J. P. Morris and B. A. K. Chibber (1988) Haemostas. 18, 15-23
233. W. F. Mangel (1991) Plasminogen Activators: from Cloning to Therapy. 77, 43-53
234. W. Nieuwenhuizen, M. Voskuilen, A. Vermond, G. H. Veeneman, J. H. van Boom, E. A. Klasen and N. D. Zegers (1987) in: Fundamental and Clinical Fibrinolysis, edited by F. J. Castellino, P. J. Gaffney, M. Samama and A. Takada, Exerpta Medica, Amsterdam
235. H. R. Lijnen and D. Collen (1982) Sem. Thrombos. Hemostas. 8, 2-10
236. R. D. Gerard, K. R. Chien and R. S. Meidell (1986) Mol, Biol, Med. 3, 449-457
237. D. A. Hart and A. Rehemtulla (1988) Comp. Biochem. Physiol. 90, 691-708 .
238. J. Krause (1988) Fibrinolysis 2, 133-142
239. L. Patthy (1987) FEBS letts 214, 1-7
240. M. Verstraete (1992) Cardiovascular Drugs and Therapy 6, 111-124
241. A. Klausner (1986) Bio/Technology 4, 706-711
242. J. Loscalzo (1990) Circulation 82, 1062-1063
243. H. R. Lijnen and D. Collen (1991) Thrombosis and Haemostasis 66, 88-110
244. H. R. Lijnen and D. Collen (1991) Biotechnology of Plasma Proteins 58, 69-72
245. M. S. Runge, C. Bode, E. Haber and T. Quertermous (1991) Molecular Biology & Medicine 8, 245-255
246. N. U. Bang (1989) Circulation 79, 1391-1392
247. J. H. Robinson and M. J. Browne (1991) TIBTECH. 9, 86-90
248. J. Krause and P. Tanswell (1989) Drug Res. 39, 632-637
249. M. Dewerchin and D. Collen (1991) Bioconjugate Chemistry 2, 293-300
250. D. J. Hayzer, I. M. Lubin and M. S. Runge (1991) Bioconjugate Chemistry 2, 301-308
251. D. E. Vaughan and J. Loscalzo (1991) Trends in Cardiovascular Medicine 1, 36-39
252. E. Haber, T. Quertermous, G. R. Matsueda and M. S. Runge (1989) Science 243, 51-56
253. E. J. Topol (1989) Seminars in Hematology 26, 25-31
254. T. Astrup (1966) Fed. Proc. 25, 42-51

255. T. Astrup (1978) Progress in chemical fibrinolysis and thrombolysis 3, 1-56
256. P. Wallén (1985) Progress in fibrinolysis 7, 3-11 (1985).
257. D. Wood (1988) in: Fibrinogen, fibrin stabilisation and fibrinolysis, edited by J. L. Francis (VCH, Weinheim
258. T. Astrup, Sem. Thromb. Hemostas. 17, 161-174
259. T. L. Yang-Feng, G. Opdenakker, G. Volckaert and U. Francke (1986) Am. J. Hum. Genet. 39, 79-87
260. P. M. Stein, S. A. Stass and J. Kagan (1993) Genomics 16, 301-302
261. K. Swisshelm, K. Dyer, E. Sadler and C. Disleche (1985) Cytogenet. Cell. Genet. 40, 756
262. A. Todd (1959) J. Pathol. Bacteriol. 78, 281-283
263. K. Tympanidis and T. Astrup (1972) J. Urol. 105, 214-219
264. D. Raum, D. Marcus and C. A. Alper (1980) Science 209, 497

Chapter 7

General Discussion

Under physiological conditions plasmin activity is localised to the fibrin surface. This is accomplished in two ways. Firstly, the plasmin inhibitor (α_2 PI) rapidly inactivates fluid phase plasmin whereas plasmin on the fibrin surface is relatively protected from inhibition. And secondly, plasminogen is preferentially activated on the fibrin surface by the plasminogen activators tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA).
During thrombolytic therapy with the plasminogen activators, t-PA or u-PA systemic plasmin formation occurs resulting in a lytic state. High concentrations of plasminogen activators, 2 - 3 orders of magnitude higher than the physiological concentration circumvent the processes responsible for the localisation of plasmin towards the fibrin clot. Although single chain t-PA (sct-PA) has fibrin binding capacity, it is no pro-enzyme and will activate plasminogen outside the vicinity of a fibrin clot. Single chain u-PA (scu-PA) is in contrast with sct-PA a proenzyme, but once activated will not be localised to the fibrin surface because it lacks fibrin binding capacity.
To counteract this disadvantage of thrombolytic therapy (induction of a lytic state) with plasminogen activators we tried to combine the fibrin specificity of t-PA with the proenzyme character of scu-PA.
Both u-PA (GKP) and t-PA (FGK1K2P) are mosaic proteins, built from highly homologous domains (Patthy, 1984; Patthy, 1990). These domains are structurally autonomous and hypothesized to be functionally autonomous. Therefore it should be possible to transpose functions from t-PA to u-PA by transposing domains. Previous studies showed that the K2 domain within t-PA plays an important role in fibrin specificity of t-PA (Verheijen et al.,1986; Zonneveld et al., 1986a; Zonneveld et al., 1986b; Munk et al., 1989). Since scu-PA contains a kringle domain and because kringle domains were shown to be structurally and functionally autonomous folding units, we focused on this domain (Trexler and Patthy, 1983 , Patthy, 1984, Zonneveld et al., 1986a; Zonneveld et al., 1986b). In this thesis it is shown that the K2 domain in the t-PA domain deletion mutant K2P, plays an important role in fibrin specificity, like fibrin binding, lysyl binding and fibrin dependent plasminogen activation. Placing the K1 domain of t-PA for the P domain of t-PA results in a molecule ,K1P, with no fibrin specificity. Since the

K1P and the K2P molecules behaved differently in fibrin binding, lysyl binding and fibrin dependent plasminogen activation, it gave us the opportunity to modify the K1 in such a way that a K2 phenotype would be created. Comparison of 15 primary kringle structures revealed two highly variable regions, which were named kringle specific sequences (KSS 1 and KSS 2). Trexler and Patthy had already hypothesized that these KSS endow a kringle structure with a specific function (Trexler and Patthy, 1983). When we studied these highly variable regions of the K1 and the K2 domain we observed in the KSS 2 of the K2 domain a unique stretch of amino acid residues $N^{69}RRLTW^{74}$. Next the aminoacid residues of the KSS2 in K1 $A^{68}GKYSS^{73}$ were replaced by the aminoacids $N^{69}RRLTW^{74}$. The resulting molecule, which we named k1P, bound to fibrin and to lysyl Sepharose and showed fibrin dependent plasminogen activation. This work supported the view that the presence of a lysyl binding site in the K2 domain of K2P plays an important role in the fibrin specificity of K2P (Verheijen et al., 1986; Zonneveld et al., 1986a; Zonneveld et al., 1986b; Munk et al., 1989). Furthermore the kringle domains within the various KP molecules seemed structurally and functionally autonomous. The KSS of kringle domains seemed to endow a kringle with a function. This result gave us the opportunity to introduce fibrin specificity in the scu-PA molecule with fairly small changes in the primary amino acid sequence. However, the introduction of the residues held responsible for lysyl binding of the K2 domain of t-PA, into the K domain of u-PA, did not result in lysyl binding of the resulting u-PA mutant. Also when the K domain of u-PA was completely replaced by the K2 domain of t-PA no lysyl binding and no fibrin dependent plasminogen activation were observed. Further biochemical analysis indicated that in this hybrid molecule a lysyl binding site is present, but not accessible. Most likely because of an lysyl binding site mediated interaction between the K2 and part of the Pdomain, since the isolated GK2 part of this hybrid molecule did bind to lysyl Sepharose and the isolated K2 domain was found to bind to u-PA (GKP) and the protease domain of u-PA (P). The binding of K2 to the P domain of u-PA could be disturbed by the lysyl analogue ϵ amino caproic acid (EACA).
When the modified u-PA kringle was placed amino-terminal of the protease domain of t-PA, a weak interaction with lysyl Sepharose was observed. This could be an indication that a weak lysyl binding

site was created in the u-PA kringle. On the other hand it could also be possible that, within this molecule domain, domain interactions influence the lysyl binding. At the present time we do not know if the isolated modified u-PA kringle binds to lysyl Sepharose. Despite the presence of a weak lysyl binding site and the high overall similarity with K2P and k1P, the u-PA molecule with the modified kringle did not show fibrin dependent plasminogen activation.

These results challenge the view that structural autonomous domains behave like functional autonomous domains, at least in hybrid plasminogen activators. The lysyl binding site in the u-PA molecule seems to function differently from the lysyl binding site present in the t-PA. Recently it was suggested that the K2 domain of t-PA interacts strongly with the linker peptide G^{144} - K^{158} of the u-PA molecule. (Liu et al., 1992; Song et al., 1992; Colucci et al., 1993; Novokhatny et al., 1994). This interaction could be responsible for the altered properties of the hybrid plasminogen activators we studied. But the presence of a lysyl binding site not always results in such an intra-molecular interaction. When the K domain of u-PA was replaced by the K1 domain of plasminogen, lysyl binding was observed despite the presence of this linker peptide. However, this molecule did not show fibrin dependent plasminogen activation (Boutaud and Castellino, 1993).

The observation that domains in hybrid plasminogen activators do not always function autonomously and the unexpected role of the lysyl binding site therein, made us reconsider the role of the lysyl binding site in t-PA. First we studied the influence of the position of the lysyl binding site (= K2) on the functions of the resulting molecule. The kringle 2 domain and the kringle 1 domain of t-PA were swapped into different protein backgrounds, FGK1K2P and GK1K2P respectively. Lysyl binding, fibrin binding, fibrin dependent plasminogen activation and the amidolytic activity of the single chain form were all affected by kringle reversal. Interestingly the two domains hypothesized to be involved in fibrin binding of t-PA, the F and the K2 domain (Verheijen et al., 1986, Zonneveld et al., 1986; Nesheim et al., 1990; Horrevoets et al., 1994) do not seem functionally not autonomous. Swapping the two kringle domains (FGK2K1P) resulted in reduced fibrin binding, which could be completely competed by the lysine analogue ϵ amino caproic acid (EACA). This indicates that the F domain mediated

interaction no longer occurs, despite the presence of the F domain. Deletion of the F domain results in a better pro-enzyme of t-PA and exchange of the kringle domains results in a molecule which is a better plasminogen activator in the absence of fibrin. These observations suggests that the position of the lysyl binding site within the t-PA molecule has a profound influence on the resulting biochemical properties of the molecule. These results could be explained by hypothesizing extensive domain-domain interactions within the t-PA molecule. Deletion of the F domain results in a better availability of the lysyl binding site and placing the K2 on the position of the K1 results in a lesser availability of the lysyl binding site. Most likely this availability of the lysyl binding site is influenced by competition of a lysyl residue within the F domain and/or the P domain and the lysyl binding site in the K2. These results extended the observation made in hybrid plasminogen activators. Also in the t-PA molecule the structurally autonomous K2 domain does not function as an autonomously domain. Recently it was shown that the F domain and the P domain interact, and that also the F and the G parts of t-PA are not independent domain. (Novokhatny et al., 1991; Smith et al., 1994; Margossian et al., 1993).

The role of the lysyl binding site was further studied using lysyl binding site mutants. Fibrin binding experiments with domain deletion mutants of t-PA and the corresponding lysyl binding site mutants resulted in a paradox. Deletion of the lysyl binding site and competition with the lysine analogue ϵ amino caproic acid (EACA) are not equivalent processes. This observation suggests a broader role for the lysyl binding site than the proposed direct interaction with a lysyl residue of the fibrin network. However, both deleting the lysyl binding site in the K2P variant of t-PA and competition of K2P binding with EACA result in abolishment of fibrin binding. This is again in agreement with the notion that the lysyl binding site directly interacts with a lysyl residue of the fibrin clot. A closer look reveals that the C_{50} of the K2P mutant (C_{50} = 1.5 μM) is far to high to explain the observed decrease in fibrin affinity of the t-PA(D236N) (t-PA C_{50} = 70 nM; t-PA(D236N) C_{50} = 169 nM). Furthermore the affinity of the interaction of ϵ amino caproic acid (EACA) with the K2 domain is about 2 - 3 orders lower than that of the interaction of K2P with fibrin. This suggests the involvement of other amino acid residues besides the lysyl residue in the

interaction of K2P with fibrin. Occupation of the lysyl binding site in the K2 domain results in steric hindrance, disturbing other interactions. However, deleting the lysyl binding site also results in deletion of fibrin binding (C_{50} > 20 μM), indicating that upon deletion of the lysyl binding site, also the fibrin binding site is destroyed in this K2P mutant molecule.
This paradox is easily explained when a lysyl binding site mediated domain-domain interaction is hypothesized. Deleting the lysyl binding site results in a conformationally changed molecule, thereby changing the fibrin binding pocket of t-PA.
At the present time there are some indications that EACA induces a conformation change in the t-PA molecule (Hasegawa and Kondo, 1985; Ichimura, et al., 1987). The solubility of t-PA is increased in the presence of EACA. However, it is difficult to discriminate between a conformational change and intermolecular interaction when solubility is concerned. For instance it is known that isolated kringle 2 domains interact via a lysyl residue of one kringle domain and the lysyl binding site of another K2 domain. This interaction is Cl^- mediated (IC_{50} = 30 mM; Vos et al., 1992). The solubility of t-PA is not influenced by the presence of Cl^- suggesting that a K2-K2 interaction is probably not responsible for the low solubility of t-PA (Novokhatny et al., 1991).

When both fibrin binding domains, the F domain and the lysyl binding site, are removed still fibrin binding is observed. This result challenges the view that the F and the K2 domain are the only domains involved in fibrin binding (Verheijen et al., 1986; Zonneveld et al., 1986; Nesheim et al., 1990; Horrevoets et. 1994) Bennet and coworkers showed with an alanine scan of t-PA that the fibrin binding property is spread over al domains, except the K2 domain (Bennet et al., 1991).
Competition experiments with t-PA and K2P molecules indicate that these molecules bind to the same location on the fibrin network. Interesting is the competition between GK1K2P(D236N) and K2P. The observed reduction of fibrin binding of t-PA in the presence (C_{50} = 70 nM) or in the absence of EACA (C_{50} = 360 nM) could reflect the action of the plasminogen molecule on the fibrin bound t-PA. Occupation of the lysyl binding site of t-PA results in the release of the K2P part from the fibrin surface, ready to hydrolyse the R^{561}-V^{562} peptide bond of plasminogen. Whether this

represents the initiation of fibrinolysis on the fibrin surface is not known.
Since K2P(D236N) no longer interacts with fibrin it was surprising to find that it still is an efficient plasminogen activator, similar in efficiency to K2P. This indicates that the fibrin dependent plasminogen activation proceeds most likely via fibrin bound plasminogen, as comparable with scu-PA (Liu and Gurewich, 1991; Fleury et al., 1993).

Conclusion

To reduce one of the disadvantages of current thrombolytic agents t-PA and u-PA (induction of a lytic state) the combination of the positive features of both plasminogen activators t-PA (fibrin specificity) and scu-PA (proenzyme) was considered. Based on two assumptions it was tried to transpose fibrin specificity of t-PA to the u-PA molecule. The first assumption was that structurally autonomous domains are also functionally autonomous domains. It is shown in this thesis that the structurally autonomous domains in hybrid plasminogen activators and in t-PA do not always function autonomously. Extensive domain-domain interaction occurs. The second assumption was that the lysyl binding site of t-PA is directly involved in fibrin binding. It is shown here that the lysyl binding site is most likely also involved in domain-domain interaction. It is hypothesized that the lysyl binding site in t-PA is involved in stabilising a conformation necessary for high affinity fibrin binding.

References

Bennett, W. F., Paoni, N. F., Keyt, B. A., Botstein, D., Jones, A. J. S., Presta, L., Wurm, F. M., and Zoller, M. J. (1991) J. Biol. Chem. 266, 5191-5201
Boutaud, A. and Castellino F. J. (1993) Archives of Biochemistry and Biophysics 303, 222 - 230
Colucci, M., Cavallo, L., G., Agnelli, G., Mele, A., Bürgi, R., Heim, J., and Semeraro, N. (1993) Thrombos. and Haemostas. 69, 446 - 472
Heim, H., Asselbergs, F., Bürgi, R., Chaudhuri, B., Küenzi, M., Meyhack, B., Fleury, V., Lijnen, H.R., Anglés-Cano (1993) J. Biol. Chem. 268, 18554 - 18559.
Hasegawa, A., Kondo, S. (1985) European patent application publication number EP0156169.
Horrevoets, A.J.G., Smilde, A., Vries, de C., and Pannekoek, H. (1994) J. Biol. Chem. 269, 12639 - 12644
Ichimura, M., Saki, K., Kunihiro, Y., and Nakashiro, T. (1987) European patent application publication number 0217379
Liu, J., and Gurewich, V. (1991) J. Clin. Invest. 88, 2012 - 2017
Liu, J., Song, A., Zhu, D., and Gurewich, V. (1992) in Peptides, Chemistry and Biology. (Smith, J., and Rivier, J.E. eds.) pp. 810 - 811, ESCOM, Leiden
Margossian, S. S., Slayter, H. S., Kaczmarek, E., and McDonagh, J. (1993) Biochimica et Biophysica Acta

1163, 250-256
Munk, de G. A. W., Caspers, M. P. M., Chang, G. T. G., Pouwels, P. H., Enger-Valk, B. E. & Verheijen, J. H. (1989) Biochemistry 28, 7318 - 7325
Nesheim, M., Fredenburgh, J. C., and Larsen, G. R. (1990) J. Biol. Chem. 265, 21541-21548
Novokhatny, V. V., Ingham, K. C., and Medved, L. V. (1991) J. Biol. Chem. 266, 12994-13002
Novokhatny, V. V., Medved, L., Lijnen, H. R., Collen, D., and Ingham, K. C. (1994) J. Fibrinolysis 8, 11 (abstr)
Patthy, L., Trexler, M., Banyai, L., and Varadi, A. (1984) FEBS Lett. 171, 131-136
Patthy, L. (1985) Cell. 41, 657 - 663
Patthy, L. (1990) Blood coagulation and fibrinolysis, 1, 153 - 166
Smith, B. O., Downing, A. K., Dudgeon, T. J., Cunningham, M., Driscol, P. C. and Campbell, I. D. (1994) Biochemistry, 33, 2422 - 2429
Song, A., Liu, J., Yu, R., Cui, D., Zhou, T., Cui, H., and Zhu, D. (1992) Science in China. 35, 975 - 973
Trexler, M., and Patthy, L. (1983) Proc. Natl. Acad. Sci. USA 80, 2457-2461
Verheijen, J. H., Caspers, M. P. M., Chang, G. T. G., Munk, de G. A. W. d., Pouwels, P. H. & Enger-Valk, B. E. (1986) EMBO Journal 5, 3525 - 3530.
Vos, de A. M., Ultsch, M. H., Kelley, R. F., Padmanabhan, K., Tulinsky, A., Westbrook, M. L. & Kossiakoff, A. A. (1992) Biochemistry 31, 270 - 279
Zonneveld, van A. J. v., Veerman, H. & Pannekoek, H. (1986a) Proc. Natl. Acad. Sci. USA. 83, 4670 - 4674
Zonneveld van, A. J., Veerman, H. & Pannekoek, H. (1986b) J. Biol. Chem. 261, 14214 - 14218

Summary

The presence of a blood clot (thrombus) within a blood vessel will deprive downstream tissue of oxygen. Without oxygen, cells within such a tissue will soon die. The longer this oxygen deprivation exists, the more cells will die. Eventually, this will result in a rapid decline of the functional features of such a tissue. Therefore to prevent loss of tissue function it is essential to remove such an obstructing clot without delay. A well-known example is myocardial infarction. A blood clot obstructing the blood flow in a blood vessel suppling the heart muscle with blood, will deprive the heartmuscle cells of oxygen. When this situation obtains for a longer period, these muscle cells will die. The more muscle cells die, the bigger the decline in heart function. As larger parts of the heart muscle become dysfunctional, it will be impossible for the heart muscle to cope with strenuous activity. When such effort is attempted, the heart responds by sending out a s.o.s. (pain in the chest).
The observation that the body possesses ways of dissolving blood clots, resulted in a search for the agents that are involved in this process. Once, these agents responsible for the dissolution of blood clots are characterized, it should be possible to use them to dissolve obstructing blood clots in blood vessels.
In this way, obstructing blood clots could be lysed and the fast decline of the functional features of tissues prevented.
At the present time, this desired fast-acting agent for dissolving blood clots has still not been found. However, research has resulted in the identification of some enzymes involved in blood clot dissolution.
The most important of these is plasmin. Plasmin degrades the protein (fibrin) which gives most of the blood clots their rigidity. (The process of fibrin degradation is referred to as fibrinolysis).
In the thirties, plasmin was used to dissolve obstructing blood clots in man. However, the result turned out to be not very promising. Not only was fibrin, the scaffold of the blood clot degraded, but also the blood proteins that are involved in the formation of blood clots, preventing the person from forming blood clots for a longer period and thereby increasing the risk of bleeding. Since bleeding is just as detrimental to tissue as deprivation of oxygen, plasmin treatment seemed not very promising as a therapeutical agent.
Under physiological conditions the mechanisms for the dissolution

of blood clots are highly blood clot specific. The mechanisms underlying this clot selectivity in healthy persons, are intricate. One clue, for this blood clot specific dissolution under physiological conditions, came from the observation that in circulation, plasmin is present as an inactive precursor molecule, plasminogen. Other proteins, referred to as plasminogen activators are needed to convert plasminogen into plasmin. These plasminogen-activators preferentially convert plasminogen into plasmin on the surface of a blood clot (fibrin). The two known plasminogen activators, tissue-type plasminogen activator (t-PA) and urokinase-type plasminogen activator (u-PA) localise the plasminogen to plasmin conversion towards the blood clot in different ways, although the exact molecular sequences leading to plasminogen activation on a blood clot (fibrin) are still not well-known.
Tissue-type plasminogen activator is present as an active plasminogen activator and possesses high affinity for a blood clot. In the presence of a blood clot, plasminogen is more efficiently converted into plasmin by tissue-type plasminogen activator. On the other hand urokinase-type plasminogen activator has no preferences for a blood clot but this molecule is present within the blood as a inactive precursor. Only in the presence of a blood clot does urokinase-type plasminogen activator convert plasminogen into plasmin (the exact molecular description is still not well-known). What is interesting, is the observation, that plasmin is capable of converting inactive urokinase-type plasminogen activator to the active form. This active urokinase-type plasminogen activator no longer needs the presence of a blood clot to convert plasminogen into plasmin.
Apart from this blood clot dependent conversion of plasminogen to plasmin, there exists another important way to localise the plasmin activity towards the fibrin clot. Outside the blood clot (fibrin), plasmin is rapidly inactivated by the plasmin inhibitor (α_2 plasmin inhibitor), and tissue-type plasminogen activator is rapidly inactivated by plasminogen activator inhibitors (PAI). So, only in the presence of a blood clot are both plasmin and tissue-type plasminogen activator relatively protected from rapid inactivation.

At the present time, both tissue-type plasminogen activator and urokinase-type plasminogen activator are used in the treatment of obstructing blood clots in blood vessels. The results vary widely.

One of the disadvantages of these agents is the induction of bleeding.
The improvement of the plasminogen activators, tissue-type plasminogen activator and urokinase-type plasminogen activator focussing on the reduction of the risk of bleeding, form the subject of this thesis. To be more specific, is it possible to modify tissue-type plasminogen activator or urokinase-type plasminogen activator in such a way that the chance of bleeding will be reduced ?.

In chapter 1 the molecular processes underlying clot lysis in a healthy person are described. From this description it becomes apparent, why in a healthy person, clot lysis is never accompanied by bleeding. The concentrations of plasminogen activator during clot lysing therapy are typically 2 to 3 orders of magnitude higher than the concentration of plasminogen activator in a normal healthy person. Under these conditions, all the mechanisms involved in localisation of the plasmin activity towards the blood clot fail. When large amounts of tissue-type plasminogen activator are given to a person, it will first deplete the plasminogen activator inhibitor pool.(Tissue-type plasminogen is always present in an active form). Despite its preference for a blood clot, high concentrations of tissue-type plasminogen activator will activate plasminogen in circulation. Since the plasmin formed is not protected by the blood clot, it will be rapidly inactivated by the plasmin inhibitor. Because of the large quantities of plasmin formed, the concentration of free plasmin inhibitor will rapidly decline and plasmin will be able to degrade other proteins some of which play a key role in the formation of blood clots. This could result in bleeding.
In contrast, the inactive urokinase-type plasminogen activator will not convert plasminogen into plasmin in circulation. However, when plasmin is formed on the blood clot, this plasmin will convert urokinase-type plasminogen activator into its active form. This form of urokinase-type plasminogen activator is blood clot independent. First the plasminogen activator inhibitor will inactivate urokinase type plasminogen activator which has formed. But as the concentration of active urokinase-type plasminogen activator rises this inhibitor pool will be depleted. Urokinase-type plasminogen activator is now able to convert plasminogen into plasmin in circulation. As with tissue-type plasminogen, too much plasmin will eventually induce bleeding. Bleeding could be prevented if tissue-

type plasminogen activator could be modified into an inactive form. The high amounts of tissue-type plasminogen, necessary during clot lysing therapy will not then convert plasminogen into plasmin. Since t-PA has a preference for blood clots, the formation of plasmin will be localised towards the blood clot.
At the beginning of the experimental work it seemed difficult to transform tissue-type plasminogen activator into an inactive molecule, which would still activate plasminogen in the presence of a blood clot. At that time it seemed more straightforward to change urokinase-type plasminogen activator, in such a way that it would obtain a preference for blood clots. Inactive urokinase-type plasminogen activator was hypothesized to activate plasminogen to plasmin only in the presence of a blood clot.
This thesis describes which approach is used to make urokinase-type plasminogen activator a blood clot binding protein. Furthermore it shows why it turned out to be more difficult than originally thought.

Tissue-type plasminogen activator and urokinase-type plasminogen activator are strongly related proteins. Both are composed of comparable parts (domains, see figure 4 and 5 , chapter 1). Two of these parts are important for this research project: the part which is referred to as kringle domain (K) and the part that is referred to as protease domain (P). One of the starting points of this research project was that the three-dimensional structure of these domains within a plasminogen activator is independent of the presence of other domains in the plasminogen activator, and more importantly that also the functions of these domains are not influenced by the molecular context.
In both plasminogen activators this P domain is involved in the conversion of plasminogen into plasmin. It makes no difference whether this P domain is part of a plasminogen activator or present as an isolated domain indicating the functional independence of this domain.
One of the domains in tissue-type plasminogen activator that is involved in the binding of t-PA to a blood clot and is involved in blood clot dependent plasmin formation, is the kringle 2 domain. Within this kringle 2 domain there exists a binding site for a lysyl residue (one specific amino acid within a protein). The presence of this lysyl binding site is associated with the binding to a blood

clot and the ability to convert efficiently plasminogen into plasmin in the presence of a blood clot. This lysyl binding is present in tissue-type plasminogen activator and in isolated kringle 2 domain, again stressing the functional independence of this lysyl binding site. Since there is also a kringle domain in urokinase-type plasminogen activator and since this kringle domain does not possess a lysyl binding site and since urokinase-type plasminogen activator has no affinity for a blood clot, our attention was focused on this lysyl binding site. We wondered whether or not it would be possible to introduce clot binding properties into the urokinase-type plasminogen activator by introducing a lysyl binding site into the kringle domain of the urokinase-type plasminogen activator. This formed the second starting point of this research project, that the lysyl binding site is directly involved in the binding of tissue-type plasminogen activator to a blood clot.

In chapter 2 it is shown that the kringle 2 domain of tissue-type plasminogen activator plays an important role in the interaction of tissue-type plasminogen activator with a blood clot and in the clot dependent plasminogen conversion. When the kringle 2 domain is placed before the protease domain of the tissue-type plasminogen activator (K2P), this molecule possesses a lysyl binding site, binds to a blood clot and efficiently converts plasminogen to plasmin in the presence of a blood clot. In contrast, the K1P (kringle 1 domain of the tissue type plasminogen activator placed aminoterminal of the protease domain of tissue-type plasminogen activator) has none of these functional features. Comparison of the K1 structure with the K2 structure (The protease domains are both from the tissue-type plasminogen activator) revealed that a part of the kringle 2 domain is not present in the K1 domain. Introduction of this part of the kringle 2 domain into the kringle 1 domain resulted in a molecule k1P (little kringle 1). The k1P contains a lysyl binding site, binds to a blood clot and converts plasminogen to plasmin on the fibrin clot. It therefore seems reasonable to assume, that the lysyl binding site plays an central role in binding to a blood clot and the efficient conversion of plasminogen to plasmin on the fibrin clot. Important for this research project is the observation that these functions can be transferred from one domain to another domain by transferring small unique pieces of kringle domain.

In chapter 3 an attempt is described to make urokinase-type plasminogen activator a clot binding agent. The kringle of urokinase-type plasminogen activator is changed in such a way that theoretically a lysyl binding site should be created. Also the functional lysyl binding site is introduced in the urokinase-type PA molecule by replacing the kringle domain of urokinase-type plasminogen activator for the K2 domain of t-PA. Amazed and a little disappointed, we found that neither molecule contained a functional lysyl binding site. Certainly for the urokinase-type plasminogen activator with the kringle 2 domain (that harbours a functional lysyl binding site) this result was unexpected.
When this kringle 2 domain was isolated from the urokinase-type plasminogen activator a lysyl binding site could be detected. Furthermore isolated kringle 2 domains interact with the P domain of urokinase type plasminogen activator. This interaction could be disturbed by lysyl-like compounds. Most likely the lysyl binding site is involved in an interaction with the protease domain of the urokinase-type plasminogen activator. These results question the functional independence of the kringle 2 domain in this urokinase-type plasminogen activator. Furthermore, this result shows that the introduction of clot binding properties in urokinase-type plasminogen activator by the construction of a lysyl binding site in the kringle domain of urokinase-type plasminogen activator is more difficult than anticipated.

Chapter 4 examines whether the result described above is an exception to the rule and the fact that within tissue-type plasminogen activator, domains, do function independent. The kringle 2 domain and the kringle 1 domain within the tissue-type plasminogen activator changed position. Because of the similarity in the overall structure of kringles, the overall structure of this tissue-type plasminogen activator with swapped kringles will not differ much from the original tissue-type plasminogen activator. However, functional features such as the interaction with a blood clot the conversion of plasminogen in the presence of a blood clot (fibrin) and the presence of a lysyl binding site are all affected, suggesting that also in tissue-type plasminogen activator, domains do in fact interact extensively, creating a unique structure responsible for a certain functional feature. The role of the lysyl binding site in these domain-domain interactions is evident.

In chapter 5 this lysyl binding site is studied in more detail. By changing two atoms (aspartic acid into asparagine) in the kringle 2 domain of tissue-type plasminogen activator, these molecules no longer possess a functional lysyl binding site.
From the studies with this tissue-type plasminogen activator variants, it becomes apparent that the lysyl binding site is not directly involved in the interaction with a blood clot (fibrin). It is hypothesized that the lysyl binding site is involved in an interaction with a lysyl residue of the protease domain of tissue-type plasminogen activator. The resulting conformation is responsible for the high fibrin affinity of the tissue-type plasminogen activator molecule. Furthermore, the binding to a blood clot (fibrin) and the enhanced converion of plasminogen into plasmin in the presence of a fibrin clot (fibrin) seem not nessecarily coupled processes. If the tissue-type plasminogen activator mutant (containing the kringle 2 domain and the protease domain) K2P contains a lysyl binding site, it interacts with a blood clot and shows enhanced conversion of plasminogen to plasmin in the presence of a blood clot. In contrast the K2P with an impaired lysyl binding site does not interact with a blood clot, but still efficiently converts plasminogen to plasmin in the presence of a blood clot.

In chapter 6 other disadvantages of the blood clot dissolving agents, tissue-type plasminogen activator and urokinase-type plasminogen activator are described.
Furthermore, some strategies to improve these disadvantages are discussed. This discussion is extended to vampire bat saliva plasminogen activator and the bacterial proteins streptokinase and staphylokinase.

In chapter 7 the results of this thesis are evaluated. This discussion centres on the role of the lysyl binding site in tissue-type plasminogen activator. It becomes clear, that the structurally independent domains do not behave independently. This is true for the urokinase-type plasminogen activator which contains the kringle 2 domain of tissue-type plasminogen activator but also for tissue-type plasminogen activator itself. The role of the lysyl binding site in tissue-type plasminogen activator in binding to a blood clot is indirect, most likely keeping the tissue-type plasminogen activator molecule in the blood clot binding form.

Samenvatting

Een bloedstolsel (thrombus) in het bloedvat kan zeer nadelige gevolgen voor een individu hebben. Wanneer het bloedvat afgesloten wordt, zullen cellen in het achterliggende weefsel een gebrek aan zuurstof krijgen. In een omgeving met te weinig zuurstof gaat een cel al snel dood. Wanneer het gebrek aan zuurstof lang duurt zullen veel cellen doodgaan. Het weefsel waarin deze cellen liggen kan dan steeds minder werk verrichten. Om verlies van cellen en daarmee verlies van functie zoveel mogelijk te voorkomen is het dus zaak om zo snel mogelijk dit stolsel te verwijderen.
Een bekend voorbeeld is het hart infarct. Een bloedstolsel in een bloedvat dat het hart van bloed voorziet, onthoudt het hart de zo belangrijke zuurstof. Afhankelijk van de plaats van zo'n stolsel sterven kleinere of grotere stukken van de hartspier af. De hartspier zal minder werk kunnen verrichten. Grote inspanning kan het hart dan niet meer aan. Wanneer dit toch verlangd wordt geeft het hart waarschuwingssignalen af (pijn op de borst).

De waarneming, dat het lichaam beschikt over een manier om bloedstolsels op te lossen (thrombolyse) heeft geleid tot een ware speurtocht naar de stoffen die betrokken zijn bij het oplossen van stolsels in het lichaam. Wanneer eenmaal de stoffen verantwoordelijk voor dit oplossen van stolsels bekend zouden zijn, zo was het idee, dan zouden deze stoffen gebruikt kunnen worden om door stolsels afgesloten vaten op een elegante manier te openen. Cellen en weefsel en daarmee functies zouden dan zoveel mogelijk behouden blijven.
Een specifiek snelwerkend stolsel oplossend middel (thrombolyticum) is tot nu toe niet gevonden. Wel zijn er een aantal enzymen ontdekt, die een rol spelen bij de afbraak van bloedstolsels. Het belangrijkste is plasmine. Plasmine breekt het eiwit (fibrine) af, waaraan vele bloedstolsels hun stevigheid ontlenen (fibrinolyse). Heel vroeger, in de jaren 30 is nog eens geprobeerd om in mensen bloedstolsels met plasmine op te lossen. Maar dit bleek niet zo succesvol. Niet alleen het fibrine, het geraamte van het stolsel, werd afgebroken maar ook tal van andere eiwitten waaronder eiwitten die een belangrijke rol spelen bij de vorming van bloedstolsels. Dit resulteerde juist in moeilijk

controleerbare bloedingen. Bloedingen vormen een net zo'n groot gevaar voor cellen en het functioneren van een weefsel, als het gebrek aan zuurstof dat onstaat na afsluiting van een bloedvat door een stolsel. Het vervangen van het ene kwaad (het stolsel in een bloedvat) door het andere (bloedingen in weefsels) lijkt niet zinvol.

Anders dan bij de bovenbeschreven effecten van plasmine, worden stolsels in gezonde personen verwijderd (onder ander door plasmine) zonder dat dit resulteert in het onstaan van bloedingsverschijnselen. Een aanwijzing waarom dat zo is, kwam met de waarneming dat plasmine in het bloed in een niet-actieve vorm voorkomt, plasminogeen. Er zijn andere eiwitten nodig, de zogenaamde plasminogeen-activatoren, om plasminogeen in plasmine om te zetten. Deze plasminogeen activatoren zorgen er nu voor, dat plasminogeen alleen in de buurt van een stolsel in plasmine wordt omgezet. De tot nu toe bekende plasminogeen activatoren, weefsel-type plasminogeen activator (t-PA) en urokinase-type plasminogeen activator (u-PA) doen dit op verschillende manieren. Weefsel-type plasminogeen activator bindt aan het stolsel (aan fibrine) en kan in aanwezigheid van dit stolsel (op een nog onbekende manier) beter plasminogeen in plasmine omzetten. Urokinase-type plasminogeen activator, komt in tegenstelling tot weefsel-type plasminogeen activator in een inactieve vorm, net als plasminogeen in het bloed voor. Alleen in de aanwezigheid van een stolsel (fibrine) is het in staat (op een nog onbekende manier) plasminogeen in plasmine om te zetten. Bijzonder is dat het op het stolsel gevormde plasmine het inactieve urokinase-type plasminogeen activator omzet in een actieve vorm. Deze actieve vorm van urokinase-type plasminogeen activator kan plasminogeen activeren in afwezigheid van een stolsel.
Naast deze stolsel afhankelijke omzetting van plasminogeen in plasmine, is er nog een belangrijke manier om de plasmine activiteit tot het stolsel te beperken en te voorkomen dat plasmine andere eiwitten aanvalt. Door de aanwezigheid van zeer snel werkende, zeer specifieke remmers van plasmine (α_2 plasmine inhibitor) en weefsel-type plasminogeen activator en urokinase-type plasminogeen activator (plasminogeen activator inhibitor) in het bloed, wordt vrij plasmine maar ook vrij weefsel-type plasminogeen activator direct geïnactiveerd. Alleen de aanwezigheid van het stolsel

beschermt de plasmine en weefsel-type plasminogeen activator tegen een snelle inactivatie.
Beide enzymen, weefsel-type plasminogeen activator en urokinase-type plasminogeen activator zijn en worden gebruikt om stolsels, die vaten afsluiten op te lossen. De resultaten, het weer open maken van een verstopt bloedvat, variëren sterk. Een van de nadelen is nog steeds het onstaan van bloedingen, ondanks de voorkeur van plasminogeen activatoren om plasmine voornamenlijk in aanwezigheid van een stolsel te vormen.
Het verbeteren van plasminogeen activatoren, waardoor er minder bloedingen onstaan bij het gebruik van deze stoffen om stolsel te verwijderen, vormt het uitgangspunt van dit proefschrift. Anders gezegd, is het mogelijk om weefsel-type plasminogeen activator en urokinase-type plasminogeen activator zo te veranderen dat er geen bloedingsneiging meer optreedt ?

In hoofdstuk 1 is geprobeerd wat dieper in te gaan op de manier waarop in een gezond persoon bloedstolsels verdwijnen. Hieruit volgt direct waarom in gezonde personen het verdwijnen van bloedstolsels niet gepaard gaat met een bloedingsneiging. De concentraties plasminogeen activator gedurende stolsel oplossende therapie zijn 100 tot 1000 maal hoger dan de concentraties in gezonde personen. Onder deze condities falen alle mechanismen, die er voor zorgen dat het oplossen van het stolsel beperkt blijft tot het stolsel. Bij hoge concentraties zal weefsel-type plasminogeen activator, dat niet voorkomt als een inactief eiwit, ondanks de voorkeur voor stolsels (fibrine) ook plasminogeen buiten het stolsel activeren. Dit plasmine, dat niet door het stolsel "beschermd" wordt, zal direct geinactiveerd worden door de plasmine remmer. Maar als de concentratie plasmine remmer daalt (heel veel weefsel-type plasminogeen activator kan heel veel plasminogeen omzetten) kan plasmine niet meer geïnactiveerd worden en zal in staat zijn andere eiwitten (onder andere eiwitten die betrokken zijn bij de bloedstolling) af te breken. Dit zal uiteindelijk leiden tot het onvermogen om gedurende enige tijd stolsels te vormen (een bloedingsneiging). Urokinase-type plasminogeen activator komt wel voor als een inactief eiwit, maar zal na activatie niet bij het stolsel blijven, daar het geen stolsel (fibrine) bindende eigenschappen bezit. Wanneer, op nog onbekende manier, urokinase-type plasminogeen activator plasminogeen op het

stolsel heeft omgezet in plasmine, zal, zoals boven al beschreven is, plasmine urokinase-plasminogeen activator omzetten in een actieve vorm. Eerst zal dit actieve urokinase-type plasminogeen activator, dat geen voorkeur heeft voor het stolsel, geïnactiveerd worden door de plasminogeen activator remmer. Deze remmer zal ook uitgeput raken (er is heel veel urokinase-type plasminogeen activator aanwezig) en actief urokinase-type plasminogeen activator begint plasminogeen buiten de thrombus te activeren. Uiteindelijk onstaat ook nu weer te veel plasmine, leidend tot een tijdelijk onvermogen om stolsels te vormen.
Bloedingsneiging zou dus beperkt kunnen worden door van weefsel-type plasminogeen activator een inactief voorloper molecuul te maken. Veel weefsel-type plasminogeen activator zal dan plasminogeen alleen op het stolsel omzetten in plasmine. Bij het begin van het onderzoek leek de constructie van inactief weefsel-type plasminogeen activator niet zo eenvoudig. Toen, begin 1990 leek het eenvoudiger om het al inactieve urokinase-type plasminogeen activator, stolsel (fibrine) bindend te maken. In dit proefschrift wordt beschreven hoe geprobeerd is urokinase-type plasminogeen activator stolsel (fibrine) bindend te maken, van welke aannames bij de vervaardiging van een stolsel bindend urokinase-type plasminogeen activator zijn uitgegaan en waarom dit stolsel (fibrine) bindend maken moeilijker gebleken is dan aanvankelijk gedacht werd.
Zowel weefsel-type plasminogeen activator als urokinase-type plasminogeen activator zijn moleculen die sterk verwant zijn en beide zijn opgebouwd uit een aantal onderdelen (domeinen, zie figuur 3 en 4 in hoofdstuk 1). Belangrijk voor dit onderzoek vormen twee van die onderdelen, het onderdeel dat kringle domein genoemd wordt en het onderdeel dat protease domein genoemd wordt. Zowel weefsel-type plasminogeen activator als urokinase-type plasminogeen activator bezitten deze onderdelen. Het protease domein wordt P genoemd en het kringle domein wordt K genoemd. Een van de uitgangspunten voor dit onderzoek was, dat deze onderdelen niet alleen een eigen vorm (structuur) hebben, onafhankelijk van het eiwit waarin ze zich bevinden, maar dat deze onderdelen ook een functie bezitten die niet beinvloed wordt door de rest van het eiwit.
Zo bezitten weefsel-type plasminogeen activator en urokinase-type plasminogeen activator een P domein. In beide plasminogeen

activatoren is dit domein betrokken bij de omzetting van plasminogeen in plasmine. Het maakt niet uit of dit domein zich in een plasminogeen activator of buiten de plasminogeen activator bevindt. Een van de domeinen van weefsel-type plasminogeen activator die betrokken is bij binding aan een stolsel en er voor zorgt dat op het stolsel plasminogeen beter omgezet wordt in plasmine, is het kringle 2 domein. Het bijzondere van dit kringle 2 domein, is de aanwezigheid van een bindingsplaats voor een lysyl residu (een specifiek aminozuur in een eiwit).
Aangezien er ook een kringle domein in urokinase-type plasminogeen activator voorkomt, en bekend is dat urokinase-type plasminogeen activator geen lysyl bindingsplaats bezit en ook niet aan fibrine bindt, is de aandacht gericht op de introductie van een lysyl bindingsplaats in het urokinase-type plasminogeen activator. Een tweede belangrijk uitgangspunt voor de constructie van een stolsel (fibrine) bindend urokinase-type plasminogeen activator, was dat deze lysyl bindingsplaats, betrokken is bij de binding van weefsel-type plasminogeen activator aan fibrine.

In hoofdstuk 2 worden de structuren (bepaalde aminozuren) in het kringle 2 domein bepaald, die een rol spelen in lysyl binding, binding aan een stolsel en de betere omzetting van plasminogeen in plasmine in aanwezigheid van een stolsel (fibrine). In weefsel-type plasminogeen activator bevindt zich naast het kringle 2 domein ook nog een kringle 1 domein (zie hoofdstuk 1 figuur 4). Wanneer het molecuul K1P (kringle 1 domein voor het protease domein van t-PA) vergeleken wordt met K2P (kringle 2 domein voor het protease domein van t-PA) dan bezit K2P, in tegenstelling tot K1P alle gewenste eigenschappen, te weten lysyl binding, het vermogen om aan een stolsel te binden en verhoogde omzetting van plasminogeen in plasmine in aanwezigheid van een stolsel (fibrine). Vergelijking van de kringle structuren (aminozuur volgorde) laat zien dat er een stukje (6 aminozuren) kringle bestaat, dat uniek is voor het kringle 2 domein. Introductie van dit kringle 2 unieke stukje in het kringle 1 domein resulteert in een molecuul k1P (k1 = "kleine" kringle 1). Dit molecuul bezit een lysyl bindingsplaats, bindt aan een stolsel en vertoont verhoogde omzetting van plasminogeen in plasmine in aanwezigheid van een stolsel (fibrine). Het lijkt er dus op, dat de aanwezigheid van een lysyl bindings plaats belangrijk is voor de binding aan een stolsel en

voor een beter omzetting van plasminogeen in plasmine op een stolsel. Belangrijk is, dat het door het uitwisselen van een kleine stukje domein deze eigenschappen overgedragen kunnen worden naar een ander domein.

In hoofdstuk 3 is dan ook geprobeerd om op een overeenkomstige manier de kringle van het urokinase-type plasminogeen activator molecuul zo te veranderen, dat er een lysyl bindingsplaats zal onstaan. Immers lysyl binding en binding aan een stolsel en verhoogde omzetting van plasminogeen in plasmine zijn gekoppelde functies. Voor de zekerheid wordt ook de kringle van de urokinase-type plasminogeen activator vervangen door het kringle 2 domein van weefsel-type plasminogeen activator (dat een lysyl bindingsplaats bevat). Geen van de gemaakte nieuwe plasminogeen activatoren (met het veranderde u-PA kringle domein of met het K2 domein) lijkt een lysyl bindingsplaats te bezitten. Dit resultaat is zeker onverwacht voor de urokinase-type plasminogeen activator waarin het kringle 2 domein van weefsel-type plasminogeen activator zit. Controle experimenten laten zien dat het kringle 2 domein wel degelijk een lysyl bindings plaats bevat, maar dat deze niet beschikbaar is. Wanneer het kringle 2 domein uit de urokinase-type plasminogeen activator wordt geknipt en dus niet meer beinvloedt kan worden door de ander onderdelen van de urokinase-type plasminogeen activator, kan er in deze losse kringle 2 weer een lysyl bindingplaats worden aangetoond. Verder onderzoek laat zien dat de lysyl bindings plaats niet beschikbaar is, omdat deze waarschijnlijk gebruikt wordt om aan een onderdeel van urokinase-type plasminogeen activator te binden. Losse kringle 2 domeinen binden aan het protease domein van urokinase-type plasminogeen activator en deze binding kan worden verstoord door stoffen die lijken op lysyl residuen. Dit onverwachte resultaat maakt het moeilijker om urokinase-type plasminogeen activator stolsel bindend te maken door de introductie van een lysyl bindings plaats in het kringle domein.

In hoofdstuk 4 wordt gekeken of boven beschreven domein domein contacten uniek zijn voor combinaties van onderdelen uit verschillende plasminogeen activatoren, of dat er ook domein domein interacties optreden in weefsel-type plasminogeen activator zelf. Door in weefsel-type plasminogeen activator de kringle 2 van

plaats te verwisselen met de kringle 1 wordt duidelijk dat in weefsel-type plasminogeen activator de positie van het kringle 2 domein invloed heeft op de binding aan een stolsel, en de verhoogde omzetting van plasminogeen in plasmine op het stolsel (fibrine). De opvatting dat domeinen functioneren als zelfstandige eenheden lijkt steeds moeilijker houdbaar. Ook nu weer speelt de lysyl bindings plaats een belangrijke rol in deze domein domein interactie.

In hoofdstuk 5 wordt de rol van de lysyl bindings plaats in het kringle 2 domein van weefsel-type plasminogeen activator, nauwkeuriger bestudeerd. Door een zeer kleine verandering (verandering van slechts twee atomen, het aminozuur asparagine-zuur wordt vervangen door asparagine) wordt de lysyl bindings plaats uitgeschakeld. De binding van deze veranderde weefsel-type plasminogeen activatoren aan een stolsel (fibrine) is alleen te verklaren wanneer aangenomen wordt, dat de lysyl bindings plaats niet direct betrokken is bij deze binding. Het lijkt er meer op dat de lysyl bindings plaats betrokken is bij het in de juiste vorm houden van weefsel-type plasminogeen activator, zodat dit goed aan een stolsel kan binden. Bestudering van de omzetting van plasminogeen in plasmine in aanwezigheid van een stolsel (fibrine), laat bovendien zien dat het hebben van een lysyl bindings plaats, fibrine binding en verhoogde omzetting van plasminogeen in plasmine in aanwezigheid van een stolsel niet noodzakelijkerwijs gekoppeld hoeft te zijn. Het eiwit K2P (kringle 2 domain en een protease domein) waarin de lysyl bindings plaats uitgeschakeld is, zet in aanwezigheid van een stolsel plasminogeen net zo goed om als K2P waarin de lysyl bindings plaats nog aanwezig is. Deze veranderde K2P heeft zijn voorkeur voor een stolsel verloren.

In hoofdstuk 6 worden ander nadelen van de plasminogeen activatoren besproken wanneer ze gebruikt worden voor het oplossen van stolsels. Sommige stolsels blijken nauwelijks oplosbaar. Andere stolsels verwijnen wel, maar nieuwe stolsels worden dan weer snel gevormd. Manieren om tot betere stolsel oplossende middelen te komen worden besproken. Ook stolsel oplossende middelen die niet in de mens voorkomen (vampier speeksel plasminogeen activator en de bacterie eiwitten streptokinase en staphylokinase) krijgen aandacht. De nadruk ligt

op de manier waarop stolsel oplossende middelen hun activiteit tot het stolsel beperken.

In hoofdstuk 7 worden de resultaten van dit proefschrift in een wat breder kader geplaatst. Hier ligt de nadruk op de rol van de lysyl bindings plaats in het kringle 2 domein van weefsel-type plasminogeen activator. Duidelijk wordt dat het kringle 2 domein in weefsel-type plasminogeen activator, en de hier bestudeerde combinatie van weefsel-type plasminogeen activator en urokinase-type plasminogeen activator zich niet als een zelfstandige domein gedraagt, maar betrokken is in interacties met andere delen van deze eiwitten. De lysyl bindings plaats in deze plasminogeen activatoren gaat zeer waarschijnlijk een interactie aan met het protease domein. In weefsel-type plasminogeen activator is deze interactie tussen de lysyl bindings plaats en het P domein belangrijk voor het in de goede vorm houden van t-PA, zodat het goed aan een stolsel kan binden.

De in dit proefschrift beschreven experimenten laten zien dat de twee genoemde aannames om het urokinase-type plasminogeen activator stolsel bindend te maken
- 1. domeinen ondervinden geen invloed van andere domeinen in de plasminogeen activatoren t-PA en u-PA -2. de lysyl bindings plaats is direct betrokken bij binding aan een stolsel, niet valide zijn.

Nawoord

Het hier beschreven onderzoek is, onder leiding van Jan Verheijen uitgevoerd op het Gaubius Laboratorium (TNO-PG) in Leiden. Aan de totstandkoming van het uiteindelijke proefschrift hebben velen een belangrijke bijdrage geleverd. Dingeman Rijken en Willem Nieuwenhuizen hebben de meeste artikelen kritisch doorgelezen. Helen Richardson heeft delen van dit proefschrift van engelse verbeteringen voorzien.
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Curriculum Vitae

De auteur van dit proefschrift is geboren op 8 maart 1960 in Den Haag. Het atheneum B werd aan de Dalton scholen gemeenschap Aronskelkweg in 1979 afgerond. In hetzelfde jaar volgde inschrijving aan de Rijksuniversiteit Leiden als student Biologie. In 1983 werd het kandidaatsexamen B1 afgelegd.
Op de bloedbank in Leiden is van 1983 - 1984 aan T cel differentiatie antigenen (naar later bleek CD45R) gewerkt bij dr. F. Koning. In dezelfde periode zijn ook, onder leiding van dr. H. W. Bruning, monoklonale antilichamen vervaardigd tegen fluoresceïne. Hierna volgde een aanstelling als student-assistent. Bij dr. H. W. Bruning is tot 1985 gewerkt aan de vervaardiging van anti-idiotypische monoklonale antilichamen tegen des-enkefaline-γ-endorfinen. Van 1985 tot 1986 is bij dr. W. de Priester onderzoek verricht aan de beweging van membraan moleculen en de invloed van het cytoskelet en het signaaltransductie systeem hierop, in de slijmschimmel D. discoïdeum. Van 1986 tot en met 1987 volgde een studie naar de cis-regulerende elementen en de trans-regulerende factoren van de transpositie reactie van het Tc-1 transposon in de nematode C. elegans bij dr. R. P. H. Plasterk. Het doctoraal examen is in 1987 afgelegd.
Bij Centocor is onder leiding van dr. P. Tetteroo tot 1989 gewerkt aan problemen, die ontstaan bij het opschalen van cel kweken. Als vervangend dienstplichtige volgde een aanstelling tot 1990 bij het Gaubius Instituut (TNO). Er is bij dr. J. H. Verheijen onderzoek gedaan aan thrombolytische middelen. Van 1990 tot 1994 werd als assistent in opleiding bij het IVVO-TNO en bij het PG-TNO onder aspiciën van dr. J. H. Verheijen het experimentele werk uitgevoerd dat in dit proefschrift beschreven is. In 1995 volgde een aanstelling als ALIFI fellow bij de afdeling Reumatologie van het Academisch Ziekenhuis Leiden. Op dit moment wordt in samenwerking met dr. C. Verwey de transcriptie factor NFmatp35 gekloneerd en de transcriptie regulatie van het TNF gen bestudeerd.