# STRUCTURAL REQUIREMENTS OF FIBRINOGEN FRAGMENTS, WHICH ENHANCE THE RATE OF PLASMINOGEN ACTIVATION BY t-PA

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**STRUCTURAL REQUIREMENTS OF FIBRINOGEN FRAGMENTS, WHICH ENHANCE THE RATE OF PLASMINOGEN ACTIVATION BY t-PA**

Een wetenschappelijke proeve op het gebied van de
**Natuurwetenschappen**

## Proefschrift

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door

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***Nil volentibus arduum***

*Aan mijn Ouders*
*en opleiders*

*Voor Marjo*

## Contents

# Chapter 1

## GENERAL INTRODUCTION

### 1.1 Haemostasis

The haemostatic balance is the equilibrium between the two opposing systems coagulation and fibrinolysis. An activated coagulation system leads to thrombin formation, and active thrombin will start the eventual formation of fibrin, the insoluble protein matrix of a blood clot. Fibrin has only a temporary function. After it has fulfilled its role, *e.g.* in tissue repair, it will be converted to soluble degradation products, and this causes lysis of the blood clot.

The degradation of fibrin is catalysed by plasmin. Plasmin, the product of an activated fibrinolytic system, is a proteolytic enzyme (not specific for fibrin) which circulates in the blood stream as an inactive precursor: plasminogen. It is activated by plasminogen activators such as tissue-type plasminogen activator (t-PA), which are highly specific proteases.

Thus, in its most elementary form, the haemostatic balance can be described as the equilibrium between fibrin formation and dissolution, *i.e.* under normal physiological conditions the fibrin concentration should be in a steady state.

In the following paragraphs a short description will be given of the structure of fibrin(ogen) and the multistep processes involved in fibrin formation and dissolution.

#### 1.1.1 Fibrinogen and fibrin

Mammalian fibrinogens consist of a double set of three polypeptide chains: Aα, Bβ, and γ. Since all three chain types are homologous, they probably evolved from a common ancestor (Doolittle 1980, Henschen 1980). The

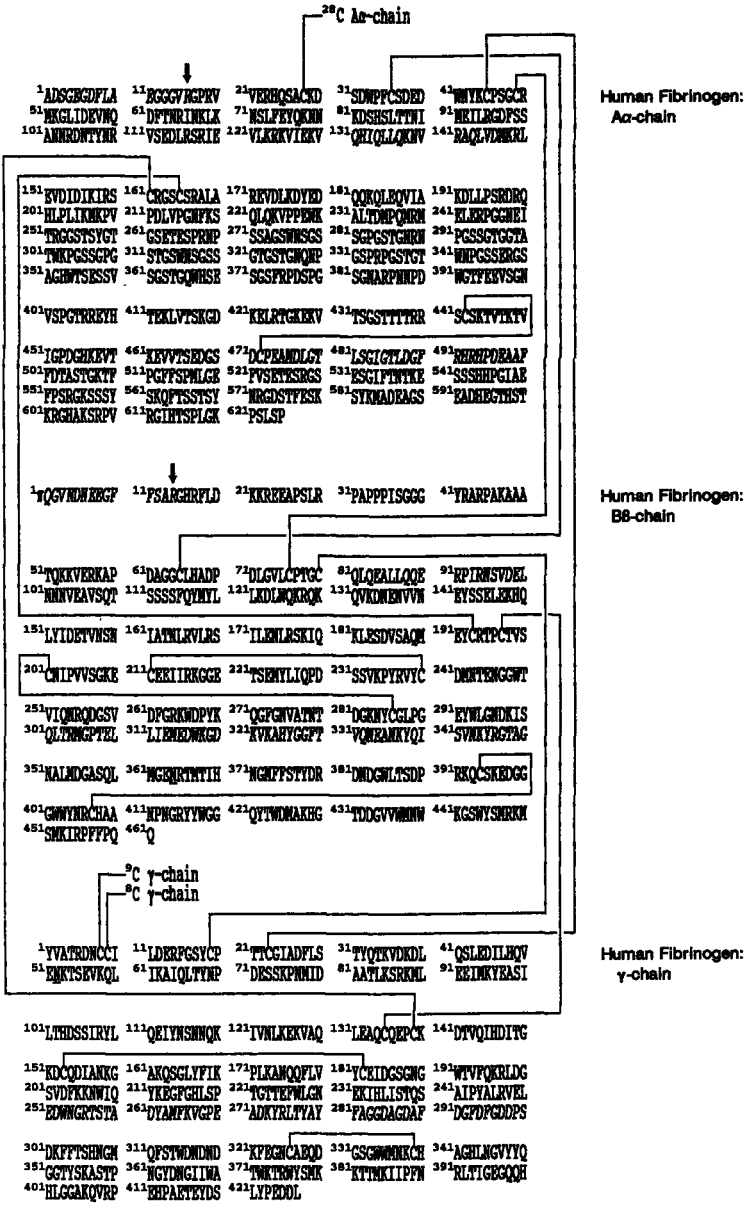


**Figure 1** Primary structure of the Aα-, Bβ- and γ-chain of human fibrinogen: C-C connections represent disulfide bonds; fibrinopeptides are in Italics; glycosylation sites are underlined; ⇓ indicates cleavage site of thrombin.

individual fibrinogen chains are synthesized by the parenchymal cells of the liver and assembled within the rough endoplasmatic reticulum.

Several reviews on the structure of fibrin(ogen) have been published (Doolittle 1981, Henschen 1981). The primary structures of the individual chains of fibrinogen has been established in several laboratories by direct sequencing of the polypeptide chains, and has also been derived from mRNA/DNA sequences. The amino acid sequences shown in figure 1 are the consensus sequences as can be found in the NBRF Protein Database (George *et al.* 1986): Aα-chain, code FGHUA, accession-number A03116; Bß-chain, code FGHUB, accession-number A03121; γ(B)-chain, code FGHUGB, accession-number A03126.

Structure of fibrinogen

The two Aα-chains, two Bß-chains and two γ-chains of fibrinogen are kept together by disulfide-bridges as depicted in figure 1, according to Henschen *et al.* (1983). A number of models has been proposed with a view to integrate the available information on the amino acid sequences, results of chemical and enzymatic fragmentation, size and shape of the molecule as derived from physicochemical measurements, and electron microscopical images. The schematic model of the fibrinogen molecule on the basis of such information is shown in figure 2, which is adapted from Doolittle (1984). In a slightly modified model, suggested by Hoeprich and Doolittle (1983) on the basis of NMR results, the two amino-terminal stretches of the two γ-chains are aligned in an antiparallel fashion.

The central domain (nodule) contains the amino termini of the six polypeptide chains. The fibrinopeptides A and B are located at the amino-terminal ends of the Aα- and Bß-chain, respectively. The two distal domains are connected to the central domain by coiled coils (*i.e.* the three protein chains twisted into an α-helix, also designated a supercoil), 112 residues in length (160 Å). The two halves of the symmetrical fibrinogen molecule are connected by a single disulfide bridge between the two Aα-chains and two disulfide bonds between the two γ-chains. Disulfide rings (*i.e.* three disulfide bridges which are not to far apart and keep the three protein chains together; for an example see figure 10) are present

on both ends of the coiled coil connector, holding all three chains in place. There are four oligosaccharide substitutions, two located on the γ-chains close to the central domain and two on the Bβ-chains, one within each distal domain. Crosslinking sites are situated near the carboxyl termini of the γ-chains, extending from the globular distal domains. On each Aα-chain (towards the carboxyl terminal end), there are two crosslinking acceptor sites (Gln) and five potential donor sites (Lys). Also in the middle of the Aα-chains crosslinking sites occur.

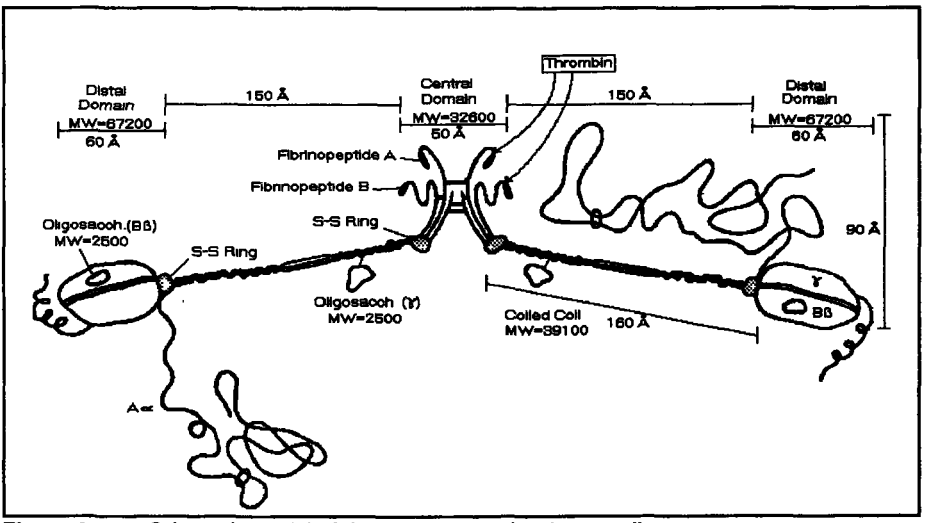


**Figure 2** Schematic model of the fibrinogen molecule according to Doolittle (1984). For explanation see text, the arrows originating from "thrombin" designate the cleavage sites of this enzyme.

The blood coagulation system

A complex cascade of reactions following an injury or trauma, eventually leads to the formation (activation) of thrombin from its zymogen prothrombin. Thrombin is a proteolytic enzyme capable of hydrolyzing specifically a few peptide bonds in fibrinogen, *i.e.* an arginyl-glycyl bond (between positions 16 and 17 of the Aα chain and between positions 14 and 15 of the Bβ-chain, see figure 1), thus liberating fibrinopeptides A and B. Only these arginyl-glycyl bonds are

hydrolysed although fibrinogen contains eleven more arginyl-glycyl bonds. Removal of the two fibrinopeptides A and B exposes new amino termini which have binding sites on the distal domains. This binding leads to polymerization.

The polymerization of the fibrin monomers to form a fibrin-gel occurs by a half-staggered overlap mechanism as evidenced by electron micrographs (Ferry *et al.* 1954). The next step in the polymerization process is the crosslinking of the subunits in the fibrin gel by the formation of isopeptide bonds between adjacent monomers, by an enzyme called factor XIII. Factor XIII is activated by thrombin from a Factor XIII precursor (a zymogen) occurring in plasma and in platelets. The active enzyme links neighbouring monomers by formation of peptide bonds between the side chains of special lysine residues and glutamine residues. In this way two γ-chains of neighbouring monomers are linked covalently and in the anti-parallel sense by two amide bonds between the side-chains of γ-Lys$^{406}$ and γ-Gln$^{398}$. The subsequent crosslinking of similar sites on the Aα-chains further completes the crosslinking of fibrin.

### 1.1.2 **Fibrinolysis**

Figure 3 shows the t-PA pathway of fibrinolysis. Fibrin is fragmented by the serine protease plasmin, which is activated from its zymogen plasminogen. The activation of plasmin is at least partly exerted by tissue-type plasminogen activator (t-PA), which is also a serine protease. Only a few other proteolytic actions of t-PA are known, which illustrate the high specificity of t-PA for plasminogen.

The specific and strong stimulation of the plasminogen activation by t-PA in the presence of fibrin (see figure 3 and paragraph 1.1.4) confines the generation of plasmin (and thus the fibrinolytic activity) to the fibrin surface.

t-PA is synthesized by vascular endothelial cells which secrete the activator into the blood stream. The amino acid sequence of t-PA has been determined partly by amino acid sequencing (Pohl *et al.* 1984) and from the cDNA sequence (Pennica *et al.* 1983). t-PA consists of 527 amino acids, which corresponds with a molecular weight of about 65,000.

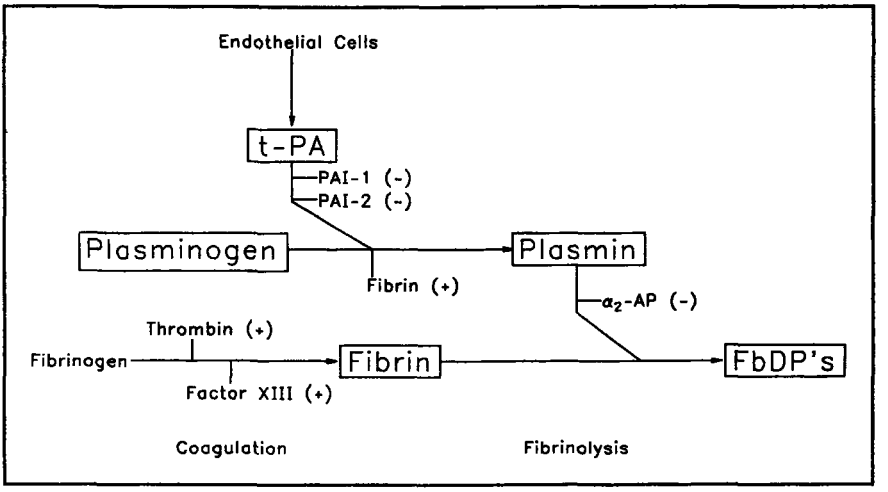


**Figure 3** Schematic pathway of the t-PA route of fibrinolysis, see text for description. (-) is inhibition, (+) is enhancement/interaction. Abbreviations: $\alpha_2$-AP, $\alpha_2$-antiplasmin; FbDP's, fibrin degradation products; PAI, plasminogen activator inhibitor; t-PA, tissue-type plasminogen activator. Adapted from Kluft (1988).

The mature protein has a single polypeptide chain (that can be cleaved by plasmin to give two-chain t-PA during fibrinolysis [Rijken *et al.* 1982]). t-PA comprises two kringle domains. Apart from those kringles there are various domains in the polypeptide structure, offering multiple possibilities for regulation of availability and activity (Kluft 1988). The primary function of t-PA is to hydrolyse one specific Arg-Val peptide bond in the plasminogen molecule, which yields the active plasmin. The biological half-life of t-PA activity in the circulation is very short (3 to 6 minutes in humans) because of the rapid clearance by the liver and the inhibition by PAI-1.
Reviews on structure/function relationships and regulation of the activity of t-PA have been presented by Kluft 1988 and by Rijken 1988.

The plasminogen structure is known and has been reviewed by Castellino (1984). Mature plasminogen (also designated Glu-plasminogen, after its amino terminal amino acid) contains 790 amino acids in a single polypeptide chain. Lys-plasminogen is formed by plasmin cleavage of the amino terminal 76 amino

acids from Glu-plasminogen. Hydrolysis of the $Arg^{560}$ - $Val^{561}$ peptide bond yields active plasmin, which is composed of a heavy chain and a light chain, connected by disulfide bridges. The heavy chain contains five homologous kringle domains.

Plasminogen has been shown to have binding sites for lysine-analogues (Christensen 1984) such as 6-amino hexanoic acid (AHA, also known as $\epsilon$-aminocaproic acid) and 6-amino hexane (AH). There are two classes of AHA binding sites, one strong and five weaker sites. The binding of fibrin to plasminogen is decreased by AHA and AHA analogues, indicating the importance of lysine residues on the fibrin molecule for binding of plasminogen.

Binding of t-PA and plasminogen to fibrin

t-PA binds to fibrin with a dissociation constant of about 150 nM (Rijken *et al.* 1982). Nieuwenhuizen *et al.* (1983) suggest that a binding site for t-PA and/or plasminogen is located in the segment A$\alpha$ 148-197 which is accessible in fibrin, but not in fibrinogen. In a later study (Yonekawa *et al.* 1989, 1990) it has been suggested that still another binding site for t-PA, also not accessible in fibrinogen, might be operative in fibrin; this site is located in fragment FCB-5 (*i.e.* $\gamma$-(311-336) disulfide linked to $\gamma$-(337-379), see paragraph 1.1.3). These binding sites become exposed upon conversion of fibrinogen to fibrin, in the course of fibrinogen degradation by plasmin (see 1.1.3), or during cleavage of fibrinogen with cyanogen bromide (see 1.1.3).

Though it is clear that plasmin must bind to fibrin for hydrolysis to occur, there is no direct evidence for the binding of plasmin to fibrin, since any free plasmin generated *in vivo* is quickly inactivated by $\alpha_2$-antiplasmin (figure 3). Fibrin-bound plasmin appears to be less susceptible to inhibition by $\alpha_2$-antiplasmin than free plasmin and this limits, to some extent, the plasmin activity to the fibrin surface.

### 1.1.3 The degradation of fibrinogen

Degradation of fibrinogen by plasmin

Plasmin cleaves fibrinogen, as well as fibrin, at several lysyl- and arginyl-bonds. In the former case soluble fibrinogen degradation products (FgDP's) and in the latter case soluble fibrin degradation products (FbDP's, figure 3) are formed. The digestion proceeds through a number of overlapping steps: The initial products are a family of fibrinogen derivatives (fragments X) in which the Aα chains have been degraded to various extents at their carboxyl terminal ends (molecular weights vary from 240,000 to 320,000).

Further digestion with plasmin results in an asymmetric cleavage of fragment X which yields one fragment Y (molecular weight 170.000) and one fragment D (molecular weights ranging from 80,000 to 93,000) per fibrinogen molecule. Fragment Y is finally split into another fragment D and one fragment E (molecular weight approximately 50,000). Fragment E comprises major parts of the central domain (figure 2) of fibrinogen, including fibrinopeptides A. One fragment D includes one distal domain (figure 2).

Depending on the conditions during digestion, different fragments D are formed. Digestion in the presence of calcium-ions leads to fragments D (designated as $D_{calc}$) with the intact carboxyl terminal part of the γ-chain. Digestion in the presence of EDTA or EGTA gives rise to fragments D (known as $D_{EGTA}$), in which residues 304 till 427 of the γ-chain are missing (Van Ruijven-Vermeer *et al.* 1979).

Degradation of fibrinogen by cyanogen bromide

Treatment with cyanogen bromide in an acidic medium is a reliable method to cleave proteins specifically at methionyl residues (Gross 1967, Needleman 1970). During the procedure the methionyl residues are transformed into carboxyl terminally located homoserine lactones (Hse). Apart from many small peptides (there are 66 methionyl residues in one molecule of fibrinogen), five larger fragments remain upon cyanogen bromide cleavage (Henschen *et al.* 1984): FCB-1, molecular weight approximately 60,000, similar to fragment E (obtained by plasmin digestion; central domain of fibrinogen); FCB-2, molecular weight

approximately 43,000; FCB-3, molecular weight approximately 27,000; FCB-4, molecular weight approximately 5000, and a fragment FCB-5 with a molecular weight of approximately 7000.
Table 1 shows the chain remnant composition of the individual fragments (adapted and extended from Nieuwenhuizen 1988), and also those of fibrinogen and of fibrin monomer.

### 1.1.4 **Inhibitors and stimulators of fibrinolysis**

Inhibition of fibrinolysis

Some of the inhibitors of fibrinolysis are depicted in figure 3. For a more complete review see Kluft (1988).

Inhibition at the t-PA level can be performed by PAI-1 (endothelial type of plasminogen activator inhibitor) or by PAI-2 (placental-type of PAI). PAI-1 binds rapidly to t-PA and forms an inactive complex. The inhibition rate of t-PA by PAI-1 is strongly reduced by binding of t-PA to structures such as fibrin. The extent of protection is not documented.

Inhibition at the level of plasmin is mainly performed by $\alpha_2$-antiplasmin. Plasmin (not protected by fibrin, see above) is rapidly inhibited by $\alpha_2$-antiplasmin. Normally, only very low levels of plasmin-$\alpha_2$-antiplasmin complexes are present in the circulation. These levels, however, can increase considerably during disease. This means that the plasminogen-to-plasmin conversion normally takes place at a relatively slow rate, but can increase strongly under special pathological conditions. Regulation of the plasmin activity or action occurs also at the level of fibrin, since $\alpha_2$-antiplasmin can be crosslinked to fibrin which is then degraded more slowly than fibrin with no crosslinked $\alpha_2$-antiplasmin.

Stimulation of fibrinolysis

All molecular models for the mechanism of fibrinolysis emphasize the stimulating and regulating role of fibrin and the binding of the fibrinolytic components to fibrin. Thus, fibrin plays a role as the substrate of fibrinolysis and as an effector in its own degradation. Activation of plasminogen by plasminogen

TABLE 1

Chain (remnant) compositions of fibrin, fibrinogen, and fragments

| Fragment | Aα chain | Bβ chain | γ-chain |
|---|---|---|---|
| Fibrinogen | $(^{1}Ala - ^{625}Pro)_2$ | $(^{1}\pi Glu - ^{461}Gln)_2$ | $(^{1}Tyr - ^{427}Leu)_2$ |
| Fibrin monomer | $(^{17}Gly - ^{625}Pro)_2$ | $(^{15}Gly - ^{461}Gln)_2$ | $(^{1}Tyr - ^{427}Leu)_2$ |
| Fragment X | $(^{1}Ala - ^{208}Lys)_2$ | $(^{1}\pi Glu - ^{461}Gln)_2$ | $(^{1}Tyr - ^{427}Leu)_2$ |
| Fragment Y | $^{1}Ala - ^{208}Lys$ | $^{1}\pi Glu - ^{461}Gln$ | $^{1}Tyr - ^{427}Leu$ |
| | $+ ^{1}Ala - ^{78}Lys$ | $+ ^{54}Lys - ^{122}Lys$ | $+ ^{1}Tyr - ^{58}Lys$ |
| Fragment $D_{calc}$ | $^{111}Val - ^{197}Arg$ | $^{134}Asp - ^{461}Glu$ | $^{86}Ser - ^{427}Leu$ |
| Fragment $D_{EGTA}$ | $^{111}Val - ^{197}Arg$ | $^{134}Asp - ^{461}Glu$ | $^{86}Ser - ^{303}Lys$ |
| Fragment E | $(^{1}Ala - ^{78}Lys)_2$ | $(^{54}Lys - ^{122}Lys)_2$ | $(^{1}Tyr - ^{58}Lys)_2$ |
| FCB-1 | $(^{1}Ala - ^{51}Hse)_2$ | $(^{1}\pi Glu - ^{118}Hse)_2$ | $(^{1}Tyr - ^{78}Hse)_2$ |
| FCB-2 | $^{148}Lys - ^{207}Hse$ | $^{191}Glu - ^{224}Hse$ | $^{95}Lys - ^{265}Hse$ |
| | | $+ ^{225}Tyr - ^{242}Hse$ | |
| | | $+ ^{243}Asn - ^{305}Hse$ | |
| FCB-3 | $^{241}Glu - ^{476}Hse$ | — | — |
| FCB-4 | — | $^{374}Phe - ^{426}Hse$ | — |
| FCB-5 | — | — | $^{311}Gln - ^{336}Hse$ |
| | | | $+ ^{337}Asn - ^{379}Hse$ |

activators (particularly t-PA) is accelerated specifically by fibrin and virtually not by fibrinogen. It is conceivable that the conversion of fibrinogen to fibrin leads to the formation or exposure of sites not operational or hidden in fibrinogen which are capable of stimulating the plasminogen activation by t-PA. A simple assay has been developed (Drapier *et al.* 1970, Verheijen *et al.* 1982) to determine the rate-enhancing capacity of fibrin and fibrin(ogen) fragments (designated as stimulators). The assay is outlined in figure 4. The concentration of *p*-nitroaniline formed per time squared is proportional to the rate of plasmin formation (activation rate). The ratio of the activation rate in the presence of the

stimulator over that in absence of a stimulator is designated as the stimulation factor (SF).

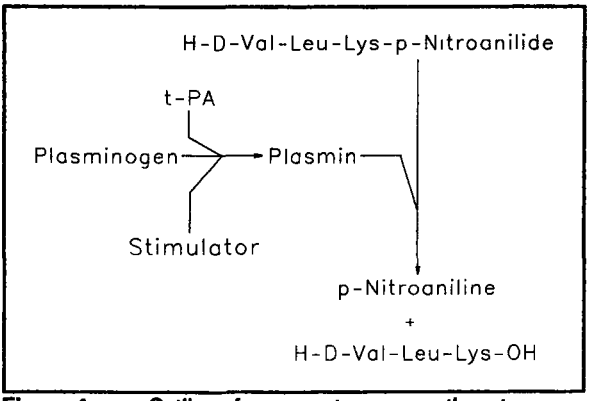


**Figure 4** **Outline of an assay to measure the rate-enhancing effect of a stimulator on the plasminogen to plasmin conversion by t-PA.**

Table 2 summarizes stimulation factors obtained for several stimulators and their concentrations which give half of the maximum stimulation factor ($c_{½SF}$). The accelerating effect on t-PA mediated plasminogen activation is not limited to fibrin and its fragments alone. Also other proteins and polymers have been shown to exert similar effects (e.g. thrombospondin, polylysine, and denatured immunoglobulins; for an overview see Nieuwenhuizen 1988).

In early publications the rate-enhancing properties in plasminogen activation have been ascribed mainly to carboxyl-terminal lysines. These seem to satisfy the requirements for binding to the lysine binding sites of plasminogen, i.e. the presence of an ϵ-amino- and an α-carboxyl group, separated by a hydrophobic carbon chain (e.g. AHA, see paragraph 1.1.2). It has been described (Voskuilen *et al.* 1987) that also a constituent lysyl residue of the chain, *i.e.* the lysyl residue at position 157 in the Aα-chain may contribute to the observed rate enhancement by fibrin. This lysyl residue (Aα-$^{157}$Lys) may interact with the postulated aminohexyl sites in plasminogen (Christensen 1984) or with t-PA. One could speculate about a spatial conformation in Aα-(148-161) in which the carboxylic side chain of an aspartyl- or glutamyl-residue in combination with the ϵ-amino group of the non-terminal lysyl-residue mimics a carboxyl terminal lysine.

TABLE 2

Stimulation factors (SF) of some fibrin(ogen) derivatives and the concentrations required for half-maximal stimulation ($c_{½SF}$)

| Stimulator | SF | $c_{½SF}$ in $\mu$M | Ref. |
|---|---|---|---|
| Fibrin monomer | 34 | 0.02 | Nieuwenhuizen *et al.* 1983[a] |
| CNBr dig. fibrinogen | 36 | 0.08 | Nieuwenhuizen *et al.* 1983[a] |
| FCB-2 | 39 | 0.12 | Nieuwenhuizen *et al.* 1983[a] |
| FCB-5 | 25 | 0.80 | Yonekawa *et al.* 1989, 1990 |
| Aα-(148-207) | 20 | 0.50 | Nieuwenhuizen *et al.* 1983 |
| Aα-(148-160) | 11.3 ± 3.2 | 12 | Voskuilen *et al.* 1987 |
| Aα-(149-160) | 9.1 ± 3.6 | 15 | Voskuilen *et al.* 1987 |

## 1.2 An introduction to Peptide Chemistry

Peptide chemistry is the part of organic chemistry that deals with the chemistry of protein fragments; one aspect is to provide tools for preparing amino acid derivatives that can be linked into peptides.

When two amino acids are to be condensed to form a dipeptide all the functions in the amino component and the carboxyl component that are not allowed to react, must be reversibly blocked by protective groups. The carboxyl function has to be activated since the hydroxyl moiety of the carboxyl group is not a leaving group. Apart from this, the integrity of the chiral centers on the two amino acids must be preserved during the coupling reaction.

The process of condensation thus consists of three stages:

(1) Protection as indicated above

(2) Activation of the carboxyl component

(3) Acylation of the amino component

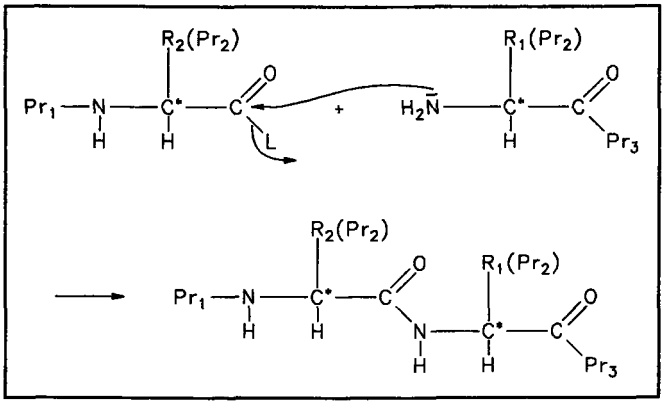

**Figure 5** Formation of an amide bond (peptide bond); L: leaving group; $Pr_1$, $Pr_2$, and $Pr_3$ are protective groups; $R_1$ and $R_2$ are the side chain of an amino acid.

*ad* 1. The applied protective groups provide for the temporary protection of the α-amino function, and the semipermanent protection of the side chain functions. For the protection of the α-amino groups a number of alkoxycarbonyl functions have been developed (for a review see Bodanszky 1984). Their applicability depends on the properties of the parent alcohols: to render their removal selective, these properties have to match. This principle has been treated by Barany and Merrifield (1977), who coined the term **orthogonality** for it.

This quality describes the nature of the protective groups with respect to each other: if $Pr_1$, for example, is a base-labile amino-protective group, $Pr_2$ and $Pr_3$ should preferably be acid-labile protective groups, *i.e.* removal of $Pr_1$ may not affect $Pr_2$ or $Pr_3$. The dipeptide derivative obtained can be extended at its amino terminus by acylation with another activated amino acid with side-chain $R_3(Pr_2)$ to form a tripeptide derivative, and so on.

*ad* 2. The α-carboxyl function of N-protected amino acid derivatives should be activated, to allow aminolysis by the amino component (*cf.* figure 5). Activation of an α-carboxyl group in an amino acid derivative includes the risk on racemization: if an intramolecular enolization can take place, *cf.* figure 6, racemization will always occur to a certain extent (Kemp 1979, Benoiton and

Chen 1981). If the α-carboxyl group forms part of a peptide, racemization can generally not be prevented, since the pertinent carboxyl group forms part of an acylamino acid.

It has been found that amino acid derivatives, belonging to the class of *t*-butyloxycarbonyl amino acids, racemize slowly or not at all (Benoiton and Chen 1981), provided an excess of nucleophilic base is absent in the reaction mixture.

The azide coupling, introduced long ago by Curtius (1902), is a well known exception: an azide usually does not cyclize to give oxazolones. This reaction is an important tool in the condensation of peptide units to longer chains (see below).

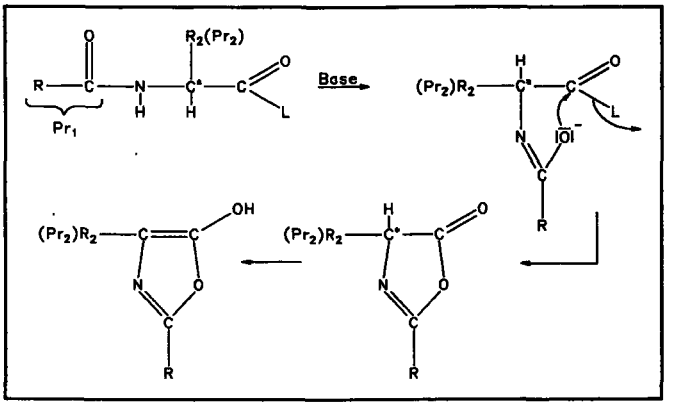


**Figure 6** Unwanted formation of an oxazolone-ring and concomitant loss of chirality if $Pr_1$ is an acyl-type amino protective group.

The acylation competes with racemization: Kisfaludy (1970) found alkoxycarbonylamino acid pentafluorphenyl esters to react so rapidly with the amino components, that, even a molar excess of triethylamine was found to be harmless.

### Strategy and tactics

*ad* 3. The consequence of these considerations is to follow the strategy of stepwise acylation using alkyloxycarbonylamino acids and to begin the synthesis of a peptide (in contrast to the *in vivo* synthesis of proteins) at the carboxyl

terminal amino acid, as advocated by Bodanszky and Du Vigneaud (1959[a,b]).

The strategy evolved to the use of benzyloxycarbonyl groups for the temporary protection of α-amino groups (removal: catalytic hydrogenolysis) in combination with protective functions derived from *tert*-butyl alcohol for semipermanent protection of side-chain functions (removal: mild acidolysis, Schwyzer and Sieber 1963). Following the introduction of functions built to undergo ß-elimination (Kader and Stirling 1964, Carpino and Han 1970, 1972) the fundamental strategies of today were defined. At present, one can choose from a wide variety of protecting groups, which respect these considerations; making a choice from them, and finding the optimal combinations, resorts under the **tactics** of a particular synthesis.

For an overview of almost all available protecting groups (some examples in figure 7), leaving groups and/or coupling reagents (some examples in figure 8) see Bodanszky 1984 and references therein.

Sometimes, in the synthesis of longer peptides, a suitable **strategy** might include one or more fragment condensations: smaller peptide derivatives are easier to purify and the synthesis of fragments allows simultaneous operations. In condensation reactions the danger of racemization (*cf.* figure 6) is immanent. As indicated, the risk of racemization may be minimized when acid azides are used, but the azide coupling has its own number of aberrations, one of them being the Curtius-rearrangement to isocyanides, leading to the loss of the activated C-terminal amino acid.

A very practical modification of the azide method eliminating most of these difficulties has been given by Honzl and Rudinger (1961). In their procedure the carboxyl terminus of a peptide is protected by an alkyl ester ($Pr_3$ in figure 5), *e.g.* a methyl ester, which is hydrazinolysed (the alkyl ester acts as a leaving group in this reaction) as usual. Upon treatment of the hydrazide with an alkyl nitrite, *e.g.* *tert.*-butylnitrite, the azide is formed. The peptide-azide is not isolated because of its instability (isocyanates may be formed) but it is brought directly into

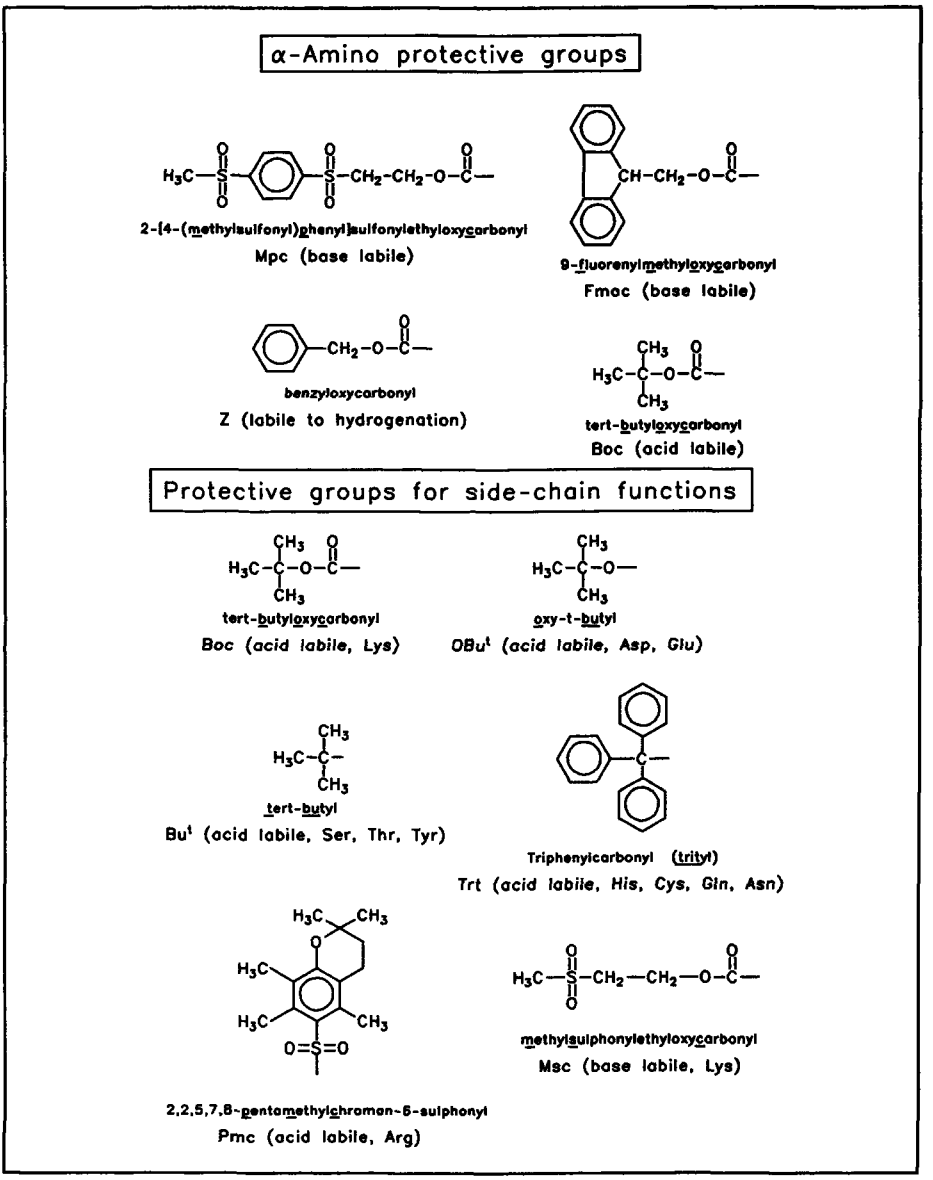


**Figure 7** Protective groups used in this dissertation: Mpc, Verhart an Tesser 1988; Fmoc, Carpino and Han 1970, 1972; Z, Bergmann and Zervas 1932; Boc, Ben-Ishai and Berger 1952; OBu$^t$, Roeske 1959; Bu$^t$, Schröder 1964; Trt, Stelakatos *et al.* 1966; Pmc, Ramage and Green 1987; Msc, Tesser and Balvert-Geers 1975.

reaction with the free amino-terminus of another peptide, usually without racemization. For a review see Meienhofer (1979).

König and Geiger (1970) have introduced the principle of racemization-inhibition during the unification of two partially protected peptides. Racemization appears to be suppressed if during the activation of the carboxyl component a modest excess of a non-acylating weak acid is added. The most versatile

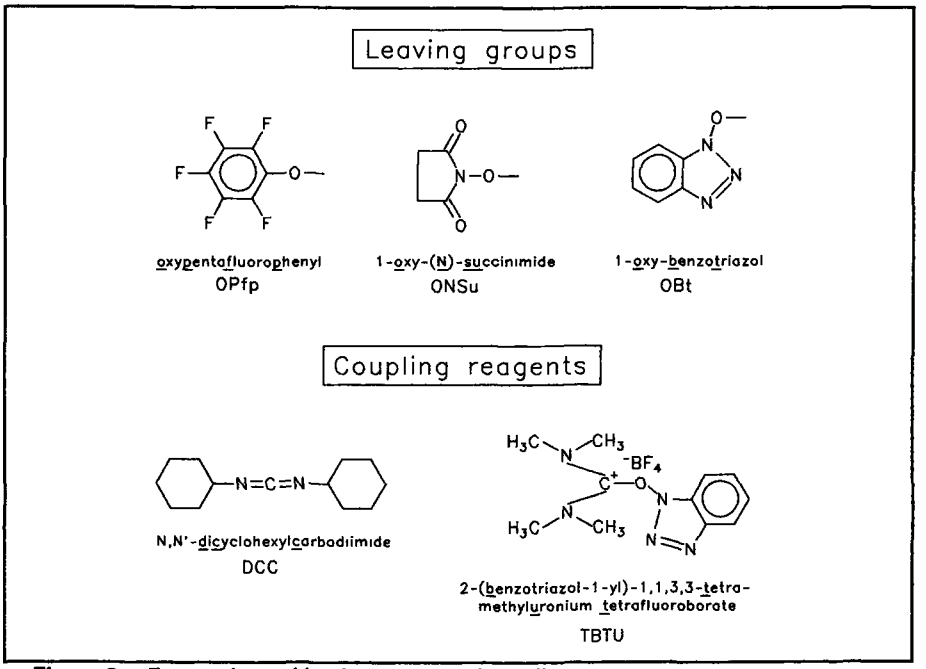


**Figure 8** Frequently used leaving groups and coupling reagents

additive turned out to be 1-hydroxybenzotriazole (König and Geiger 1970), since it: 1. donates protons (pKa=4.1), reducing the chance of racemization; 2. potentiates the action of the condensing agent (*i.e.* DCC) by formation of active esters, and 3. prevents formation of N-acylurea's by reduction of the lifetime of the (presumed) O-acylurea intermediate.

Thus, an alternative for the azide-method, a route for the condensation of two peptide fragments is the *in situ* activation with DCC (Sheehan and Hess 1955) or

TBTU (Knorr *et al.* 1989) in an excess of HOBt. This route almost always leads to by-products (racemization). The only two C-terminal amino acid residues that are completely inert as far as racemization is concerned, in either method for fragment condensation, are the glycyl or prolyl residue.

Solid Phase Peptide Synthesis (SPPS)

The most important application of the stepwise strategy for synthesis of peptides is the solid-phase method of Merrifield (1963). In SPPS the protecting group $Pr_3$ in figure 5 is not an ordinary protecting group, like -OMe or -OBu$^t$, but a linker attached to a solid support. If the rules of orthogonality are obeyed, then the protecting group $Pr_1$ can be removed while $Pr_2$ and $Pr_3$ remain intact. The soluble reaction products simply can be washed away and a second amino acid derivative can be introduced as depicted in figure 5, either via an active ester or via an *in situ* coupling. After a predefined number of cycles the fully protected target peptide can be cleaved from the resin in a medium which attacks $Pr_3$. This approach is the ideal course of a peptide synthesis on a solid support.

According to the protective group tactics, SPPS can be divided into two major classes. One class (designated the "Boc-chemistry" in SPPS) uses an acid labile $\alpha$-amino protection ($Pr_1$, *i.e.* Boc) and a more acid stable side chain protection ($Pr_2$, benzyl-type ethers and esters, substituted with groups that have a negative inductive effect, such as Cl or $NO_2$), while the linker ($Pr_3$) is much more acid stabile than $Pr_1$ and $Pr_2$. Resin-bound peptides prepared via the "Boc-chemistry" must be cleaved from the resin using extremely strong acids like liquid hydrogen fluoride (HF) or trifluoromethanesulfonic acid (TFMSA). For a comprehensive overview of techniques, protecting groups, linkers, and solid supports used in the SPPS "Boc-chemistry", see Stewart and Young (1984) and references therein.

Another class (called the "Fmoc-chemistry" in SPPS) uses the base labile N$\alpha$-Fmoc protecting group and *tert.*-butyl type protecting groups (acid labile, *i.e.* -Bu$^t$, -OBu$^t$ and Boc; see figure 7) for side chain protection. The solid phase linker ($Pr_3$) is also acid labile. Most frequently used in the "Fmoc-chemistry" is 4-alkoxybenzyl alcohol, (the "Wang"-linker [Wang 1973] endowed with a stability

comparable to that of the *tert.*-butyl functions). The 2-methoxy-4-alkoxybenzyl alcohol linker, "SASRIN"-linker (Mergler *et al.* 1988[a,b]), sensitive to dilute (0.5 - 1.0%) trifluoroacetic acid, is ideally suited for the preparation of fully protected peptides. An overview of techniques, protecting groups, linkers, and solid supports in the SPPS "Fmoc-chemistry" is given in Fields and Noble (1990) and references therein.

The concept of SPPS lends itself for automation. Two types of automated processors are being developed; a continuous-flow processor and a batch processor. The latter can be operated in a semi-automatic way and leaves the opportunity to monitor the progress of the coupling reaction via, for instance, a ninhydrin-test for free amino-groups; this so-called Kaiser Test (Kaiser *et al.* 1970), is the most frequently used technique for monitoring completeness of acylation in SPPS.

### 1.3 Conjugation of a peptide to a carrier protein

Peptide-carrier conjugates have to be used to raise antipeptide antibodies, since generally, non-conjugated peptides consisting of 10-25 amino acid residues do not elicit antibodies following injection in animals. To induce immunogenicity, peptides are coupled to macromolecular carriers, usually proteins (such as bovine serum albumin (BSA) or keyhole limpet haemocyanin (KLH)), to synthetic carriers (*i.e.* multi-chain poly(DL-Ala)-(L-Lys), [Audibert *et al.* 1982]), or they are incorporated in liposomes, micelles or immuno-stimulating complexes known as iscoms (Morein *et al.* 1984). Since peptides and proteins contain several functional groups, conjugation using carbodiimide (Goodfriend *et al.* 1964, Davis *et al.* 1984) or homobifunctional reagents (*e.g.* glutardialdehyde, [Avrameas 1969, Pfaff *et al.* 1982]) generate a great number of different products (and is therefore known as the "chaos" coupling, Peeters *et al.* 1989). In order to obtain the best defined product, heterobifunctional cross-linkers should be used in such a way that the peptide couples specifically, and in a predictable fashion to the carrier. Some aspects of chemical cross-linkers and the modification of proteins have been reviewed by Han *et al.* (1984) and Feeney (1987).

In a useful method for preparing peptide-carrier conjugates advantage is taken of the fast nucleophilic addition of a thiol group to the double bond of a maleimide (Marrian 1949). The amino groups of a carrier can be acylated to a predefined extent by the addition of an active ester bearing a maleimide moiety such as succinimidyl *m*-maleimidobenzoate, MBS (Kitagawa and Aikawa 1976); succinimidyl 6-(*N*-maleimido)-*n*-hexanoate, MHS (Keller and Rudinger 1975); succinimidyl 4-(*N*-maleimidomethyl)-*cyclo*-hexane-1-carboxylate, SMCC (Yoshitake *et al.* 1979). A peptide that does not contain a sulfydryl group can be functionalized during its synthesis at the amino terminus by acylation with S-acetylthioacetic acid (Ata-OH) or the succinimidyl ester of Ata-OH (Sata) (Duncan *et al.* 1983). An alternative is the insertion of a protected cysteinyl residue as carboxyl-terminal residue (the synthesis of the peptide starts with a carboxy-terminal cysteine) or at any other position in the chain. Treating an Ata-peptide with base, even hydroxylamine, results in the liberation of the sulfydryl group. Subsequently the maleylated carrier is allowed to react with the HS-peptide, see figure 9 route A.

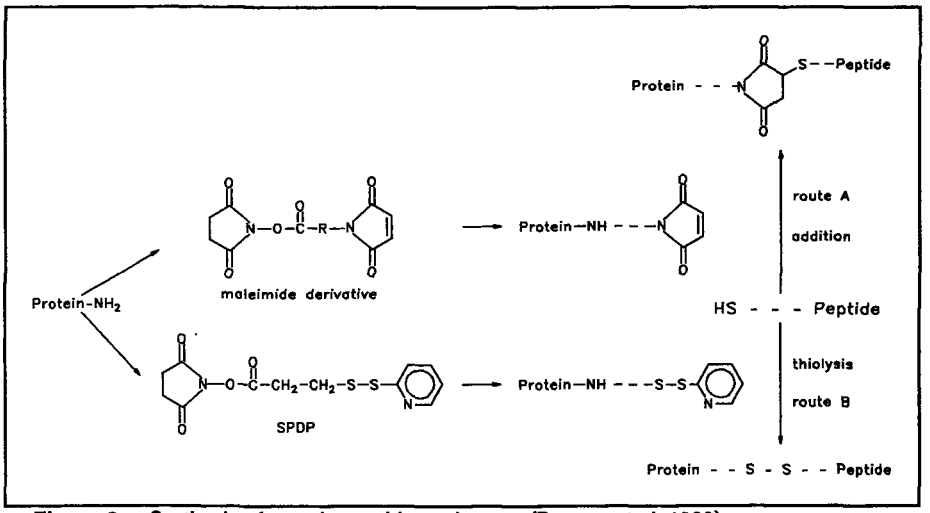


**Figure 9** Synthesis of protein-peptide conjugates (Peeters *et al.* 1989)

Since both the peptide and the protein are covalently connected through acylation of an amino group in both of them, it is evident but nonetheless important to note that also the protein can be thiolated and the peptide maleylated.

In another method of controlled conjugation S-S links are formed between the peptide and a carrier protein by the thiolysis of activated disulfides introduced by the acylation of a protein or a peptide with succinimidyl 3-(2-pyridyldithio)propionate (SPDP) (Carlsson *et al.* 1978), see figure 9 route B.

When carrier protein - peptide conjugates are used as immunogens, the potential immunogenicity of the linker groups has to be considered. Peeters *et al.* (1989) described a comparison of four bifunctional reagents for coupling peptides to proteins and the effect of the three moieties (protein, linker, peptide) on the immunogenicity of the conjugates. The results from this study clearly show a very high antibody-level against the rigid linkers produced by SMCC and MBS, and very low levels against the flexible linker produced by MHS. The lowest level was obtained by the aliphatic disulfide originating from SPDP. However, a major drawback of SPDP coupling is the resulting disulfide linkage, which confers a susceptibility to reductive cleavage by ubiquitously occurring thiol compounds. In contrast, the thio-ether linkage resulting from MHS is very stable. Therefore, Peeters *et al.* (1989), conclude that MHS is the bifunctional reagent of choice for coupling peptides to proteins.

## 1.4 Aim of the investigations

As pointed out in paragraph 1.1.4 the sequence Aα-(148-160) is the shortest sequence known at present to enhance the rate of plasminogen to plasmin activation by t-PA. The location and position relative to the Bß- and γ-chain of this site in the fibrin(ogen) molecule is outlined in figure 10. The fact that fibrinogen (in contrast to fibrin) does not act as a stimulator led to the hypothesis that the sequence Aα-(148-160) in fibrinogen is not accessible to plasminogen and/or t-PA, and that, upon conversion of fibrinogen to fibrin, this site apparently would become exposed. This theory was proven by making monoclonal antibodies against synthetic Aα-(148-160). These monoclonal antibodies react .

with fibrin and not with fibrinogen. Chapter 2 is an account of the synthesis, conjugation to bovine serum albumin, of Aα-(148-160), and production and characterization of monoclonal antibodies against Aα-(148-160).

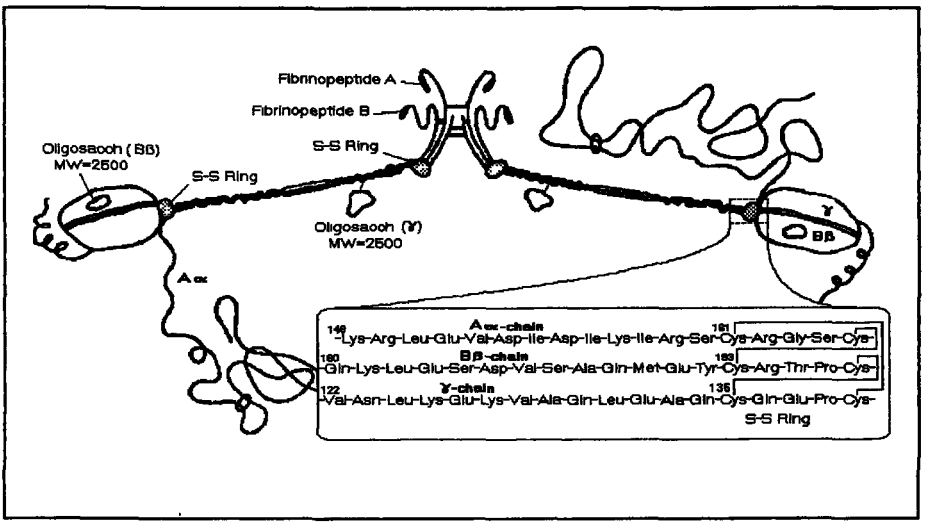


**Figure 10** Schematic model of the fibrinogen molecule (Doolittle 1984), with (inset) an enlarged detail, which is a schematic representation of the distal disulfide ring and a small part of the coiled-coil, showing the exact position of the sequence Aα-(148-160) in the coiled-coil relative to short stretches of the β- and γ-chain

Homology searches show that a charge distribution similar to that found in Aα-(148-160) (see below) is also present in the γ-chain, more specifically in the sequence γ-(316-322). This sequence is a part of FCB-5, which has been shown to have stimulating capacity (Yonekawa *et al.* 1989, 1990). During the fibrinogen-to-fibrin conversion, Aα-(148-160) appears to become accessible to monoclonal antibodies elicited against synthetic Aα-(148-160) (see Chapter 2). Since a similar situation may exist for (at least parts of) FCB-5, we have prepared monoclonal antibodies against a part (*i.e.* γ-(312-324)) of FCB-5, and found that these are fibrin-specific and do not bind fibrinogen.

Chapter 3 is an account of the synthesis, conjugation to bovine serum albumin of fibrinogen-γ-(312-324)-tridecapeptide, and production and characterization of the obtained monoclonal antibodies.

Chapter 4 describes the synthesis and the stimulation factors of successive linear hexapeptides and of a tetrapeptide within the sequence Aα-(148-160), in order to further locate the stimulating site within Aα-(148-160).

The results described by Voskuilen *et al.* (1987) suggest that not only carboxyl-terminally located lysine-residues, but also Aα-Lys$^{157}$, contribute considerably to the rate-enhancing effect of fibrin.
Chapter 5 describes the synthesis and the stimulating capacity of several peptide-analogues of Aα-(148-160) in which Aα-Lys$^{157}$ has been replaced by some lysine-analogues and several other amino acid residues, on the conversion of plasminogen to plasmin.

The sequence of Aα-(148-160) shows an interesting charge distribution,

| Lys | Arg | Leu | Glu | Val | Asp | Ile | Asp | Ile | Lys | Ile | Arg | Ser |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| + | + | 0 | - | 0 | - | 0 | - | 0 | + | 0 | + | 0 |

*i.e.* negative and neutral amino acid residues alternate, and are embraced by two sets of positively charged residues. This sequence appears to be highly conserved in fibrinogen of several species.
Chapter 6 deals with the synthesis and the stimulating capacities of synthetic peptide analogues, derived from Aα-(148-160), in which the typical charge distribution is interrupted by exchanging Aα-Val$^{152}$ by charged (and some uncharged) amino acid residues.

Ten Kortenaar *et al.* (1985) describe that an azide-coupling involving an amino-terminal aspartyl residue with an unprotected carboxylic side-chain, proceeds with high yield and purity.
If this phenomenon would be generally applicable such a coupling procedure could be a new tool in the methodology of peptide-fragment condensation (see paragraph 1.2).
Chapter 7 describes the synthesis of protected peptide azides with similar

substitutions of Val[152] as those in chapter 6. To check the reaction described by ten Kortenaar *et al.* (1985), the protected peptide azides were allowed to react with the amino-terminal (ß-unprotected) Asp[153] of the fully protected Aα-(153-160) fragment.

In chapter 8, the use in solid phase peptide chemistry of a new amino-protective group, the Mpc-group (which is devoid of the disadvantages of the Fmoc-group), is described. Also, an improved synthesis of Mpc-ONSu and of some Mpc-amino acids will be described in detail in chapter 8.

## Chapter 2

# THE SEQUENCE Aα-(148-160) IN FIBRIN, BUT NOT IN FIBRINOGEN, IS ACCESSIBLE TO MONOCLONAL ANTIBODIES

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ABSTRACT

Fibrin, but not fibrinogen, accelerates the t-PA catalyzed activation of plasminogen. We have shown before (J. Biol. Chem., 1987; 262: 5944-5946) that essential information for this accelerating capacity of fibrin resides in the sequence Aα-(148-160). Our working hypothesis, based on those findings, is that Aα-(148-160) is buried in fibrinogen and becomes accessible to proteins such as plasminogen and/or t-PA, when fibrinogen is converted to fibrin.

To test this hypothesis we have raised a monoclonal antibody (MoAb) against synthetic Aα-(148-160) and found that this MoAb reacts with fibrin and not with fibrinogen. This finding shows that Aα-(148-160) becomes accessible when fibrinogen is converted to fibrin, and that Aα-(148-160) is a fibrin-specific neoantigenic determinant.

INTRODUCTION

When the coagulation system is activated, thrombin is generated. Thrombin will convert circulating fibrinogen to fibrin monomer by the cleavage of fibrinopeptides, A and B, from the amino-terminal ends of the two fibrinogen Aα and Bß chains, respectively.

The fibrin monomers will aggregate through the resulting new amino-termini of the α and ß chains which constitute sites, that bind to complementary sites in the carboxyl-terminal domains (Doolittle 1981, Laudano and Doolittle 1981) of other fibrin(ogen) molecules.

The carboxyl-terminal sites already exist in fibrinogen, and at low concentrations fibrin will be kept in solution by complexation with fibrinogen. Beyond a critical (local) concentration fibrin will aggregate to form a fibrin gel *i.e.* a thrombus will be formed.

With the advent of the MoAb technology it has become possible to raise antibodies against molecular features, which are specific for fibrin and do not occur in fibrinogen (neoantigenic determinants). Examples are MoAb's which have been raised against the new amino-termini of the fibrin α-chains (Scheefers-Borchel *et al.* 1985, Liau *et al.* 1987) and ß-chains (Hui *et al.* 1983). Those antibodies were elicited using conjugates of a carrier protein and synthetic

peptides with an amino acid sequence, identical to that occurring in the amino-terminal stretches of the fibrin α and ß chains, respectively.

The activation of plasminogen by tissue-type plasminogen activator (t-PA) is greatly accelerated by fibrin, but not by fibrinogen. We have shown before (Nieuwenhuizen *et al.* 1983[b]) that the sequence Aα-(148-197) and in particular Aα-(148-160) (Voskuilen *et al.* 1987) and lysine Aα 157 (Voskuilen *et al.* 1987) play an essential role in this acceleration. Our working hypothesis based on those findings is, that this part of the Aα chain is buried in fibrinogen and becomes accessible to proteins such as plasminogen and t-PA during the fibrinogen-to-fibrin conversion. If this hypothesis is correct, Aα-(148-160) would also become accessible to antibodies upon fibrin formation, and antibodies against Aα-(148-160) would be expected to react with fibrin and not with fibrinogen.

In this paper we describe the production of MoAb's against this part of the Aα chain using a conjugate of bovine serum albumin and a synthetic peptide with the sequence of Aα-(148-160). The resulting antibodies react exclusively with fibrin, and not with fibrinogen. This demonstrates that Aα-(148-160) is a fibrin-specific neoantigenic determinant, and supports our hypothesis that the sequence Aα-(148-160) is inaccessible in fibrinogen and becomes accessible in fibrin.

## MATERIALS AND METHODS

### Synthesis of [Ata-D,L-Nle$^{147}$ ]-Fibrinogen-Aα-(147-160)-tetradecapeptide

Fibrinogen-Aα-(148-160)-tridecapeptide (Lys-Arg-Leu-Glu-Val-Asp-Ile-Asp-Ile-Lys-Ile-Arg-Ser) was extended with acetylthioacetyl-D,L-norleucine at the amino-terminus. Thus, methionine present in fibrinogen at Aα 147 is replaced by D,L-Nle. This procedure allows the coupling to a carrier protein, and (via the Nle-content) the subsequent determination of the number of coupled peptides per carrier protein molecule. The [Ata-D,L-Nle$^{147}$]-Fibrinogen-Aα-(147-160)-tetradecapeptide (Ata-$P_{14}$) was synthesized by Solid Phase Peptide Synthesis (SPPS) using the p-alkoxybenzyl alcohol resin (Wang 1973) and $N^{\alpha}$-(9-fluorenyl)methyloxycarbonyl amino acids (Fmoc amino acids) (ten Kortenaar *et al.*

1986). During SPPS the amino acid side chains were protected with acid-labile protecting groups: the ϵ-amino group of Lys with Boc, the δ-guanidino group of Arg with Pms, the γ-carboxyl group of Glu and the β-carboxyl group of Asp with $OBu^t$ and the β-hydroxyl group of Ser with $Bu^t$. Fmoc-Ser ($Bu^t$)-OH (2 eq.) was coupled to the resin (1.07 mMoles/g), by *in situ* activation with dicyclohexyl-carbodiimide (DCC, 2 eq.) and 4-(dimethylamino)pyridine (DMAP, 2 eq.). To suppress racemization, 1-hydroxy-1-benzotriazole (HOBt, 4 eq.) was added (van Nispen *et al.* 1985). All reactants were dissolved in DMF. After 16 hrs at 5°C the remaining alcohol groups of the resin were capped with benzoylchloride and pyridin in DMF for 1 hour. The subsequent cleavage of the Fmoc groups was carried out in 20% piperidine/DMF. Coupling of Fmoc-Arg(Pms)-OH (3 eq.) was performed by *in situ* activation with DCC (3.3 eq.)/HOBt (3.6 eq.) in DMF. The Fmoc group was cleaved off with 20% piperidine/DMF. Finally, norleucine was introduced as Fmoc-D,L-Nle-OH and the Ata group as described above.
The fully protected Ata-$P_{14}$ was cleaved from the resin during a two hour reaction with 55% trifluoroacetic acid in dichloromethane; all side chain protecting groups (except the Pms group) are cleaved in this mixture simultaneously. After evaporation *in vacuo* the residue was treated with a mixture of TFA/methanesulfonic acid/thioanisole (10/1/1) for 2 hrs at ambient temperature to remove the Pms group.
The crude Ata-$P_{14}$ was purified via countercurrent distribution using the extraction system n-butanol/acetic acid/water (5/1/4) over 200 transfers. A chromatographically pure fraction was obtained. Except for serine, this fraction had the expected amino acid composition (moles/mole; Nle defined as 1; theoretical composition in parentheses) *i.e.* Arg 2.15 (2.00), Asp 2.14 (2.00), Glu 1.03 (1.00), Ile 3.04 (3.00), Leu 0.98 (1.00), Lys 2.14 (2.00), Nle 1.00 (1.00), Ser 0.56 (1.00), Val 1.01 (1.00).
The low amount of Ser is due to deterioration in the very strongly acidic conditions during hydrolysis before amino acid analysis.

Coupling of the tetradecapeptide to BSA

To 50 mg BSA (Carl Roth, Karlsruhe, FRG; 99% pure) dissolved in 1 ml buffer

(phosphate, pH = 8.0), 4.5 mg 6-(1-maleimido)hexanoic acid 1-succinimide ester was added (Peeters *et al.* 1989). After 5 minutes the reaction mixture was subjected to gel filtration on a Sephadex G-25 column (Pharmacia, Uppsala, Sweden), run in phosphate buffer pH = 6.0.
The peptide derivative was activated by cleaving the acetyl group from the Ata-moiety by dissolution in a mixture of 4 M NaOH/methanol/dioxane (1/5/14) for exactly 15 seconds and subsequent neutralization with acetic acid. The solution containing the neutralized thioacetyl-tetradecapeptide (Tha-$P_{14}$) was added to the functionalized BSA and allowed to react for two hours at ambient temperature (Keller and Rudinger 1975). The reaction mixture was dialyzed against distilled water for 24 hours and lyophilized. The amount of incorporated Nle, as determined by amino acid analysis, showed that on average 4 to 5 Tha-$P_{14}$ molecules were linked per BSA molecule.

Antigens

Human fibrinogen was purified as described before (van Ruijven-Vermeer and Nieuwenhuizen 1978).
Fibrin monomers were prepared according to Haverkate and Timan (1976) and stored as a 13.4 mg/ml solution in 20 mM acetic acid at -20°C. Prior to use they were diluted to 0.45 mg/ml with 20 mM acetic acid (fibrin monomer solution, see below).
Fibrinogen A$\alpha$ chain was obtained by reduction and carboxymethylation of fibrinogen and purified according to Doolittle and coworkers (1977).
Cyanogen bromide digestion of fibrinogen was performed according to Blombäck *et al.* (1968). CNBr fragment FCB-2 (comprising the sequence A$\alpha$-(148-207)) was isolated from the digest as described by us before (Nieuwenhuizen *et al.* 1983[b]) by a method adapted partly from the procedure of Olexa *et al.* (1979).
Soluble fibrin was generated in plasma by addition of thrombin (Leo, Ballerup, Denmark; 10 $\mu$l, 2 NIH/ml) to 0.1 ml portions of normal pooled citrated plasma. At timed intervals (between 0-75 seconds) 40 $\mu$l aliquots were taken in which the thrombin reaction was quenched by 20-fold dilution in PBS containing 0.05% Tween 20 and hirudin (0.2 U/ml).

In some experiments Arvin (= *Agkistrodon rhodostoma* venom protease) (Defibrase, Pentapharm, Basel, Switzerland) was used instead of thrombin, under the same conditions. In these experiments, however, the reaction was quenched by the addition of diisopropylfluorophosphate (DFP) to a final concentration of 1 mM.

Immunization scheme

Female BALB/c mice were injected intraperitoneally with 125 μg immunogen (corresponding with approximately 10 μg of BSA-linked peptide) in Freund complete adjuvant (Difco, Amsterdam, The Netherlands) and then twice at 2 weeks intervals with 125 μg immunogen in Freund incomplete adjuvant. An intraperitoneal injection of 250 μg immunogen in 0.15 M NaCl was given three days before fusion.

Fusion

The immunized mice were killed in ether vapor and spleen cells were harvested. Spleen cells ($7.8*10^7$) were fused with $1.4*10^7$ non-producing myeloma cells (Sp2/0 AG14) in the presence of 40% polyethyleneglycol 4000 (Baker, Deventer, The Netherlands), essentially as described by Köhler and Milstein (1975). The cell suspension was diluted and divided over 96 wells microtitration plates (Costar, Cambridge, Mass.). Media of growing cells were screened for antibody production as described below. Cell lines producing reactive antibodies were subcloned twice by limiting dilution (0.5 cells/well) as described by Oi and Herzenberg (1980).

Hybridoma selection procedure

Hybridoma's were selected using an enzyme-linked immunosorbent assay (ELISA). The ELISA was performed essentially as described before (Koppert *et al.* 1985 and 1986) with purified Aα chains or FCB-2 as immobilized antigens. Immunoreactive mouse immunoglobulin was visualized with horse-radish peroxidase (HRP) labelled rabbit antimouse immunoglobulin (from Nordic, Tilburg, The Netherlands) using 3,3',5,5'-tetramethyl benzidine (TMB, from Aldrich

Chemical Co, Milwaukee), and $H_2O_2$ as the substrate mixture (Bos *et al.* 1981).

Subclass assessment and purification of the MoAb's

Subclass assessment was carried out by immunoelectrophoresis (IEF) as described by Radl (1981). Antisera against mouse α-, μ-, δ-, ε-, γ-, κ-, and λ-chain were gifts from Dr. Radl (Institute for Experimental Gerontology TNO, Rijswijk, The Netherlands).

Since the MoAb appeared to precipitate at low ionic strength, purification was carried out by simply dialysing of the ascites fluids against 0.005 M sodium phosphate pH 6.0.

RESULTS

Preparation of the monoclonal antibodies

Two weeks after fusion and selection, cell growth was observed in 260 of the 380 seeded wells. The media of 4 wells reacted with Aα chain in the ELISA system. The cells from two of the positive wells were cloned and recloned. After cloning and recloning 4 clones were chosen for further study. One is described in this paper. This line has been in culture for 2 months and has been injected into the peritoneal cavity of BALB/c mice for *in vivo* production of ascites fluid. Also *in vivo* the clone appeared to be stable *i.e.* more than 2 months (3 passages).

We will refer to the MoAb produced by this clone as anti-Fb-1/2. Subclass assessment showed that anti-Fb-1/2 is of the IgM/κ subclass. Therefore it could not be purified by protein A-Sepharose chromatography. It precipitates, however, during dialysis against a low ionic strength buffer (0.005 M sodium phosphate, pH 6), and can easily be redissolved by addition of a high ionic strength buffer (1.7 M NaCl, 0.05 M sodium phosphate, pH 6) and remains in solution upon dilution to physiological ionic strengths.

Specificity of the monoclonal antibodies

In purified systems anti-Fb-1/2 showed the following characteristics in the ELISA system (see above). It did not react with BSA which was used as a carrier

protein, nor with fragment E, and only weakly with pure fibrinogen. It reacted, however, strongly with fibrinogen Aα chain and FCB-2. Apparently, Aα-(148-160) in FCB-2 and in Aα-chain, but not in fibrinogen, can interact with the MoAb.

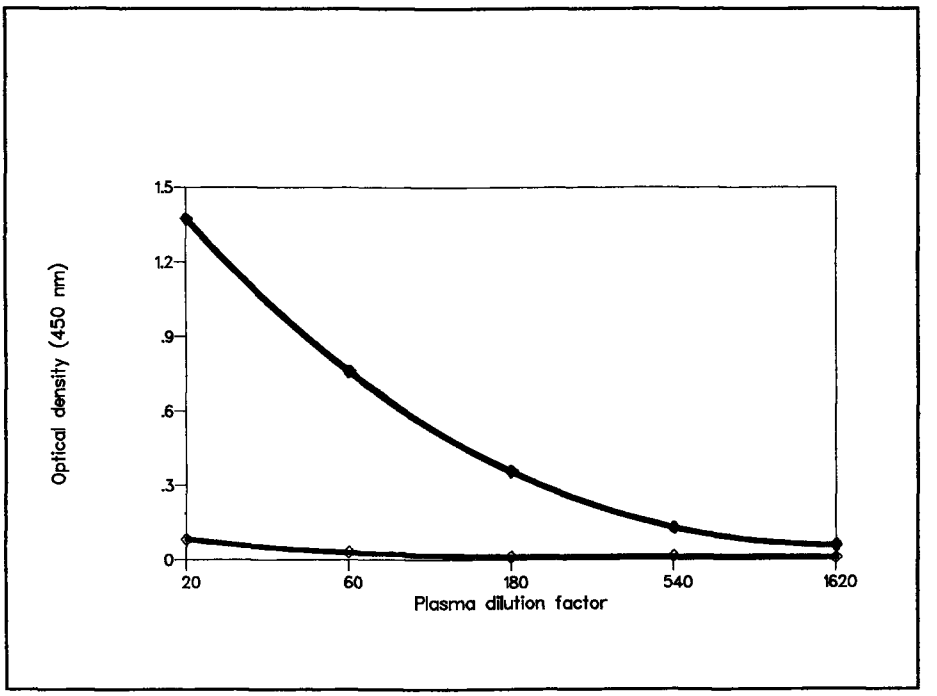


**Figure 1** Dose-response curves of 20-fold diluted plasma with a fibrinogen concentration of 2.4 mg/ml (◊) and of the same 20-fold diluted normal plasma but with fibrin monomer added to a final concentration of 15 μg/ml (♦)

Anti-Fb-1/2 is able to interact with fibrin (in the μg/ml range) in a plasma milieu *i.e.* in excess fibrinogen, as shown in figures 1 and 2. (The fibrinogen concentration of the plasma was 2.4 mg/ml). In figure 1, results of an experiment are depicted in which microtitration plates coated with anti-Fb-1/2 were used. The coated wells were incubated with serial dilutions of a pooled normal plasma which was spiked with fibrin monomer solution. Captured antigen was tagged with rabbit polyclonal anti-FDM conjugated with HRP (Koppert *et al.* 1985 and 1986), and visualized by incubation with TMB/$H_2O_2$ substrate. Fibrin concentrations down to 1 μg/ml are readily detectable.

Virtually identical dose-response curves were obtained when the fibrin monomer concentration was varied and the plasma dilution kept constant (not shown). In figure 2 a similar experiment is shown. Now the soluble fibrin was generated in plasma by treating the plasma for different periods of time with thrombin. A progressive increase in response with time of thrombin treatment is seen. Treatment with Arvin, which releases only fibrinopeptide A, yields comparable results. Normal plasma gives virtually no response.

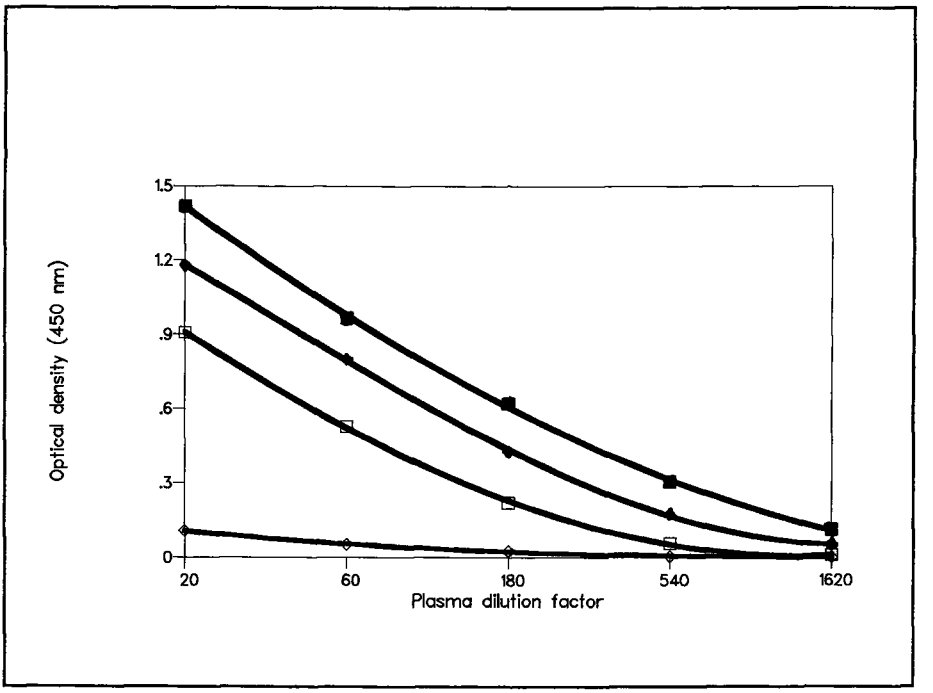


**Figure 2** Dose-response curves of normal plasma with a fibrinogen concentration of 2.4 mg/ml (◇) and of the same normal plasma treated with thrombin for 25 sec (□), 50 sec (♦), or 75 sec (■)

## DISCUSSION

Other investigators have shown that the new amino-terminal ends, which result from cleavage of the fibrinopeptides A and B from the amino-terminal ends of the fibrinogen Aα- and Bß-chains, respectively, constitute fibrin-specific epitopes (Scheefers-Borchel *et al.* 1985, Liau *et al.* 1987 and Hui *et al.* 1983).

Several fibrin-specific antibodies of this type are currently available (for review see Nieuwenhuizen 1987[a]).

Over the last years we have studied in detail the accelerating effect exerted by fibrin on the t-PA catalyzed plasminogen activation.
We found that not only fibrin, but also non-physiological fragments of fibrinogen (but not intact fibrinogen) such as the plasmin fragment $D_{EGTA}$ (Verheijen *et al.* 1982[a]) and the CNBr fragment FCB-2 (Nieuwenhuizen *et al.* 1983[b]) accelerate the plasminogen activation by t-PA (Verheijen *et al.* 1982[b] and Henschen 1981). The activity of FCB-2 and $D_{EGTA}$ resides in their Aα-chain remnants. Since the Aα-chain remnants of $D_{EGTA}$ and FCB-2 are composed of the stretches Aα-(111-197) and Aα-(148-207), respectively, we concluded that essential information must reside within Aα-(148-197) (Nieuwenhuizen *et al.* 1983[b]). In a more recent study (Voskuilen *et al.* 1987), we could delineate the essential structure even further *i.e.* to Aα-(148-160), and we could show that lysine Aα 157 plays a crucial role.

On these results we based the working hypothesis, that the stretch Aα-(148-160) is buried in fibrinogen, and is exposed upon transformation of fibrinogen to fibrin, and by digestion of fibrinogen with plasmin or with CNBr.
This concept is supported by our present findings *i.e.* that MoAb's directed against Aα-(148-160) react with fibrin and not with fibrinogen. The weak reaction, which is observed with purified fibrinogen and with 20-fold diluted plasma (Figures 1 and 2) is probably caused by trace contaminations of soluble fibrin. It has been reported that normal plasma levels of soluble fibrin are between 0.5 and 13.5 μg/ml (Nieuwenhuizen *et al.* 1987[b]). It is very unlikely, therefore, that fibrinogen preparations can be obtained with lower fibrin concentrations.

Our results show for the first time, that Aα-(148-160) is a fibrin-specific epitope. At present the applicability of anti-Fb-1/2 as capture antibody in a sandwich-type EIA for soluble fibrin in plasma, is investigated.

# Chapter 3

## EVIDENCE THAT THE SEQUENCE γ-(312-324) IS A FIBRIN-SPECIFIC EPITOPE

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ABSTRACT

Fibrin, but not fibrinogen, accelerates the activation of plasminogen catalyzed by t-PA. Detailed studies showed that (part of) this rate-enhacing effect of fibrin is brought about by two sites in the fibrin molecule: one in Aα-(148-160) and one in FCB-5. During the fibrinogen-to-fibrin conversion, Aα-(148-160) appears to become accessible, since monoclonal antibodies against synthetic Aα-(148-160) react with fibrin, but not with fibrinogen. Since a similar situation may exist for (at least parts of) FCB-5, we have prepared monoclonal antibodies against a part (*i.e.* γ-(312-324)) of FCB-5, and found that these are fibrin-specific and do not bind fibrinogen.

We conclude that γ-(312-324) is hidden in fibrinogen and is exposed by the formation of fibrin.

INTRODUCTION

Thrombin, formed from its precursor prothrombin by an activated coagulation system, converts the soluble blood protein fibrinogen to insoluble fibrin. Fibrin is known to play an important role as the protein matrix of a blood clot. Fibrin has only a temporary function; after it has fulfilled its role, *e.g.* in tissue repair, it will be converted to soluble degradation products, with concomitant lysis of the blood clot. The crucial step in this fibrinolytic process is the activation of plasminogen to plasmin by plasminogen activators such as t-PA (Rijken *et al.* 1979, Collen 1980). The formed plasmin converts fibrin to soluble degradation products.

The activation of plasminogen by t-PA is greatly accelerated by fibrin, but virtually not by fibrinogen (Wallén 1977, Allan and Pepper 1981, Hoylaerts *et al.* 1982). Not only fibrin, but also plasmin-generated fragments of fibrin (D-dimer) and of fibrinogen ($D_{EGTA}$) accelerate the t-PA catalysed plasminogen activation (Verheijen *et al.* 1982[a]). Also, cyanogen bromide digests of fibrin(ogen) accelerate the plasminogen activation and the effect is partly recovered in FCB-2 (Nieuwenhuizen *et al.* 1983[b]). Further studies (Voskuilen *et al.* 1987) showed that part of the rate-enhancing effect of fibrin is brought about by Aα-(148-160) which is a constituent of FCB-2. On the basis of these findings and the fact that

monoclonal antibodies (MoAbs) against synthetic Aα-(148-160) are fibrin-specific, we concluded that the stretch Aα-(148-160) is buried in fibrinogen but becomes exposed upon the fibrinogen-to-fibrin conversion (Schielen *et al.* 1989).

Recently, another fragment of the fibrinogen molecule was found that is capable of accelerating the plasminogen activation by t-PA; *i.e.* FCB-5 (Yonekawa *et al.* 1989, 1990). FCB-5 consists of γ-(311-336) disulfide linked to γ-(337-379). Both γ-chain remnants and the disulfide bridge seem to be involved since reduced FCB-5 nor γ-(311-336) or γ-(337-379) have rate-enhancing capacity.

It is interesting to note that γ-(315-322) has a charge distribution analogous to that of Aα-(150-158), *i.e.*

```
Aα-(150-158):  Leu-Glu-Val-Asp-Ile-Asp-Ile-Lys-Ile
                0   -   0   -   0   -   0   +   0

γ-(315-322):   Trp-Asp-Asn-Asp-Asn-Asp-Lys-Phe
                0   -   0   -   0   -   +   0
```

We reasoned that, like we have shown (Schielen *et al.* 1989) for Aα-(148-160), also a part of FCB-5, *e.g.* γ-(315-322), may be buried in fibrinogen, and become exposed upon the fibrin formation. To assess this hypothesis we elicited monoclonal antibodies (MoAbs) against synthetic γ-(312-324).

In this paper we describe the production of MoAbs against this sequence of FCB-5. A conjugate of BSA and a synthetic peptide with the sequence γ-(312-324) was used as immunogen. The resulting antibodies react exclusively with fibrin, and not with fibrinogen. This clearly demonstrates that like Aα-(148-160), γ-(312-324) is a fibrin-specific neo-epitope. This finding supports our hypothesis that (at least parts of) the rate-enhancing fragment FCB-5 exists in a latent form in fibrinogen, but becomes exposed in fibrin.

## MATERIALS AND METHODS

### Synthesis of [Ata-Nle$^{311}$]-fibrinogen-γ-(311-324)-tetradecapeptide

Fibrinogen-γ-(312-324)-tridecapeptide (Phe-Ser-Thr-Trp-Asp-Asn-Asp-Asn-Asp-Lys-Phe-Glu-Gly) was extended with Ata-Nle at the amino-terminus. Thus, Gln

present in fibrinogen at γ 311 is replaced by Nle. This procedure allows the coupling to a carrier protein and (via the norleucine content) the subsequent determination of the number of coupled peptides per carrier protein molecule. The [Ata-Nle$^{311}$]-fibrinogen-γ-(311-324)-tetradecapeptide was synthesized on *p*-alkoxybenzyl alcohol resin (Wang 1973) using $N^{\alpha}$-Fmoc amino acid derivatives (ten Kortenaar *et al.* 1986) with the aid of a half-automated peptide synthesizer (SP 640, Labortec, Bubendorf, Switzerland).

During solid phase peptide synthesis the amino acid side chains were protected with acid-labile protecting groups: the ε-amino group of lysine with Boc, the γ-carboxyl group of glutamic acid and the ß-carboxyl group of aspartic acid with $OBu^t$, and the ß-hydroxyl group of serine and threonine with $Bu^t$. The carboxyl terminal amino acid derivative, Fmoc-Gly-OH [0.9 equivalents (eq.)] was coupled to the resin (1.07 mmol/g) by *in situ* activation with DCC (0.9 eq.) and DMAP (0.9 eq.). To suppress racemization, HOBt (1.8 eq.) was added (van Nispen *et al.* 1985). All reactants were dissolved in DMF. After 16 hours at 5 °C the resin was washed three times with successively each of DMF, dichloromethane, and isopropyl alcohol. Finally, the resin was washed with diisopropyl ether and dried *in vacuo*. The amount of Fmoc-amino acid attached to the resin was determined by elemental analysis of the amount of nitrogen in a sample of the resin. The cleavage of the Fmoc groups was carried out with 20% (vol/vol) piperidine in DMF, during three consecutive cycles of 6 minutes. Coupling of the second amino acid derivative (3 eq. relative to the amount of first amino acid attached to the resin) was performed by *in situ* activation with TBTU (3 eq.) (Knorr *et al.* 1989), HOBt (3 eq.) and N-methylmorfoline (4.5 eq.). After coupling of each amino acid derivative, completion of the acylation reaction was monitored by a ninhydrin test on free amino groups (Kaiser *et al.* 1970), followed by acylation of any remaining free amino groups with 10% (vol/vol) acetic acid anhydride in DMF. The fully-protected peptide was cleaved from the resin during a 4 h reaction with 2.5% (vol/vol) ethanedithiol and 2.5% (vol/vol) water in trifluoroacetic acid followed by precipitation in diethyl ether. The crude peptide was washed several times with diethyl ether and dried at the air.

Coupling of the tetradecapeptide to BSA

To 50 mg BSA (Carl Roth, Karlsruhe, FRG; 99% pure) dissolved in 1 ml buffer (phosphate, pH = 8.0), 4.5 mg 6-(1-maleimido)hexanoic acid 1-succinimide ester was added (MHS, Peeters *et al.* 1989). After 5 minutes the reaction mixture was subjected to gel filtration on a Sephadex G-25 column (Pharmacia, Uppsala, Sweden), run in phosphate buffer pH = 6.0.

The peptide derivative was activated by cleaving the acetyl group from the Ata-moiety by dissolution in a mixture of 4 M NaOH/methanol/dioxane (1/5/14) for exactly 15 seconds and subsequent neutralization with acetic acid. The solution containing the neutralized thioacetyl-tetradecapeptide (Tha-$P_{14}$) was added to the functionalized BSA and allowed to react for two hours at ambient temperature (Keller and Rudinger 1975). The reaction mixture was dialyzed against distilled water for 24 hours and lyophilized. The amount of incorporated Nle, as determined by amino acid analysis, showed that on average 4 to 5 Tha-$P_{14}$ molecules were linked per BSA molecule.

Antigens

Human fibrinogen was purified as described before (van Ruijven-Vermeer and Nieuwenhuizen 1978).

Fibrin monomers were prepared according to Haverkate and Timan (1976) and stored as a 13.4 mg/ml solution in 20 mM acetic acid at -20°C. Prior to use they were diluted to 0.45 mg/ml with 20 mM acetic acid (fibrin monomer solution, see below).

Fibrinogen γ-chain was obtained by reduction and carboxymethylation of fibrinogen and purified according to Doolittle and coworkers (1977).

Cyanogen bromide digestion of fibrinogen was performed according to Blombäck *et al.* (1968). CNBr fragment FCB-5 was isolated from the digest as described by Yonekawa *et al.* (1989, 1990).

Soluble fibrin was generated in plasma by addition of thrombin (Leo, Ballerup, Denmark; 10 μl, 2 NIH/ml) to 0.1 ml portions of normal pooled citrated plasma. At timed intervals (between 0-60 seconds) 40 μl aliquots were taken in which the thrombin reaction was quenched by 20-fold dilution in PBS containing 0.05%

Tween 20 and hirudin (0.2 U/ml).
In some experiments Arvin (= *Agkistrodon rhodostoma* venom protease) (Arwin, Knoll AG Ludwigshafen) was used instead of thrombin, under the same conditions. In these experiments, however, the reaction was quenched by the addition of diisopropylfluorophosphate (DFP) to a final concentration of 1 mM.

Immunization scheme

Female BALB/c mice were injected intraperitoneally with 62.5 $\mu$g immunogen (corresponding with approximately 5 $\mu$g of BSA-linked peptide) in Freund complete adjuvant (Difco, Amsterdam, The Netherlands) and then 6 times at 1 week intervals with 62.5 $\mu$g immunogen in Freund incomplete adjuvant. An intraperitoneal injection of 125 $\mu$g immunogen in 0.15 M NaCl was given three days before fusion.

Fusion

The immunized mice were killed in carbondioxide vapor and spleen cells were harvested. Spleen cells ($9.8*10^7$) were fused for 1 minute with $2.4*10^7$ non-producing myeloma cells (Sp2/0 AG14) in the presence of 40% polyethyleneglycol 1500 (Boehringer Mannheim), essentially as described by Köhler and Milstein (1975). The cell suspension was washed, diluted, and divided over four 96 wells microtitration plates (Costar, Cambridge, Mass.). Media of growing cells were screened for antibody production as described below. The cell line producing reactive antibody was subcloned three times by limiting dilution (0.5 cells/well) as described by Oi and Herzenberg (1980).

Hybridoma selection procedure

Hybridoma's were selected using an enzyme-linked immunosorbent assay (ELISA). The ELISA was performed essentially as described before (Koppert *et al.* 1985 and 1986) with thrombin treated purified fibrinogen as immobilized antigen. Immunoreactive mouse immunoglobulin was visualized with horse-radish peroxidase (HRP) labelled rabbit antimouse immunoglobulin (from Nordic, Tilburg, The Netherlands) using 3,3',5,5'-tetramethyl benzidine (TMB, from Aldrich

Chemical Co, Milwaukee), and $H_2O_2$ as the substrate mixture (Bos *et al.* 1981).

Subclass assessment and purification of the MoAb's

Subclass assessment was carried out with a isotyping kit for monoclonal antibodies (Serotec, Oxford, England).

Since the MoAb appeared to precipitate at low ionic strength, purification was carried out by simply dialysing of the ascites fluids against 0.005 M sodium phosphate pH 6.0.

Sandwich-type EIA

Wells of polystyrene microtitration plates were coated with anti-γ-(312-324) by overnight incubation at 4 °C with a 10 μg/ml solution of the antibody in 0.1 M sodium carbonate buffer, pH 9.6. The anti-γ-(312-324) coated wells were incubated with serial dilutions of a pooled normal plasma which was spiked with fibrin monomer solution. Captured antigen was tagged with G8 conjugated with HRP (Hoegee *et al.* 1988), and visualized by incubation with TMB/$H_2O_2$ substrate.

RESULTS

Preparation of the monoclonal antibodies

Two weeks after fusion and selection, cell growth was observed in most of the seeded wells. The medium of one well reacted with the thrombin treated fibrinogen in the ELISA system. The cells from the positive well were cloned and recloned. After cloning and recloning one clone was chosen for further study and is described in this paper. This line has been in culture for 2 months and has been injected into the peritoneal cavity of BALB/c mice for *in vivo* production of ascites fluid. Also *in vivo* the clone appeared to be stable *i.e.* more than 2 months (3 passages).

We will refer to the MoAb produced by this clone as anti-γ-(312-324). Subclass assessment showed that anti-γ-(312-324) is of the IgM/κ subclass. Therefore it could not be purified by protein A-Sepharose chromatography. It precipitates, however, during dialysis against a low ionic strength buffer (0.005 M sodium phosphate, pH 6), and can easily be redissolved by addition of a high

ionic strength buffer (1.7 M NaCl, 0.05 M sodium phosphate, pH 6) and remains in solution upon dilution to physiological ionic strengths.

Specificity of the monoclonal antibodies

In purified systems anti-γ-(312-324) showed the following characteristics in the ELISA system (see above). It did not react with BSA which was used as a carrier protein. It reacted, however, strongly with coated, thrombin treated fibrinogen and with purified γ-chain. As illustrated by figures 1 and 2, in a sandwich-type EIA, anti-γ-(312-324) shows virtually no reaction with normal plasma with a

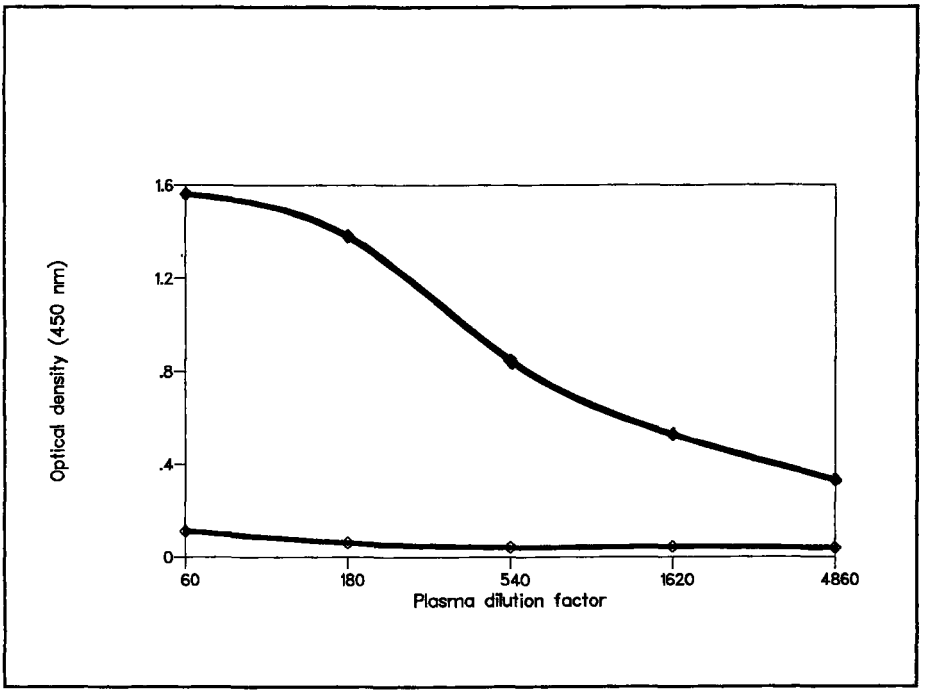


**Figure 1** Dose-response curves of 60-fold diluted plasma with a fibrinogen concentration of 2.4 mg/ml (◊) and of the same 60-fold diluted normal plasma but with fibrin monomer added to a final concentration of 5 μg/ml (♦)

fibrinogen concentration of 2.4 mg/ml. Anti-γ-(312-324) is able to interact with fibrin (in the μg/ml range) in a plasma milieu *i.e.* in excess fibrinogen, as shown in figures 1 and 2. In figure 1, results of an experiment are depicted in which

microtitration plates coated with anti-γ-(312-324) were used (sandwich-type EIA). Fibrin concentrations in the 0.1-1 μg/ml range are readily detectable.

Virtually identical dose-response curves were obtained when the fibrin monomer concentration was varied and the plasma dilution kept constant (not shown). In figure 2 a similar experiment is shown. Now the soluble fibrin was generated in plasma by treating the plasma for different periods of time with thrombin. A progressive increase in response with time of thrombin treatment is seen. Treatment with Arvin, which releases only fibrinopeptide A, yields comparable results. Normal plasma gives virtually no response.

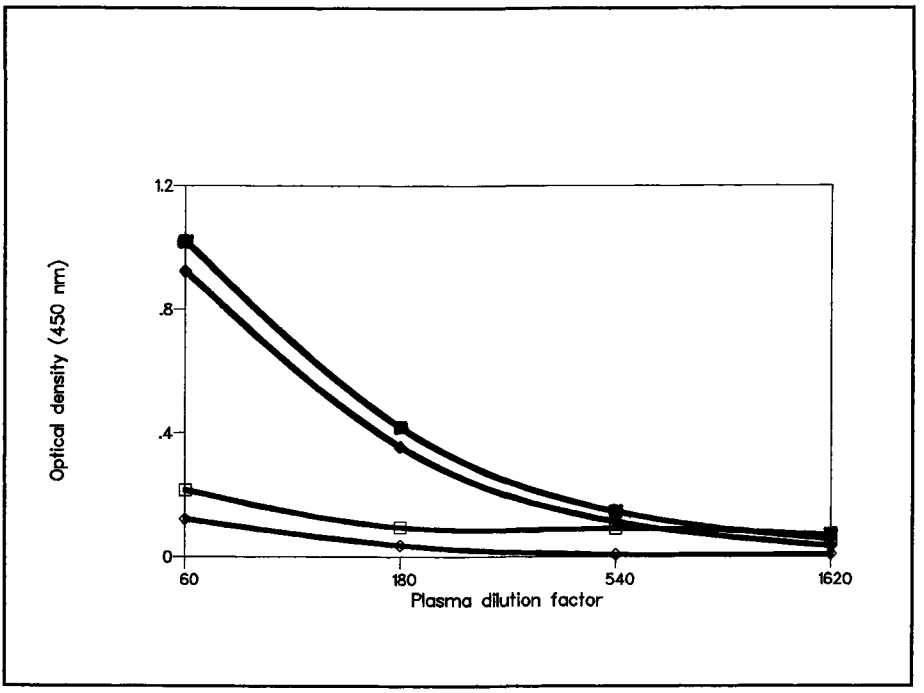


**Figure 2** Dose-response curves of normal plasma with a fibrinogen concentration of 2.4 mg/ml (◇) and of the same normal plasma treated with thrombin for 20 sec (□), 40 sec (◆), or 60 sec (■)

## DISCUSSION

The activation of plasminogen, as catalyzed by t-PA, is accelerated in the presence of fibrin. Fibrinogen has virtually no effect on the plasminogen

activation (Wallén 1977). Fragments derived of fibrin(ogen) also can have an accelerating effect on the plasminogen activation by t-PA (Verheijen *et al.* 1982[a]). Such fragments can be generated from fibrin(ogen) by digestion with plasmin or, chemically, with cyanogen bromide. It was concluded from these experiments that latent stimulatory sites pre-exist in fibrinogen which become exposed upon the conversion to fibrin or upon fragmentation of fibrinogen by plasmin or CNBr. Voskuilen *et al.* (1987) showed that the peptide Aα-(148-160), which is part of FCB-2, plays an important role in the acceleration of the plasminogen activation by t-PA.

A second type of stimulatory site was discovered by Yonekawa *et al.* 1989, 1990) in a region of the γ-chain, known as FCB-5. The FCB-5 fragment constitutes two γ-chain fragments (*i.e.* γ-(311-336) and γ-(337-379)), linked by one disulfide bond. Both γ-chain remnants and the disulfide bridge are required for rate enhancement.

In the case of Aα-(148-160), we have shown that this sequence is buried in fibrinogen and is exposed upon transformation of fibrinogen to fibrin, or by fibrinogen digestion with plasmin or with CNBr. The approach was to elicit MoAbs against synthetic Aα-(148-160). These anti-Aα-(148-160) MoAbs react with fibrin and not with fibrinogen (Schielen *et al.* 1989). Like Aα-(148-160), at least part(s) of FCB-5 may constitute a fibrin-specific epitope, *i.e.* be buried in fibrinogen and exposed in fibrin.

The sequence Aα-(150-158) in Aα-(148-160) has an interesting charge distribution, *i.e.*:

```
Leu-Glu-Val-Asp-Ile-Asp-Ile-Lys-Ile
 0   -   0   -   0   -   0   +   0
```

Negative and neutral amino acid residues alternate and the sequence ends with a lysine followed by a neutral residue. The sequence γ-(315-322) in γ-(311-336) has a similar charge distribution:

```
Trp-Asp-Asn-Asp-Asn-Asp-Lys-Phe
 0   -   0   -   0   -   +   0
```

The major differences are that the neutral amino acid residues in γ-(315-322) are much more polar (Asn instead of Ile) than in Aα-(150-158), and that the neutral

residue preceeding the lysyl residue is missing in the γ-chain.

Because of these analogous charge patterns of γ-(315-322) and Aα-(150-158) we selected a stretch of FCB-5, which includes γ-(315-322) to be part of a synthetic peptide, against which monoclonal antibodies were elicited. The obtained MoAbs appear to react only with fibrin and not with fibrinogen, thus providing evidence for our hypothesis that, like Aα-(148-160), γ-(312-324) which is part of the stimulatory fragment FCB-5, is buried in fibrin but becomes exposed upon the fibrinogen-to-fibrin conversion.

In summary, we have shown that γ-(312-324) is a fibrin-specific epitope.

## Chapter 4

# THE SEQUENCE Aα-(154-159) OF FIBRINOGEN IS CAPABLE OF ACCELERATING THE t-PA CATALYZED ACTIVATION OF PLASMINOGEN

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ABSTRACT

The rate of activation of plasminogen by tissue-type plasminogen activator is greatly increased by fibrin, but not by fibrinogen. We have previously shown that fibrin(ogen) fragments such as the fibrin(ogen) cyanogen bromide fragment 2 and a synthetic peptide with the sequence of fibrinogen Aα-(148-160) also have rate-enhancing properties. It was suggested that $Lys^{157}$ in the Aα-chain plays an important role. In order to find a possibly smaller, still stimulating site within Aα-(148-160) we synthesized successive linear amino-terminally acylated hexapeptides [*i.e.* Aα-(148-153), Aα-(149-154),....., Aα-(155-160)] and a tetrapeptide [*i.e.* Aα-(155-158)] from the sequence Aα-(148-160). The only hexapeptide capable of enhancing the rate of plasminogen-to-plasmin conversion by t-PA appears to be the amino-terminally acylated peptide comprising the sequence Aα-(154-159).

INTRODUCTION

Upon activation of the coagulation system, thrombin is generated from its zymogen prothrombin. Thrombin converts circulating fibrinogen molecules to fibrin monomers by the cleavage of fibrinopeptides, A and B, from the amino-terminal ends of the two Aα and Bβ chains of fibrinogen, respectively. The resulting new amino-termini of the α and β chain contain sites that are capable of binding to complementary sites in the carboxyl-terminal domains (Doolittle 1981, Laudano and Doolittle 1981) of other fibrin(ogen) molecules. At low concentrations fibrin monomer is kept in solution by complexing with fibrinogen. At a certain critical (local) concentration, fibrin aggregates to form a fibrin gel, that will eventually form the water-insoluble protein matrix of a blood clot. After a clot has fulfilled its role in the haemostatic process, *e.g.* in tissue repair, the matrix is dissolved by proteolysis by plasmin. Plasmin can be generated from its zymogen plasminogen by several plasminogen activators, such as tissue-type plasminogen activator (t-PA) (Rijken *et al.* 1979, Collen 1980).

Fibrin, and not fibrinogen, has the capacity to accelerate the plasminogen-to-plasmin conversion, mediated by t-PA. Fragments of fibrin and fibrinogen that enhance the rate of plasmin formation can be generated by digestion with

plasmin or, chemically, with cyanogen bromide. In particular the plasmin-generated fibrinogen fragment $D_{EGTA}$ (Verheijen *et al.* 1982[a]) and CNBr fragment FCB-2 (Nieuwenhuizen *et al.* 1983[b]) can mimic the effect of fibrin. More detailed studies have shown that the sequence Aα-(148-197) (Nieuwenhuizen *et al.* 1983[a]) and in particular the sequence Aα-(148-160) and $Lys^{157}$ in the Aα-chain (Voskuilen *et al.* 1987) play an essential role in this activation. The sequence Aα-(148-160) seems to be buried in fibrinogen and becomes exposed upon the fibrinogen-to-fibrin conversion by thrombin as evidenced by the reactivity with fibrin (and not with fibrinogen) of monoclonal antibodies raised against a synthetic peptide with the sequence Aα-(148-160) (Schielen *et al.* 1989).

In order to localize the stimulating site within Aα-(148-160) more precisely we describe in this paper the synthesis and the accelerating capacity, on the plasminogen-to-plasmin conversion by t-PA of successive linear amino-terminally acylated hexapeptides (*i.e.* a window of six residues moving from the amino terminus to the carboxyl terminus) covering the sequence Aα-(148-160). The results clearly demonstrate that the hexapeptide comprising the sequence Aα-(154-159) is the only one that is capable of accelerating the plasminogen activation by t-PA.

## MATERIALS AND METHODS

### Tissue-type plasminogen activator

Two-chain t-PA was purified from large scale melanoma cell culture according to Rijken *et al.* (1979), as modified by Kluft *et al.* (1983).

### Plasminogen

Native plasminogen (glu-plasminogen) was purified from fresh human plasma by affinity chromatography (Deutsch and Mertz 1970) on lysine-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden), precipitated with ammonium sulfate and dialysed extensively against 0.1 M Tris.HCl, pH 7.5, containing 0.1% (vol/vol) Tween 80 (Baker Chemicals).

### Peptides

The hexapeptides, Ata-Aα-(148-153); Ata-Aα-(149-154); Ata-Aα-(150-155); Ata-Aα-(151-156); Ata-Aα-(152-157); Ata-Aα-(153-158); Ata-Aα-(154-159), and Ata-Aα-(155-

160) were synthesized simultaneously on *p*-alkoxybenzyl alcohol resin (Wang 1973) using Fmoc amino acid derivatives (ten Kortenaar *et al.* 1986) and a multiple peptide synthesis system (RaMPS, Du Pont de Nemours, Boston, Mass., USA). The tetrapeptide Aα-(155-158) was synthesized on a half-automated peptide synthesizer (SP 640, Labortec, Bubendorf, Switzerland).

During solid phase peptide synthesis the amino acid side chains were protected with acid-labile protecting groups: the ϵ-amino group of lysine with Boc, the δ-guanidino group of arginine with Pmc, the γ-carboxyl group of glutamic acid and the ß-carboxyl group of aspartic acid with $OBu^t$, and the ß-hydroxyl group of serine with $Bu^t$. The carboxyl terminal amino acid [2 equivalents (eq.)] was coupled to the resin (1.07 mmol/g) by *in situ* activation with DCC (2 eq.) and DMAP (2 eq.). To suppress racemization HOBt (4 eq.) was added (van Nispen *et al.* 1985). All reactants were dissolved in DMF. After 16 hours at 5 °C the resin was washed three times with successively each of DMF, dichloromethane, and isopropyl alcohol. Finally, the resin was washed with diisopropyl ether and dried *in vacuo*. The amount of Fmoc-amino acid attached to the resin was determined by elemental analysis of the amount of nitrogen in a sample of the resin. The cleavage of the Fmoc groups was carried out with 50% (vol/vol) piperidine in DMF. Coupling of the second amino acid derivative (3 eq. relative to the amount of first amino acid attached to the resin) was performed by *in situ* activation with TBTU (3 eq.) (Knorr *et al.* 1989), HOBt (3 eq.) and NMM (4.5 eq.). After coupling of each amino acid derivative, completion of the acylation reaction was monitored by a ninhydrin test on free amino groups (Kaiser *et al.* 1970), followed by acylation of any remaining free amino groups with 10% (vol/vol) acetic acid anhydride in DMF. Finally, the Ata group was introduced via an active ester coupling with Ata-N-hydroxysuccinimide. The fully protected peptides were cleaved from the resin during a 4 h reaction with 2.5% (vol/vol) ethanedithiol and 2.5% (vol/vol) water in trifluoroacetic acid followed by precipitation in diethyl ether. The crude peptides were washed several times with diethyl ether, dried at the air, and analyzed by HPLC, specific rotation and amino acid composition.

HPLC analyses

HPLC analyses were performed on a RP-18 column (Techsil 5 C18, 250 x 4.6 mm, HPLC Technology, Cheshire, UK) at a flow-rate of 1.0 ml/min., using a 5 min. isocratic elution with 7% (vol/vol) acetonitrile and 0.1% (vol/vol) trifluoroacetic acid in water followed by a 30 min. linear gradient from 7% (vol/vol) acetonitrile and 0.1% (vol/vol) trifluoroacetic acid in water to 70% (vol/vol) acetonitrile and 0.08% (vol/vol) trifluoroacetic acid in water. Peptides were detected by UV measurement at 215 nm. The UV data were analyzed using JCL 6000 Chromatography Data System software (Jones Chromatography, Mid Glan, UK).

Amino acid analyses

Peptides were hydrolysed in 5.7 M HCl (Merck Suprapur) in evacuated sealed glass tubes for 24 hours at 120 °C. The hydrolysates were lyophilized and analyzed with a Varian 9095 amino acid analyzer using the Fmoc-protocol.

Specific rotations

The specific rotations were measured with a Perkin Elmer Polarimeter 241. All peptides were dissolved in DMF and centrifuged for 10 minutes at 4500 rpm prior to the measurement.

Assay system for the assessment of the rate-enhancing properties of the peptides

In total volumes of 0.250 ml 0.1 M Tris.HCl, containing 0.1% (vol/vol) Tween 80 were present 0.11 $\mu$M glu-plasminogen, 0.3 mM H-D-Val-Leu-Lys-*p*-nitroanilide, 300 mIU t-PA, and varying concentrations of the synthetic peptide to be tested for stimulating activity. In this assay, the concentration of *p*-nitroaniline formed per time-squared is proportional to the rate of plasmin formation (activation rate) (Drapier *et al.* 1979). The ratio of the activation rate in the presence of the peptide over that in the absence of peptide was designated as stimulation factor.

RESULTS

Table I shows that the amino acid compositions are as expected.

Table II summarizes the HPLC data and specific rotations obtained for each of the crude peptides. As indicated by the data, the purity of the peptides was

TABLE I

Amino acid compositions of the synthetic peptides prepared in this study. Expected values between parentheses.

| peptide | mole/mole of peptide | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Arg | Asp | Glu | Ile | Leu | Lys | Ser[a] | Val |
| Ata-Aα-(148-153) | 1.00(1) | 0.95(1) | 0.93(1) | —— | 1.03(1) | 1.00(1) | —— | 1.01(1) |
| Ata-Aα-(148-154) | 1.00(1) | 1.02(1) | 0.99(1) | 1.03(1) | 0.97(1) | —— | —— | 1.01(1) |
| Ata-Aα-(150-155) | —— | 2.00(2) | 0.98(1) | 1.03(1) | 1.00(1) | —— | —— | 0.98(1) |
| Ata-Aα-(151-156) | —— | 2.00(2) | 0.92(1) | 2.02(2) | —— | —— | —— | 0.95(1) |
| Ata-Aα-(152-157) | —— | 1.95(2) | —— | 2.02(2) | —— | 0.97(1) | —— | 1.00(1) |
| Ata-Aα-(153-158) | —— | 1.92(2) | —— | 3.00(3) | —— | 1.05(1) | —— | —— |
| Ata-Aα-(154-159) | 0.92(1) | 1.00(1) | —— | 3.07(3) | —— | 1.02(1) | —— | —— |
| Ata-Aα-(155-160) | 1.00(1) | 1.05(1) | —— | 2.07(2) | —— | 1.03(1) | 0.84(1) | —— |
| Aα-(155-158) | —— | 1.00(1) | —— | 2.09(2) | —— | 0.95(1) | —— | —— |

[a]The value for Ser has not been corrected for losses during hydrolysis

such that no purification of any of the synthetic peptides was required. Table III summarizes the stimulation factors found for the individual peptides, and the concentration of peptides needed for half-maximal stimulation. From table III it is clear that the synthetic amino-terminally acylated peptide with the sequence comprising fibrinogen-Aα-(154-159) has rate-enhancing potency. This shows that the rate-enhancing potency resides within Aα-(154-159). The tetrapeptide Aα-(155-158) also shows some rate-enhancing capacity, which is just significant.

## DISCUSSION

It was concluded from previous work (Nieuwenhuizen *et al.* 1983[a,b], 1985, Verheijen *et al.* 1982[a,b], 1983[a,b], 1985, Voskuilen *et al.* 1987) that the sequence fibrinogen-Aα-(148-160) plays an important role in the fibrin-induced rate enhancement of the plasminogen activation by t-PA. Aα-(148-160) is localized near the outer disulfide rings which terminate the coiled-coil regions, connecting the two fibrinogen D-domains with the central E-domain (Doolittle *et al.* 1978, Doolittle 1981). Fibrin, FCB-2 (Nieuwenhuizen *et al.* 1983[b]), some

TABLE II

Reversed phase HPLC data and $[\alpha]_D$-values of the synthetic peptides used in this study

| peptide | ret.time (mins) | % main peak | $[\alpha]_D$ | |
|---|---|---|---|---|
| Ata-Aα-(148-153) | 19.76 | 89.4 | -21.25 | (c=0.08, DMF) |
| Ata-Aα-(149-154) | 23.15 | 83.2 | - 6.25 | (c=0.16, DMF) |
| Ata-Aα-(150-155) | 24.99 | 85.3 | -19.23 | (c=0.13, DMF) |
| Ata-Aα-(151-156) | 24.89 | 92.7 | -29.47 | (c=0.10, DMF) |
| Ata-Aα-(152-157) | 23.70 | 93.8 | -14.40 | (c=0.13, DMF) |
| Ata-Aα-(153-158) | 24.50 | 87.1 | - 9.60 | (c=0.13, DMF) |
| Ata-Aα-(154-159) | 23.89 | 89.4 | -15.20 | (c=0.13, DMF) |
| Ata-Aα-(155-160) | 23.82 | 89.8 | - 8.00 | (c=0.05, DMF) |
| Aα-(155-158) | 18.43 | 96.4 | - 2.82 | (c=0.43, DMF) |

plasmin degradation products, such as $D_{EGTA}$ (Verheijen *et al.* 1982[a]) and fibrinogen-Aα-(148-197) (Nieuwenhuizen *et al.* 1983[a]), but not intact fibrinogen, all have rate enhancing properties. These facts led to the conclusion that the sites involved in the rate-enhancing properties exist in fibrinogen in a buried form. The hidden rate enhancing capacity can apparently be brought to expression upon the fibrinogen-to-fibrin conversion by thrombin, or by chemical or enzymatic digestion of fibrinogen.

Radcliff (1983) presented evidence that lysine residues in fibrin are essential for the accelerating capacity of fibrin on the plasminogen activation by t-PA. Plasmin is known to have lysine binding sites which are localized in the kringle I-IV domain. These lysine binding sites are crucial to the rate enhancing effect of FCB-2 (Verheijen *et al.* 1983[b]) as evidenced by the fact that the activation of mini-plasminogen ($Val^{442}$-plasminogen), lacking the kringle I-IV domain, is not

TABLE III

Stimulation factors of the synthetic peptides (obtained with the assay system described in the Materials and Methods section), and the peptide concentrations required for half-maximal stimulation.

| peptide | stimulation factor | half-maximal stimulation at |
|---|---|---|
| Ata-Aα-(148-153) | 1.3 ± 0.2 | ----[a] |
| Ata-Aα-(149-154) | 1.1 ± 0.1 | ----[a] |
| Ata-Aα-(150-155) | 1.8 ± 0.6 | ----[a] |
| Ata-Aα-(151-156) | 2.2 ± 1.4 | ----[a] |
| Ata-Aα-(152-157) | 1.1 ± 0.3 | ----[a] |
| Ata-Aα-(153-158) | 1.3 ± 0.2 | ----[a] |
| Ata-Aα-(154-159) | 6.0 ± 2.0 | 56 ± 50 μM |
| Ata-Aα-(155-160) | 1.9 ± 0.5 | ----[a] |
| Aα-(155-158) | 3.2 ± 0.7 | 715 ± 439 μM |

[a]Since the stimulation factors of these peptides are very low and probably not significant, the concentrations at half-maximal stimulation are not given.

accelerated by FCB-2. The rate enhancement of fibrin is diminished by the lysine analogue 6-amino hexanoic acid, which is an other indication of the importance of the lysine binding sites in the plasminogen molecule. The rate enhancement of the plasminogen activation by t-PA has been ascribed mainly to carboxyl-terminal lysines (Christensen 1984, Suenson *et al.* 1984, Norrman *et al.* 1985). The carboxyl-terminal lysines seem to fulfil the postulated requirements for binding to the lysine binding sites of plasminogen, *i.e.*, an ϵ-amino- and an α-carboxyl group separated by an aliphatic carbon chain. However, Voskuilen *et al.* (1987) have shown that $Lys^{157}$ in the Aα-chain plays an essential role in the rate-enhancement, since succinylation of this lysine residue annihilates the

stimulating effect of Aα-(148-160) completely. This lysine residue, however, is not a carboxyl-terminal residue and, therefore, also non-carboxyl-terminal lysine residues may contribute to the observed rate enhancement by fibrin.

The present study indicates that the orientation of this lysine side chain must meet special requirements. Only in the synthetic hexapeptide Aα-(154-159) the lysine side chain appears to be properly exposed to the environment, since the other $Lys^{157}$ containing hexapeptides [*i.e.* Aα-(152-157), Aα-(153-158) and Aα-(155-160)] do not stimulate.

It is likely that the typical charge distribution in the sequence Aα-(148-160), *i.e.*

```
-Lys-Arg-Leu-Glu-Val-Asp-Ile-Asp-Ile-Lys-Ile-Arg-Ser-
  +   +   0   -   0   -   0   -   0   +   0   +   0
```

where negative and neutral amino acid residues alternate, and are embraced by two sets of positively charged residues, induces (or perhaps could be a part of) a typical local conformation. Only if the local conformation fulfils special requirements, is the $Lys^{157}$ side chain exposed properly to allow acceleration of the plasminogen activation. Voskuilen *et al.* (1987) suggest that the interaction of $Lys^{157}$ with the postulated "aminohexyl sites" (Christensen 1984) in plasminogen, or with t-PA, might be explained by a spatial conformation of *e.g.* Aα-(148-160) in which the side-chain carboxyl function of one of the aspartic acid residues or the glutamic acid residue, together with the ϵ-amino group of the lysine side chain are in a position in which a carboxyl-terminal lysine can be mimicked. Implicit in this suggestion is a conformation in which the lysine side chain is oriented properly relative to other residues (*e.g.* Asp) in the same conformation.

As reported before (Nieuwenhuizen *et al.* 1983[8], Voskuilen *et al.* 1987) the effectivity drops with decreasing molecular weight of the stimulator, while the concentration needed for half-maximal stimulation increases. In this assay system, fibrin and FCB-2 enhance the activation rate of plasminogen about 35-fold, with a concentration needed for half maximal stimulation of 0.02 μM for fibrin monomers and 0.12 μM for FCB-2. The maximum stimulation by the Aα-

chain remnant of FCB-2 [*i.e.* Aα-(148-207)] is about 20-fold (concentration at half maximal stimulation is 0.5 μM), by Aα-(148-160) it is about 11-fold (concentration at half maximal stimulation is 12 μM). For Aα-(154-159) the stimulation factor is 6; the half maximal concentration is 50 μM. The observed decrease in accelerating capacity (and increase in the concentration needed for half maximal stimulation) has been ascribed (Voskuilen *et al.* 1987) to the possibility that amino acid residues other than those in Aα-(148-160) are also involved in the rate enhancing effect of fibrin monomer, FCB-2 and its Aα-chain remnant. These observations would fit in the assumption of the existence of a local conformation of which the sequence Aα-(148-160) is a component. In fibrin monomers this local conformation is fully expressed and stabilized, and would thus give an optimal rate enhancement (at the lowest concentration) of the plasminogen activation. When a part of the fibrin molecule (*e.g.* FCB-2 or its Aα-chain remnant) is taken, the expression of the local conformation decreases (the stimulation factor decreases, and the required concentrations increases). The conformations of the smaller peptides such as Aα-(148-160) or Aα-(154-159) are little or not stabilized (since other supporting parts of the fibrin molecule are absent) and this leads to a low accelerating capacity, and the need for relatively high concentrations.

Although the non-stimulating hexapeptides Ata-Aα-(152-157), Ata-Aα-(153-158), and Ata-Aα-(155-160) are part of the proposed secondary structure element, they seem not to be able to adopt the required conformation.

The synthetic tetrapeptide Aα-(155-158) appears to have a weak stimulating capacity. If the aforementioned arguments are correct, this could indicate that the lysine side chain exposure is far from optimal in Aα-(155-158).

In conclusion, we have presented evidence that the peptide with the fibrinogen sequence Aα-(154-159) contains the structural information required for enhancement of the plasminogen activation by t-PA.

# Chapter 5

## STRUCTURAL REQUIREMENTS OF POSITION Aα-157 IN FIBRINOGEN FOR THE FIBRIN-INDUCED RATE ENHANCEMENT OF THE PLASMINOGEN ACTIVATION BY t-PA

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ABSTRACT

The sequence fibrinogen-Aα-(148-160) can mimic part of the fibrin-induced rate enhancement of the activation of plasminogen by t-PA. Previously, we have reported that the lysyl residue at Aα-157 is crucial. During our further investigations on Aα-157 we found that Lys at position Aα-157 may be replaced by Glu. This unexpected finding prompted us to re-investigate the requirements of this position.

We prepared analogues of Aα-(148-160) in which the lysyl residue at Aα-157 was replaced by lysine-derivatives [Lys(Ac), Lys(Z), Lys(Msc)]; acidic residues (Asp, Glu); basic residues (Arg, Orn); polar residues [Gln, Orn(Msc)]; apolar residues [Ala, Val, Nle, Glu(ONb)], and Gly. These analogues were tested for their stimulatory activity. When Asp, Glu(ONb), Val, or Nle is present at position Aα-157 in Aα-(148-160) virtually all stimulatory capacity is lost. None of the other replacements of Aα-157 caused loss of rate-enhancing properties. From these results we conclude that for the rate-enhancing effect of Aα-(148-160), the side chain of the amino acid residue at position Aα-157 must fulfil some requirements: there must be one (as in Ala) or no (as in Gly) carbon atom in the side chain, or at least two carbons and a polar group (charged or uncharged) to which a rather bulky group (such as the Z-group) or a polar group (like the Msc group) may be attached. The highest activity [even higher than native Aα-(148-160)] was obtained with Orn, Orn(Msc), or Lys(Msc) at position Aα-157.

INTRODUCTION

Activation of the coagulation system leads to the formation of thrombin, which converts circulating fibrinogen molecules to fibrin by cleaving off fibrinopeptides A and B from the amino-terminal ends of the two Aα- and Bβ-chains of fibrinogen, respectively. The new amino-termini of the α and β chain contain sites that bind to complementary sites in the carboxyl-terminal domains (Doolittle 1981, Laudano and Doolittle 1981) of other fibrin(ogen) molecules. At low concentrations fibrin monomers are kept in solution by complexation to fibrinogen until a certain critical (local) concentration is reached. Beyond this critical concentration, fibrin monomers start to form a fibrin gel which constitutes

the protein matrix of a blood clot. After such a blood clot has fulfilled its role in the haemostatic process, the fibrin matrix is dissolved by plasmin. The proteolytic enzyme plasmin can be generated from its zymogen plasminogen by several activators, such as tissue-type plasminogen activator (t-PA) (Rijken *et al.* 1979, Collen 1980).

The plasminogen-to-plasmin conversion, as mediated by t-PA, is greatly enhanced by fibrin, but virtually not by fibrinogen (Wallén 1977, Allan and Pepper 1981, Hoylaerts *et al.* 1982). Detailed studies (Nieuwenhuizen *et al.* 1983[a], Voskuilen *et al.* 1987, Yonekawa *et al.* 1989, 1990) showed that part of this rate-enhancing effect of fibrin can be ascribed to two sites on the fibrin molecule: one in Aα-(148-160) and one in FCB-5. The sequence Aα-(148-160) seems to be buried in fibrinogen and becomes exposed upon the fibrinogen-to-fibrin conversion by thrombin as evidenced by the reactivity with fibrin (and not fibrinogen) of monoclonal antibodies raised against a synthetic peptide with the sequence Aα-(148-160) (Schielen *et al.* 1989). Also, at least part of the FCB-5 fragment [*i.e.* γ-(312-325)] seems to be buried in fibrinogen and becomes exposed by the fibrinogen-to-fibrin conversion, since monoclonal antibodies raised against synthetic γ-(312-325) are fibrin-specific (Schielen *et al.*, Chapter 3).

Voskuilen *et al.* (1987) presented evidence that $Lys^{157}$ in Aα-(148-160) is essential for the rate-enhancing effect of fibrin since succinylation of synthetic Aα-(148-160) annihilated the accelerating capacity of this peptide completely. Moreover, replacing $Lys^{157}$ by Val yielded a synthetic peptide that also showed no rate enhancement of the plasminogen activation as mediated by t-PA. However, our recent investigations revealed that Aα-(148-160) in which $Lys^{157}$ was replaced by Glu maintained its rate-enhancing capacity. This indicates that lysine at position Aα-157 is not an absolute prerequisite for rate enhancement. This unexpected finding prompted us to re-investigate the requirements of the side-chain of the residue in position Aα-157. We prepared analogues of Aα-(148-160) in which the lysyl residue at Aα-157 was replaced by lysine-analogues [Lys(Ac), Lys(Z), Lys(Msc)]; acidic residues (Asp, Glu); basic residues (Arg, Orn); polar residues [Gln, Orn(Msc)]; apolar residues [Ala, Val, Nle, Glu(ONb)], and Gly. These analogues were tested for their stimulatory activity. With Asp, Glu(ONb),

Val, and Nle at position Aα-157 in Aα-(148-160) virtually all stimulatory capacity is lost. None of the other replacements caused loss of rate-enhancing properties.

From these results we conclude that for the rate-enhancing effect of Aα-(148-160), the side chain of the amino acid residue at Aα-157 must fulfil some requirements: there must be one (as in Ala) or no (as in Gly) carbon atom in the side chain, or at least two carbons and a polar group (charged or uncharged) to which a rather bulky group (such as the Z-group) or a polar group (like the Msc group) may be attached. The highest activity [even higher than native Aα-(148-160)] was obtained with Orn, Orn(Msc), or Lys(Msc) at position Aα-157.

## MATERIALS AND METHODS

### Tissue-type plasminogen activator

Two-chain t-PA was purified from large scale melanoma cell culture according to Rijken *et al.* (1979), as modified by Kluft *et al.* (1983).

### Plasminogen

Native plasminogen (glu-plasminogen) was purified from fresh human plasma by affinity chromatography (Deutsch and Mertz 1970) on lysine-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden), precipitated with ammonium sulfate and dialysed extensively against 0.1 M Tris.HCl, pH 7.5, containing 0.1% (vol/vol) Tween 80 (Baker Chemicals).

### Peptides

The peptide analogues [$Xxx^{157}$]-Aα-(148-160), where $Xxx^{157}$ = Lys(Ac), Lys(Msc), Lys(Z), Asp, Glu, Arg, Orn, Gln, Orn(Msc), Ala, Val, Nle, Glu(ONb), or Gly, were synthesized on *p*-alkoxybenzyl alcohol resin (Wang 1973) using $N^{\alpha}$-Fmoc amino acid derivatives (ten Kortenaar *et al.* 1986) with the aid of a half-automated peptide synthesizer (SP 640, Labortec, Bubendorf, Switzerland).

During solid phase peptide synthesis the amino acid side chains were protected with acid-labile protecting groups: the ε-amino group of lysine with Boc, the δ-guanidino group of arginine with Pmc, the γ-carboxyl group of glutamic acid and the ß-carboxyl group of aspartic acid with $OBu^t$, and the ß-hydroxyl group of serine with $Bu^t$. The carboxyl terminal amino acid [0.9 equivalents (eq.)] was coupled to the resin (1.07 mmol/g) by *in situ* activation with DCC (0.9 eq.) and

DMAP (0.9 eq.). To suppress racemization, HOBt (1.8 eq.) was added (van Nispen *et al.*, 1985). All reactants were dissolved in DMF. After 16 hours at 5 °C the resin was washed three times with successively each of DMF, dichloromethane, and isopropyl alcohol. Finally, the resin was washed with diisopropyl ether and dried *in vacuo*. The amount of Fmoc-amino acid attached to the resin was determined by elemental analysis of the amount of nitrogen in a sample of the resin. The cleavage of the Fmoc groups was carried out with 20% (vol/vol) piperidine in DMF, during three consecutive cycles of 6 minutes. Coupling of the second amino acid derivative (3 eq. relative to the amount of first amino acid attached to the resin) was performed by *in situ* activation with TBTU (3 eq.) (Knorr *et al.* 1989), HOBt (3 eq.) and N-methylmorfoline (4.5 eq.). After coupling of each amino acid derivative, completion of the acylation reaction was monitored by a ninhydrin test on free amino groups (Kaiser *et al.* 1970), followed by acylation of any remaining free amino groups with 10% (vol/vol) acetic acid anhydride in DMF. The fully protected peptides were cleaved from the resin during a 4-hr reaction with 2.5% (vol/vol) ethanedithiol and 2.5% (vol/vol) water in trifluoroacetic acid followed by precipitation in diethyl ether. The crude peptides were washed several times with diethyl ether, dried at the air, and analyzed by HPLC, specific rotation and amino acid composition.

HPLC analyses

HPLC analyses were performed on a RP-18 column (Techsil 5 C18, 250 x 4.6 mm, HPLC Technology, Cheshire, UK) at a flow-rate of 1.0 ml/min., using a 5 min. isocratic elution with 7% (vol/vol) acetonitrile and 0.1% (vol/vol) trifluoroacetic acid in water followed by a 30 min. linear gradient from 7% (vol/vol) acetonitrile and 0.1% (vol/vol) trifluoroacetic acid in water to 70% (vol/vol) acetonitrile and 0.08% (vol/vol) trifluoroacetic acid in water. Peaks were detected by UV measurement at 215 nm. The UV data were analyzed using JCL 6000 Chromatography Data System software (Jones Chromatography, Mid Glan, UK).

TABLE I

Amino acid compositions of the synthetic peptides used in this study. Expected values between parentheses.

| | mole/mole of peptide | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| [Xxx$^{157}$]-Ac-(148-160) Xxx= | Ala | Arg | Asp | Glu | Gly | Ile | Leu | Lys | Nle | Orn | Ser[a] | Val |
| Ala | 1.06 (1) | 2.07 (2) | 1.94 (2) | 1.02 (1) | --- | 3.05 (3) | 0.94 (1) | 1.09 (1) | --- | --- | 0.71 (1) | 0.96 (1) |
| Arg | --- | 3.00 (3) | 2.08 (2) | 1.03 (1) | --- | 3.02 (3) | 1.06 (1) | 1.00 (1) | --- | --- | 0.82 (1) | 1.04 (1) |
| Asp | --- | 2.02 (2) | 3.06 (3) | 1.00 (1) | --- | 2.97 (3) | 1.09 (1) | 1.03 (1) | --- | --- | 0.82 (1) | 1.06 (1) |
| Gln | --- | 2.09 (2) | 2.00 (2) | 2.04 (2) | --- | 3.01 (3) | 1.00 (1) | 1.05 (1) | --- | --- | 0.80 (1) | 0.98 (1) |
| Glu | --- | 2.05 (2) | 2.00 (2) | 2.03 (2) | --- | 3.01 (3) | 1.04 (1) | 0.98 (1) | --- | --- | 0.81 (1) | 0.94 (1) |
| Glu(ONb) | --- | 2.00 (2) | n.d. | 1.98 (2) | --- | 3.06 (3) | 1.07 (1) | 1.02 (1) | --- | --- | n.d. | 0.95 (1) |
| Gly | --- | 2.07 (2) | 2.02 (2) | 1.01 (1) | 1.02 (1) | 3.00 (3) | 1.01 (1) | 0.97 (1) | --- | --- | 0.85 (1) | 1.00 (1) |
| Lys | --- | 2.04 (2) | 1.96 (2) | 1.00 (1) | --- | 2.98 (3) | 1.04 (1) | 2.07 (2) | --- | --- | 0.83 (1) | 1.08 (1) |
| Lys(Ac) | --- | 1.89 (2) | n.d. | 0.97 (1) | --- | 3.04 (3) | 1.07 (1) | 2.00 (2) | --- | --- | n.d. | 0.99 (1) |
| Lys(Msc) | --- | 2.11 (2) | 1.96 (2) | 1.00 (1) | --- | 3.13 (3) | 1.04 (1) | 2.00 (2) | --- | --- | 0.81 (1) | 0.97 (1) |
| Lys(Z) | --- | 1.91 (2) | n.d. | 1.00 (1) | --- | 2.93 (3) | 1.09 (1) | 2.12 (2) | --- | --- | n.d. | 1.08 (1) |
| Nle | --- | 2.00 (2) | 1.97 (2) | 1.00 (1) | --- | 3.24 (3) | 1.02 (1) | 0.95 (1) | 1.02 (1) | --- | 0.83 (1) | 0.97 (1) |
| Orn | --- | 1.97 (2) | 2.03 (2) | 1.02 (1) | --- | 3.04 (3) | 1.12 (1) | 1.00 (1) | --- | 1.06 (1) | 0.74 (1) | 1.03 (1) |
| Orn(Msc) | --- | 2.12 (2) | 2.05 (2) | 0.98 (1) | --- | 3.07 (3) | 1.07 (1) | 1.87 (2) | --- | 1.05 (1) | 0.80 (1) | 1.00 (1) |
| Val | --- | 2.00 (2) | 1.93 (2) | 1.02 (1) | --- | 3.08 (3) | 1.05 (1) | 0.98 (1) | --- | --- | 0.73 (1) | 2.05 (2) |

[a]The value for Ser has not been corrected for losses during hydrolysis
n.d.: not determined

Amino acid analyses

Peptides were hydrolysed in 5.7 M HCl (Merck Suprapur) in evacuated sealed glass tubes for 24 hours at 120 °C. The hydrolysates were lyophilized and analyzed with a Varian 9095 amino acid analyzer using the Fmoc-protocol.

Specific rotations

The specific rotations were measured with a Perkin Elmer Polarimeter 241. All peptides were dissolved in DMF and centrifuged for 10 minutes at 4500 rpm prior to the measurement.

Assay system for the assessment of the rate-enhancing properties of the peptides

In total volumes of 0.250 ml 0.1 M Tris.HCl, containing 0.1% (vol/vol) Tween 80 were present 0.11 $\mu$M glu-plasminogen, 0.3 mM H-D-Val-Leu-Lys-*p*-nitroanilide, 300 mIU t-PA, and varying concentrations of the synthetic peptide to be tested for stimulating activity. In this assay, the concentration of *p*-nitroaniline formed per time-squared is proportional to the rate of plasmin formation (activation rate) (Drapier *et al.* 1979). The ratio of the activation rate in the presence of the peptide over that in the absence of peptide was designated as stimulation factor.

RESULTS

Table I shows that the amino acid compositions are as expected.
Table II summarizes the HPLC data and specific rotations obtained for each of the crude peptides. As indicated by the data, the purity of the peptides was such that no purification of any of the synthetic peptides was required.
Table III summarizes the stimulation factors found for the individual peptides. The concentrations needed for half-maximal stimulation were around 50 $\mu$M.

DISCUSSION

It was concluded from previous work (Nieuwenhuizen *et al.* 1983[a,b], 1985, Verheijen *et al.* 1982, 1983[a,b], 1985, Voskuilen *et al.* 1987) that the sequence fibrinogen-A$\alpha$-(148-160) plays an important role in the fibrin-induced rate enhancement of the plasminogen activation by t-PA. A$\alpha$-(148-160) is localized near the outer disulfide rings which terminate the coiled-coil regions,

TABLE II

Reversed phase HPLC data and $[\alpha]_D$-values of the synthetic peptides used in this study.

| [Xxx$^{157}$]-Aα-(148-160) Xxx= | ret.time (mins) | % main peak | $[\alpha]_D$ |
|---|---|---|---|
| Ala | 22.70 | 87.6 | - 8.3 (c=1.01, DMF) |
| Arg | 20.10 | 81.8 | - 3.5 (c=1.08, DMF) |
| Asp | 22.26 | 90.1 | -23.3 (c=1.07, DMF) |
| Gln | 22.20 | 74.0 | - 5.0 (c=1.07, DMF) |
| Glu | 23.49 | 93.3 | - 9.6 (c=1.11, DMF) |
| Glu(ONb) | 25.71 | 85.6 | - 6.8 (c=0.60, DMF) |
| Gly | 21.12 | 85.7 | -18.3 (c=0.99, DMF) |
| Lys | 21.00 | 90.1 | - 7.3 (c=1.00, DMF) |
| Lys(Ac) | 22.59 | 88.3 | - 5.8 (c=1.06, DMF) |
| Lys(Msc) | 23.28 | 84.6 | - 3.3 (c=1.15, DMF) |
| Lys(Z) | 23.64 | 93.0 | - 5.2 (c=1.10, DMF) |
| Nle | 24.78 | 71.6 | - 4.8 (c=1.02, DMF) |
| Orn | 21.75 | 88.4 | - 2.9 (c=1.12, DMF) |
| Orn(Msc) | 22.80 | 89.9 | - 6.6 (c=1.05, DMF) |
| Val | 23.70 | 86.3 | - 6.1 (c=1.05, DMF) |

connecting the two distal fibrinogen D-domains with the central E-domain (Doolittle *et al.* 1978, Doolittle 1981). Fibrin, FCB-2 (Nieuwenhuizen *et al.* 1983[b]), some plasmin degradation products, such as $D_{EGTA}$ (Verheijen *et al.* 1982[a]) and fibrinogen-Aα-(148-197) (Nieuwenhuizen *et al.* 1983[a]), but not intact fibrinogen, all have rate enhancing properties. These facts led to the conclusion that the sites involved in the rate-enhancing properties exist in fibrinogen in a buried form.

TABLE III

Stimulation factors of the synthetic peptides, obtained with the assay system described in the Materials and Methods section

| peptide | stimulation factor |
|---|---|
| [Ala$^{157}$]-Aα-(148-160) | 17.0 ± 0.3 |
| [Arg$^{157}$]-Aα-(148-160) | 14.9 ± 2.1 |
| [Asp$^{157}$]-Aα-(148-160) | 2.4 ± 0.1 |
| [Gln$^{157}$]-Aα-(148-160) | 10.6 ± 1.9 |
| [Glu$^{157}$]-Aα-(148-160) | 8.6 ± 1.6 |
| [Glu(ONb)$^{157}$]-Aα-(148-160) | 2.0 ± 0.6 |
| [Gly$^{157}$]-Aα-(148-160) | 5.6 ± 1.6 |
| [Lys$^{157}$]-Aα-(148-160)<br>native Aα-(148-160) | 14.9 ± 3.3 |
| [Lys(Ac)$^{157}$]-Aα-(148-160) | 14.4 ± 2.4 |
| [Lys(Msc)$^{157}$]-Aα-(148-160) | 17.3 ± 0.4 |
| [Lys(Z)$^{157}$]-Aα-(148-160) | 9.1 ± 1.3 |
| [Nle$^{157}$]-Aα-(148-160) | 1.8 ± 1.0 |
| [Orn$^{157}$]-Aα-(148-160) | 20.3 ± 2.7 |
| [Orn(Msc)$^{157}$]-Aα-(148-160) | 23.7 ± 4.4 |
| [Val$^{157}$]-Aα-(148-160) | 3.7 ± 0.2 |

The hidden rate enhancing capacity can apparently be brought to expression upon the fibrinogen-to-fibrin conversion, e.g. by thrombin, or by chemical or enzymatic digestion of fibrinogen.

Radcliff (1983) presented evidence that lysine residues in fibrin are essential for the accelerating capacity of fibrin on the plasminogen activation by t-PA. Plasmin is known to have lysine binding sites which are localized in the kringle I-IV domain. These lysine binding sites are crucial to the rate enhancing effect of

FCB-2 (Verheijen *et al.* 1983[b]) as evidenced by the fact that the activation of mini-plasminogen (Val[442]-plasminogen), lacking the kringle I-IV domain, is not accelerated by FCB-2. The rate enhancement of fibrin is diminished by the lysine-analogue 6-amino hexanoic acid, which is another indication for the importance of the lysine binding sites in the plasminogen molecule. The rate enhancement of the plasminogen activation by t-PA have been ascribed mainly to carboxyl-terminal lysines (Christensen 1984, Suenson *et al.* 1984, Norrman *et al.* 1985). The carboxyl-terminal lysines seem to fulfil the requirements for binding to the lysine binding sites of plasminogen, *i.e.*, an $\epsilon$-amino- and an $\alpha$-carboxyl group separated by an aliphatic carbon chain. However, Voskuilen *et al.* (1987) showed that Lys[157] in the A$\alpha$-chain plays an essential role in the rate-enhancement, since succinylation of this lysine residue annihilates the stimulating effect of A$\alpha$-(148-160) completely. This lysine residue at A$\alpha$-157 is not a carboxyl-terminal residue and, therefore, also some non-carboxyl-terminal lysine residues may contribute to the observed rate enhancement by fibrin.

In an other study (Schielen *et al.*, Chapter 4), in which we prepared the amino-terminally acylated hexapeptides A$\alpha$-(152-157), A$\alpha$-(153-158), A$\alpha$-(154-159), and A$\alpha$-(155-160), we showed that only the hexapeptide A$\alpha$-(154-159) is capable of stimulating the plasminogen-to-plasmin conversion. Since all of these peptides comprise Lys at position A$\alpha$-157, we concluded that the orientation of Lys[157] is important and we assumed that the stretch A$\alpha$-(148-160) is involved in inducing (or perhaps is part of) a typical (local) conformation that pre-exists in fibrinogen in a latent form and is exposed in fibrin.

The general believe is that lysine residues play a crucial role in the rate-enhancing properties of fibrin. Therefore it was surprising to find that when we replaced Lys at position A$\alpha$-157 by Glu in the peptide A$\alpha$-(148-160), the resulting peptide induced a significant rate enhancement.

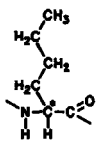
The results of the present study clearly show that Lys is not the only residue that is allowed at position A$\alpha$-157 in the peptide A$\alpha$-(148-160) to maintain stimulatory capacity. In fact, from table III it seems that many different residues are allowed in this position. These findings support the hypothesis of a certain typical (local) conformation, in which not the lysyl residue at A$\alpha$-157 *per se* is

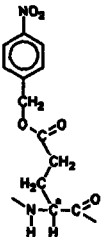
important, but rather the degree in which the inferred conformation is stabilized by the residue at position Aα-157. Introduction of Nle, Glu(ONb), Asp, or Val at position Aα-157 in the peptide Aα-(148-160) probably induces a conformation which does not fulfil the requirements for rate enhancement. All other peptide analogues tested in this study show a significant stimulating capacity, indicating that the rate-enhancing conformation is maintained by the introduction of the residues at position Aα-157, listed in figure 1.

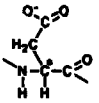
In a previous paper (Voskuilen *et al.* 1987) we showed that [Arg$^{157}$]-Aα-(148-160) did not exert any stimulating capacity. We now found (table III) that [Arg$^{157}$]-Aα-(148-160) stimulates equally well as native Aα-(148-160). The difference is probably due to the fact, that we used high concentrations of [Arg$^{157}$]-Aα-(148-160) in the rate enhancement experiments. It now appears, that (too) high concentrations of the peptide inhibit the plasminogen activation by t-PA.

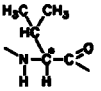
In figure 1 the structures of the residues that were incorporated at position Aα-157 in the peptide Aα-(148-160) are listed in order of increasing stimulating capacity. From figure 1 we conclude that for the rate-enhancing effect of Aα-(148-160), the side chain of the amino acid residue at Aα-157 must fulfil some requirements: there must be one (as in Ala) or no (as in Gly) carbon atom in the side chain, or at least two carbons and a polar group (charged or uncharged) to which a rather bulky group (such as the Z-group) or a polar group (like the Msc-group) may be attached. Introduction of the ONb-group on the Glu side chain annihilates the rate enhancement completely, possibly due to the apolar nature of the resulting side chain, which may be similar to that of Val or Nle. The peptides [Glu(ONb)$^{157}$]-, [Val$^{157}$]-, and [Nle$^{157}$]-Aα-(148-160) tend to have a high affinity for the column material (C18) in reversed phase HPLC, as evidenced by the long retention times of these peptides (table II). Introduction of a succinyl group at the ε-amino group of Lys may also give rise to an apolar side chain, since the results of Voskuilen *et al.* (1987) show that succinylation of synthetic Aα-(148-160) annihilates the stimulating effect of Aα-(148-160) completely. On the other

No significant stimulation


Nle

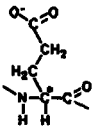

Glu(ONb)

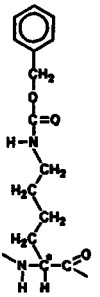

Asp

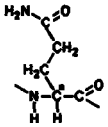

Val

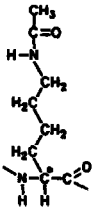
Stimulation


Gly


Glu


Lys(Z)


Gln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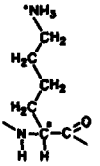
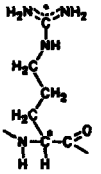
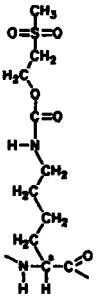
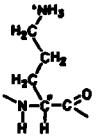
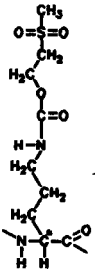

Lys


Arg


Ala


Lys(Msc)


Orn


Orn(Msc)

hand, introduction of the rather apolar Z-group at the ϵ-amino group of Lys at Aα-157 has only little effect on the stimulating capacity of Aα-(148-160), possibly due to compensation by the polar ϵ-amido group.

From table III and figure 1 it is clear that increases in stimulating capacity of Aα-(148-160) can be obtained, which is largest by introduction of Orn, or the Msc-group at the ϵ-amino group of Lys or the δ-amino group of Orn at position Aα-157.

In conclusion, we have shown that the lysyl residue at position Aα-157 can be replaced by a variety of other residues or amino acid derivatives, indicating that not the residue at position Aα-157 is important, but the degree to which a presumed typical (local) conformation in Aα-(148-160) is induced, maintained, or stabilized by the residue at Aα-157. This typical (local) conformation is recognized by plasminogen or t-PA and can be regarded as crucial in the rate enhancement of the plasminogen-to-plasmin activation by t-PA.

← **Figure 1** Structures of Xxx in the peptide analogues [Xxx$^{157}$]-Aα-(148-160), listed in order of increasing stimulating capacity

# Chapter 6

## THE ROLE OF $^{152}$Val OF THE FIBRINOGEN Aα-CHAIN IN THE FIBRIN-INDUCED RATE ENHANCEMENT OF THE PLASMINOGEN ACTIVATION BY t-PA

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Submitted for publication

ABSTRACT

Fibrin, but not fibrinogen, enhances the rate of plasminogen activation by t-PA. We have described a site in fibrin(ogen), *i.e.* Aα-(148-160), which can mimic part of the rate enhancement induced by fibrin. During the fibrinogen-to-fibrin conversion, Aα-(148-160) appears to become accessible to proteins, since monoclonal antibodies against synthetic Aα-(148-160) react with fibrin, but not with fibrinogen. In previous publications we have reported on the role of position Aα-157. In this study we investigated the influence of the typical charge distribution in Aα-(148-160), *i.e.*

**Lys-Arg-Leu-Glu-Val-Asp-Ile-Asp-Ile-Lys-Ile-Arg-Ser**

**+ + 0 - 0 - 0 - 0 + 0 + 0**

on the rate-enhancing effect. To disturb the charge distribution in the peptide $^{152}$Val was replaced by charged residues (Arg, Lys, Glu). Also incorporated were some uncharged polar residues (Ser, Tyr), uncharged nonpolar residues (Ala, Nle), and Gly and Pro. The results clearly indicate that, to maintain stimulatory activity, Val (which possesses an uncharged nonpolar side chain) at position Aα-152 may only be exchanged by another uncharged nonpolar residue, *e.g.* Ala or Nle. With tyrosine (with its aromatic, though polar side chain) at position Aα-152 the peptide also retains some rate-enhancing capacity. With the imino acid Pro (of which the side chain is nonpolar in nature) at position Aα-152 the peptide becomes inactive, possibly due to the known structure breaking capacity of proline. Our results indicate that the typical charge distribution in Aα-(148-160) is essential for the stimulatory action.

INTRODUCTION

An activated coagulation system leads to the formation of thrombin from its precursor prothrombin. Thrombin converts the soluble blood protein fibrinogen to insoluble fibrin. Up to a limited concentration fibrin is, however, kept in solution by complexing with fibrinogen. At higher concentrations, fibrin starts to aggregate and forms the insoluble protein matrix of a blood clot. Fibrin has only a temporary function; after it has fulfilled its role, *e.g.* in tissue repair, it will be converted to soluble degradation products, with concomitant lysis of the blood

clot. The degradation of fibrin is catalysed by plasmin, the product of an activated fibrinolytic system. Plasminogen, the precursor of plasmin, is activated by plasminogen activators such as tissue-type plasminogen activator (t-PA) (Rijken *et al.* 1979, Collen 1980). Fibrin is not merely the substrate of fibrinolysis, but is also a cofactor in the fibrinolytic system *i.e.* it enhances the rate of plasmin formation. Both t-PA and plasminogen interact with fibrin, and the accelerating effect of fibrin on the plasminogen activation by t-PA may be explained by these interactions (Nieuwenhuizen 1988). Fibrinogen has been reported to have virtually no rate-enhancing capacity (Wallén 1977, Allan and Pepper 1981, Hoylaerts *et al.* 1982).

Detailed studies (Nieuwenhuizen *et al.* 1983[a], Voskuilen *et al.* 1987, Yonekawa *et al.* 1989, 1990) showed that (part of) this rate-enhancing effect of fibrin is brought about by two sites in the fibrin molecule: one in Aα-(148-160) and one in the cyanogen bromide fragment FCB-5 [*i.e.* γ-(311-336), disulfide linked to γ-(337-379)]. The sequence Aα-(148-160) seems to be buried in fibrinogen, and to become exposed upon the fibrinogen-to-fibrin conversion as evidenced by the reactivity with fibrin (and not fibrinogen) of monoclonal antibodies raised against a synthetic peptide with the sequence Aα-(148-160) (Schielen *et al.* 1989). The rate enhancement of the plasminogen activation by t-PA has been ascribed mainly to carboxyl-terminal lysines (Christensen 1984, Suenson *et al.* 1984, Norrman *et al.* 1985). Voskuilen *et al.* (1987) have shown that $Lys^{157}$ in the Aα-chain plays an essential role in rate-enhancement. This lysine residue, however, is not a carboxyl-terminal residue and, therefore, non-carboxyl-terminal lysine residues may contribute also to the observed rate enhancement by fibrin.

In another study (Schielen *et al.*, Chapter 4), in which we prepared the hexapeptides Aα-(152-157), Aα-(153-158), Aα-(154-159), and Aα-(155-160), we have shown that Aα-(154-159) is capable of stimulating the plasminogen-to-plasmin conversion. Only in the synthetic hexapeptide Aα-(154-159) does the lysine side chain appear to be properly exposed to the environment, since the other $Lys^{157}$ containing hexapeptides [*i.e.* Aα-(152-157), Aα-(153-158) and Aα-(155-160)] do not stimulate.

It is likely that the typical charge distribution in the sequence Aα-(148-160), *i.e.*

```
-Lys-Arg-Leu-Glu-Val-Asp-Ile-Asp-Ile-Lys-Ile-Arg-Ser-
  +   +   0   -   0   -   0   -   0   +   0   +   0
```

where negative and neutral amino acid residues alternate, and are embraced by two sets of positively charged residues, induces (or perhaps could be a part of) a typical local conformation. Only if the local conformation fulfils special requirements, is the $Lys^{157}$ side chain properly exposed to allow acceleration of the plasminogen activation.

In the present study we have investigated the influence of the typical charge distribution and, hence, the assumed (local) conformation in Aα-(148-160) on the rate-enhancing effect. To disturb the charge distribution in the peptide, $^{152}$Val was replaced by charged residues (Arg, Lys, Glu), uncharged polar residues (Ser, Tyr), uncharged nonpolar residues (Ala, Nle), and Gly and Pro were incorporated.

Because we intended to compare some of the synthetic peptides prepared in this study with the same peptides (synthesized in a different manner and extended at their carboxyl-terminus with a glycine residue) from another study (Schielen *et al.*, Chapter 7), the synthesized analogues were carboxyl-terminally elongated with a glycyl residue.

The results of the present investigations clearly indicate that, in order to maintain stimulatory activity, Val (which has an uncharged nonpolar side chain) may only be replaced at position Aα-152 by another uncharged nonpolar residue, *e.g.* Ala or Nle. With tyrosine (with its aromatic, though polar side chain) at position Aα-152 the peptide also retains some rate-enhancing capacity.

Our results indicate that the typical charge distribution in Aα-(148-160) is essential for the stimulatory action.

## MATERIALS AND METHODS

### Tissue-type plasminogen activator

Two-chain t-PA was purified from large scale melanoma cell culture according to Rijken *et al.* (1979), as modified by Kluft *et al.* (1983).

### Plasminogen

Native plasminogen (glu-plasminogen) was purified from fresh human plasma by affinity chromatography (Deutsch and Mertz 1970) on lysine-Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden), precipitated with ammonium sulfate and dialysed extensively against 0.1 M Tris.HCl, pH 7.5, containing 0.1% (vol/vol) Tween 80 (Baker Chemicals).

### Peptides

The peptide analogues [$Xxx^{152}$],[$Gly^{161}$]-Aα-(148-161), where $Xxx^{152}$ = Ala, Arg, Glu, Gly, Lys, Nle, Pro, Ser, Tyr, and Val, were synthesized on *p*-alkoxybenzyl alcohol resin (Wang 1973) using $N^{\alpha}$-Fmoc amino acid derivatives (ten Kortenaar *et al.* 1986) with the aid of a half-automated peptide synthesizer (SP 640, Labortec, Bubendorf, Switzerland).

During solid phase peptide synthesis the amino acid side chains were protected with acid-labile protecting groups: the ε-amino group of lysine with Boc, the δ-guanidino group of arginine with Pmc, the γ-carboxyl group of glutamic acid and the ß-carboxyl group of aspartic acid with $OBu^t$, and the ß-hydroxyl group of serine with $Bu^t$. The carboxyl terminal amino acid [0.9 equivalents (eq.)] was coupled to the resin (1.07 mmol/g) by *in situ* activation with DCC (0.9 eq.) and DMAP (0.9 eq.). To suppress racemization, HOBt (1.8 eq.) was added (van Nispen *et al.* 1985). All reactants were dissolved in DMF. After 16 hours at 5 °C the resin was washed three times with successively each of DMF, dichloromethane, and isopropyl alcohol. Finally, the resin was washed with diisopropyl ether and dried *in vacuo*. The amount of Fmoc-amino acid attached to the resin was determined by elemental analysis of the amount of nitrogen in a sample of the resin. The cleavage of the Fmoc groups was carried out with 20% (vol/vol) piperidine in DMF, during three consecutive cycles of 6 minutes. Coupling of the second amino acid derivative (3 eq. relative to the amount of first amino acid attached to the resin) was performed by *in situ* activation with TBTU

(3 eq.) (Knorr *et al.* 1989), HOBt (3 eq.) and N-methylmorfoline (4.5 eq.). After coupling of each amino acid derivative, completion of the acylation reaction was monitored by a ninhydrin test on free amino groups (Kaiser *et al.* 1970), followed by acylation of any remaining free amino groups with 10% (vol/vol) acetic acid anhydride in DMF. The fully protected peptides were cleaved from the resin during a 4 h reaction with 2.5% (vol/vol) ethanedithiol and 2.5% (vol/vol) water in trifluoroacetic acid followed by precipitation in diethyl ether. The crude peptides were washed several times with diethyl ether, dried at the air, and analyzed by HPLC, specific rotation and amino acid composition.

HPLC analyses

HPLC analyses were performed on a RP-18 column (Techsil 5 C18, 250 x 4.6 mm, HPLC Technology, Cheshire, UK) at a flow-rate of 1.0 ml/min., using a 5 min. isocratic elution with 7% (vol/vol) acetonitrile and 0.1% (vol/vol) trifluoroacetic acid in water, followed by a 30 min. linear gradient from 7% (vol/vol) acetonitrile and 0.1% (vol/vol) trifluoroacetic acid in water to 70% (vol/vol) acetonitrile and 0.08% (vol/vol) trifluoroacetic acid in water. Peaks were detected by UV measurement at 215 nm. The UV data were analyzed using JCL 6000 Chromatography Data System software (Jones Chromatography, Mid Glan, UK).

Amino acid analyses

Peptides were hydrolysed in 5.7 M HCl (Merck Suprapur) in evacuated sealed glass tubes for 24 hours at 120 °C. The hydrolysates were lyophilized and analyzed with a Varian 9095 amino acid analyzer using the Fmoc-protocol.

Specific rotations

The specific rotations were measured with a Perkin Elmer Polarimeter 241. All peptides were dissolved in DMF and centrifuged for 10 minutes at 4500 rpm prior to the measurement.

Assay system for the assessment of the rate-enhancing properties of the peptides

In total volumes of 0.250 ml 0.1 M Tris.HCl, containing 0.1% (vol/vol) Tween 80 were present 0.11 $\mu$M glu-plasminogen, 0.3 mM H-D-Val-Leu-Lys-*p*-nitroanilide,

## TABLE I

Amino acid compositions of the synthetic peptides prepared in this study. Expected values between parentheses.

| | mol/mol peptide | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| [Xxx$^{152}$],[Gly$^{161}$]-Ac-(148-161) Xxx= | Ala | Arg | Asp | Glu | Gly | Ile | Leu | Lys | Nle | Pro | Ser[a] | Tyr | Val |
| Ala | 0.99 (1) | 2.11 (2) | 2.07 (2) | 1.00 (1) | 0.99 (1) | 3.14 (3) | 0.95 (1) | 2.08 (2) | --- | --- | 0.71 (1) | --- | --- |
| Arg | --- | 3.11 (3) | 2.11 (2) | 1.00 (1) | 1.07 (1) | 3.15 (3) | 0.92 (1) | 2.05 (2) | --- | --- | 0.77 (1) | --- | --- |
| Glu | --- | 2.03 (2) | 1.98 (2) | 2.01 (2) | 1.00 (1) | 3.01 (3) | 1.05 (1) | 1.97 (2) | --- | --- | 0.88 (1) | --- | --- |
| Gly | --- | 2.00 (2) | 2.08 (2) | 1.02 (1) | 1.97 (2) | 3.00 (3) | 1.02 (1) | 1.97 (2) | --- | --- | 0.87 (1) | --- | --- |
| Lys | --- | 2.13 (2) | 2.08 (2) | 0.98 (1) | 0.96 (1) | 3.00 (3) | 1.00 (1) | 2.89 (3) | --- | --- | 0.93 (1) | --- | --- |
| Nle | --- | 1.92 (2) | 1.94 (2) | 1.00 (1) | 1.01 (1) | 3.20 (3) | 1.02 (1) | 1.93 (2) | 1.03 (1) | --- | 0.69 (1) | --- | --- |
| Pro | --- | 1.99 (1) | 2.02 (2) | 0.94 (1) | 1.00 (1) | 3.02 (3) | 1.03 (1) | 2.00 (2) | --- | 0.96 (1) | 0.73 (1) | --- | --- |
| Ser | --- | 2.07 (2) | 2.00 (2) | 1.03 (1) | 1.00 (1) | 3.13 (3) | 1.02 (1) | 2.03 (2) | --- | --- | 1.69 (2) | --- | --- |
| Tyr | --- | 2.04 (2) | 2.00 (2) | 1.01 (1) | 1.00 (1) | 2.99 (3) | 1.04 (1) | 2.03 (2) | --- | --- | 0.84 (1) | 1.01 (1) | --- |
| Val | --- | 2.01 (2) | 1.96 (2) | 1.00 (1) | 1.07 (1) | 3.06 (3) | 1.03 (1) | 2.03 (2) | --- | --- | 0.79 (1) | --- | 1.01 (1) |

[a]The value for Ser has not been corrected for losses during hydrolysis

300 mIU t-PA, and varying concentrations of the synthetic peptide to be tested for stimulating activity. In this assay, the concentration of *p*-nitroaniline formed per time-squared is proportional to the rate of plasmin formation (activation rate) (Drapier *et al.* 1979). The ratio of the activation rate in the presence of the peptide to that in the absence of peptide was designated as stimulation factor.

## RESULTS

Table I shows that the amino acid compositions were as expected.
Table II summarizes the HPLC data and specific rotations obtained for each of the crude peptides. As indicated by the data, the purity of the peptides was such that no purification of any of the synthetic peptides was required.
Table III summarizes the stimulation factors found for the individual peptides. The concentrations needed for half-maximal stimulation were around 50 $\mu$M.

## DISCUSSION

Fibrin is known to accelerate the plasminogen-to-plasmin conversion by t-PA. This acceleration can not be induced by fibrinogen (Wallén 1977), or only to a limited extent. Verheijen *et al.* (1982[a]) have demonstrated that fragments derived from fibrin or fibrinogen by digestion with plasmin, or, chemically, with cyanogen bromide, also enhance plasminogen activation. It was concluded, that stimulatory sites pre-exist in fibrinogen and become exposed upon the fibrinogen-to-fibrin conversion, or fragmentation by plasmin and cyanogen bromide.

Two of these stimulatory sites have been so far identified. One site lies within the sequence A$\alpha$-(148-160) (Nieuwenhuizen *et al.* 1983[a], Voskuilen *et al.* 1987) and the other within a fibrin(ogen) fragment known as cyanogen bromide fragment FCB-5 [*i.e.* $\gamma$-(311-336), disulfide linked to $\gamma$-(337-379)] (Yonekawa *et al.* 1989, 1990).

The sequence A$\alpha$-(148-160) seems to be buried in fibrinogen, and to become exposed upon the fibrinogen-to-fibrin conversion as evidenced by the reactivity with fibrin (and not fibrinogen) of monoclonal antibodies raised against a synthetic peptide with the sequence A$\alpha$-(148-160) (Schielen *et al.* 1989). Also, at

TABLE II

Reversed phase HPLC data and $[\alpha]_D$-values of the peptides used in this study

| [Xxx$^{152}$],[Gly$^{161}$]-Aα-(148-160) Xxx= | ret.time (mins) | % main peak | $[\alpha]_D$ |
|---|---|---|---|
| Ala | 20.81 | 85.9 | - 8.4 (c=0.56, DMF) |
| Arg | 19.56 | 94.4 | -10.8 (c=0.38, DMF) |
| Glu | 20.28 | 84.5 | -10.7 (c=0.28, DMF) |
| Gly | 19.42 | 88.4 | - 9.2 (c=0.26, DMF) |
| Lys | 19.41 | 86.2 | -10.7 (c=0.59, DMF) |
| Nle | 20.57 | 89.8 | -9.8 (c=0.54, DMF) |
| Pro | 20.37 | 77.1 | -30.3 (c=0.31, DMF) |
| Ser | 20.07 | 85.6 | - 11.9 (c=0.57, DMF) |
| Tyr | 20.98 | 81.3 | - 11.4 (c=0.58, DMF) |
| Val | 22.06 | 78.1 | - 10.4 (c=0.52, DMF) |

least part of the FCB-5 fragment [*i.e.* γ-(312-324)] seems to be buried in fibrinogen, and becomes exposed by the fibrinogen-to-fibrin conversion, since monoclonal antibodies raised against synthetic γ-(312-324) are fibrin-specific (Schielen *et al.*, Chapter 3).

In another study (Schielen *et al.*, Chapter 4), in which the amino-terminally acylated hexapeptides Aα-(152-157), Aα-(153-158), Aα-(154-159), and Aα-(155-160) were prepared, it was shown that only the hexapeptide Aα-(154-159) is capable of stimulating the plasminogen-to-plasmin conversion. As indicated in

TABLE III

Stimulation factors of the synthetic peptides, obtained with the assay system described in the Materials and Methods section

| peptide | stimulation factor |
|---|---|
| [Ala$^{152}$],[Gly$^{161}$]-Aα-(148-161) | 4.9 ± 0.1 |
| [Arg$^{152}$],[Gly$^{161}$]-Aα-(148-161) | 1.9 ± 0.5 |
| [Glu$^{152}$],[Gly$^{161}$]-Aα-(148-161) | 2.2 ± 0.7 |
| [Gly$^{152}$],[Gly$^{161}$]-Aα-(148-161) | 2.4 ± 0.9 |
| [Lys$^{152}$],[Gly$^{161}$]-Aα-(148-161) | 2.3 ± 0.8 |
| [Nle$^{152}$],[Gly$^{161}$]-Aα-(148-161) | 9.0 ± 1.0 |
| [Pro$^{152}$],[Gly$^{161}$]-Aα-(148-161) | 2.8 ± 0.7 |
| [Ser$^{152}$],[Gly$^{161}$]-Aα-(148-161) | 3.7 ± 1.2 |
| [Tyr$^{152}$],[Gly$^{161}$]-Aα-(148-161) | 4.3 ± 0.8 |
| [Val$^{152}$],[Gly$^{161}$]-Aα-(148-161) | 8.3 ± 2.3 |

the introduction, the general belief is that lysine residues are important in the plasminogen-to-plasmin conversion by t-PA. All of the aforementioned hexapeptides contain a lysine residue. The fact that only Aα-(154-159) enhances the plasminogen-to-plasmin conversion led to the assumption that the lysine residue at position Aα-157 has to be exposed in a proper manner to the milieu. We hypothesized that the stretch Aα-(148-160) is involved in inducing (or perhaps is part of) a typical (local) conformation that exists in fibrinogen in a latent form, and is exposed in fibrin. The sequence Aα-(154-159) probably contains the minimum information for this (local) conformation to be induced.

In yet an other study (Schielen *et al.*, Chapter 5) we have shown that the lysine residue at position Aα-157 *per se* is not important, since this lysine residue can be replaced by several other amino acid residues, such as Gly or Ala, virtually without loss of stimulating capacity. These findings support the hypothesis of a certain typical (local) conformation, in which not the lysyl residue at Aα-157 *per se* is important, but rather the degree in which the assumed rate-enhancing conformation is maintained (stabilized) by the residue at position Aα-157. The proposed typical (local) conformation can probably be stabilized by the typical charge distribution in the sequence Aα-(148-160), *i.e.*

```
Lys-Arg-Leu-Glu-Val-Asp-Ile-Asp-Ile-Lys-Ile-Arg-Ser
 +   +   0   -   0   -   0   -   0   +   0   +   0
```

where negative and neutral amino acid residues alternate, and are embraced by two sets of positively charged residues.

The aim of the present study was to disrupt this typical charge pattern, by replacing valine at position Aα-152 by various charged and uncharged amino acid residues with different polarities.

From the results (table III) it is clear that introduction of charged residues such as Arg, Glu and Lys at position Aα-152, or a known structure-breaking residue such as the imino acid Pro at position Aα-152, probably disturbs the hypothesized conformation such that the resulting peptides show virtually no rate-enhancing properties. Introduction of uncharged polar residues such as Ser and Tyr at position Aα-152 yields peptides with some stimulatory capacity, although the stimulation by the peptide with Ser at position Aα-152 is hardly significant. The only residues that can replace Val at position Aα-152, without loss of the rate enhancement of the peptides, are Nle and Ala. We conclude that the apolar $Val^{152}$ can only be replaced by another apolar residue (Nle or Ala). From table III it is also clear that the peptide with Nle (which is known to be as apolar as Val) at position Aα-152, is as equally active as the native peptide.

In conclusion, we have shown that the typical charge distribution in Aα-(148-160) can not be disturbed by the introduction of charged (such as Arg, Glu, or

Lys), uncharged polar (such as Ser), or known structure-breaking (as Pro) residues at position Aα-152 without loss of rate enhancement.

This typical charge distribution probably underlies a certain (local) conformation that is essential for the rate-enhancing properties of fibrin in the plasminogen activation by t-PA.

# Chapter 7

## THE EFFICIENT ACYLATION OF AN N-TERMINALLY LOCATED ASPARTYL RESIDUE; CONTRIBUTION TO THE DEVELOPMENT OF A NEW STRATEGY FOR FRAGMENT CONDENSATIONS

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ABSTRACT

The full solid phase synthesis of peptides longer than ten amino acid residues usually yields a preparation contaminated with peptides with an aberrant sequence. A way to avoid the full solid phase synthesis of such longe sequences is to first synthesize short protected peptide fragments on a solid support. Isolation of protected peptides requires a resin which binds the peptide chain through a very acid labile linker. The obtained peptide derivatives can then be converted in solution to activated compounds, which can directly be condensed to give the protected form of the target peptide. Our results show that condensations of peptide azides to a ß-unprotected amino-terminal aspartyl peptide derivative is quite generally applicable and proceeds in high yields. The peptides, analogues of [Gly$^{161}$]-fibrinogen-A$\alpha$-(148-161), prepared following this condensation strategy, appeared to be significantly purer on HPLC than those prepared entirely via solid phase peptide synthesis.

INTRODUCTION

The concept of solid phase peptide synthesis (SPPS), developed by Merrifield (1963), underwent many methodical improvements and can be considered now to be the method of choice for synthesizing peptides and small proteins in a fast and convenient manner. The most successful and extensively explored method employs the base labile Fmoc-group (Carpino and Han 1970, 1972) for temporary N$^{\alpha}$-amino protection (Atherton *et al.* 1978[a,b], Chang and Meienhofer 1978). A comprehensive review about SPPS utilizing Fmoc-amino acids has been given by Fields and Noble (1990).

The synthesis of peptides on a solid support is subject to several peculiarities arising from the rigid (polystyrene) matrix. The degree of permeation of protected amino acids into the matrix affects the coupling rate and, hence, the coupling efficiency. The density of loading of the first amino acid residue (the carboxyl-terminal) on the solid support may cause spatial problems during the progression of the synthesis and yield truncated sequences.

Especially the last step in a solid phase synthesis is critical: during the simultaneous cleavage/deprotection procedure in acidic media, high local

concentrations of carbonium ions in the restricted space within the solid matrix can be considered as the cause of considerable damage to the peptide.

During the synthesis of fibrinogen-Aα-(148-160) (*i.e.* H-Lys-Arg-Leu-Glu-Val-Asp-Ile-Asp-Ile-Lys-Ile-Arg-Ser-OH) and many analogues thereof we encountered some of the aforementioned problems. Loadings of Fmoc-Ser($Bu^t$) on a *p*-alkoxybenzyl alcohol resin (Wang 1973) amounting to 0.5 mmol and higher per gram resin caused very low coupling efficiencies around both arginyl residues. Loadings of 0.2-0.3 mmol Fmoc-Ser($Bu^t$) per gram resin caused low coupling efficiencies around the 11$^{th}$ amino acid derivative, $Arg^{149}$, during the syntheses of analogues of fibrinogen-Aα-(148-160), *i.e.*, [$Nle^{157}$]- and [$Val^{157}$]-fibrinogen-Aα-(148-160) and [$Xxx^{152}$],[$Gly^{161}$]-fibrinogen-Aα-(148-161) where Xxx=Lys, Arg, Glu, Pro, and Ser. Most of the difficulties with regard to low coupling efficiencies in the area of $Arg^{149}$ could be solved by repetition of the cycle, adding dichloromethane (up to 50%) to the reaction mixture, and/or by gentle heating (40 °C) of the reaction mixture. The introduction of Mpc-Arg(Pmc)-OH at position Aα-149 in the synthesis of [$Nle^{157}$]-fibrinogen-Aα-(148-160) instead of Fmoc-Arg(Pmc)-OH gave a coupling efficiency of almost 100% within 30 min. at ambient temperature (Schielen *et al.*, Chapter 8). Generally, the coupling efficiencies improve by utilising the relatively slim Mpc-group for the $N^{\alpha}$-amino protection of amino acids and amino acid derivatives.

A relatively new approach to the construction of longer peptides is to synthesize first small protected peptides (up to 9 residues) starting from a solid support bearing a very acid labile linker (such as the SASRIN resin, *i.e.* polystyrene functionalyzed with 2-methoxy-4-alkoxybenzyl alcohol, Mergler 1988[a,b]). This approach offers rapid access to fully protected peptide fragments that can subsequently be coupled in solution to larger fragments. The final deprotection of the target peptide can then be performed in a homogeneous solution, thus shortening the contact time between the carbonium ions and the peptide and increasing the chance of an effective encounter between the liberated carbonium ions and the scavengers present in the deprotection medium.

Ten Kortenaar *et al.* described in 1985 a highly efficient procedure in which the condensation of a peptide azide and a fragment bearing H-Asp(OH)- as the amino terminal proceeded without any detectable by-products. They assumed that the coupling proceeds via an intramolecular reaction of the free amino terminus and the mixed anhydride formed at the β-carboxyl group of the aspartyl residue. Such a mixed anhydride seems to be easily formed (Bodanszky and Natarjan 1975, Natarjan and Bodanszky 1976), since the reaction of a peptide azide (carboxyl component) with a peptide derivative of which the β-unprotected aspartyl residue is not located amino-terminally (amino component), yields a reaction product in which the ratio amino-component/carboxyl-component is roughly 1/2 (ten Kortenaar *et al.* 1985).

At present we studied the applicability of this reaction to some fragment condensations in the syntheses of peptide analogues of the type [Xxx$^{152}$],[Gly$^{161}$]-fibrinogen-Aα-(148-161) (*i.e.* Lys-Arg-Leu$^{150}$-Glu-Xxx$^{152}$-Asp-Ile-Asp-Ile-Lys-Ile-Arg-Ser$^{160}$-Gly). For Xxx, the carboxyl-terminal residue of the peptide azide, we have chosen Arg, Glu, Gly, Lys, Pro, Ser, Phe, and Val.

Most of these peptides have also been synthesized completely by SPPS (Schielen *et al.*, Chapter 6). Thus, the peptides obtained via the present procedure for fragment condensation can be compared with peptides that were prepared fully stepwise on a solid support.

The results indicate that the reaction of most peptide azides (*i.e.* [Xxx$^{152}$]-fibrinogen-Aα-(148-152)-azide) with the fragment whose amino-terminal residue is H-Asp(OH)- (*i.e.* [Gly$^{161}$]-fibrinogen-Aα-(153-161)) proceeds in high yields. The peptide azides in which the carboxyl-terminus was Ser(Bu$^t$) or Phe did not couple at all. During the solid phase syntheses of the constituent peptide fragments to be condensed no incomplete couplings occurred and the purity of the target peptides was significantly higher compared to the same peptides synthesized fully stepwise.

MATERIALS AND METHODS

Amino acid analyses

Peptides were hydrolysed in 5.7 M HCl (Merck Suprapur) in evacuated sealed glass tubes for 24 hours at 120 °C. The hydrolysates were lyophilized and analyzed with a Varian 9095 amino acid analyzer using the Fmoc-protocol.

HPLC analyses

HPLC analyses of fully deprotected peptides were performed on a RP-18 column (Techsil 5 C18, 250 x 4.6 mm, HPLC Technology, Cheshire, UK) at a flow-rate of 1.0 ml/min., using a 5 min. isocratic elution with 7% (vol/vol) acetonitrile and 0.1% (vol/vol) trifluoroacetic acid in water followed by a 30 min. linear gradient from 7% (vol/vol) acetonitrile and 0.1% (vol/vol) trifluoroacetic acid in water to 70% (vol/vol) acetonitrile and 0.08% (vol/vol) trifluoroacetic acid in water. Peaks were detected by UV measurement at 215 nm. The UV data were analyzed using JCL 6000 Chromatography Data System software (Jones Chromatography, Mid Glan, UK).

Melting points were determined with a Büchi melting point apparatus and are uncorrected.

Specific rotations

The specific rotations were measured with a Perkin Elmer Polarimeter 241.

Thin-layer chromatography (t.l.c.) was performed on precoated t.l.c. plates (Merck, silica gel 60, F-254, 0.2 mm). Solvent systems used were: $CHCl_3$/MeOH/AcOH (95/20/3) (system A), $CHCl_3$/MeOH (4/1) (system B), and *n*-butanol/acetic acid/water (4/1/1) (system C). Spots were visualized using one or more of the following methods: UV-fluorescence quenching 254 nm, Barton's reagent spray (a mixture of equal volumes of $FeCl_3$ (30 g) in water (170 ml) and $K_3Fe(CN)_6$ (2 g) in water 200 ml), ninhydrin spray (240 mg in 400 ml n-butanol and 16 ml acetic acid).

Assay system for the assessment of the rate-enhancing properties of the peptides

In total volumes of 0.250 ml 0.1 M Tris.HCl, containing 0.1% (vol/vol) Tween 80 were present 0.11 $\mu$M glu-plasminogen, 0.3 mM H-D-Val-Leu-Lys-*p*-nitroanilide, 300 mIU t-PA, and varying concentrations of the synthetic peptide to be tested

for stimulating activity. In this assay, the concentration of *p*-nitroaniline formed per time-squared is proportional to the rate of plasmin formation (activation rate) (Drapier *et al.* 1979). The ratio of the activation rate in the presence of the peptide over that in the absence of peptide was designated as stimulation factor.

Peptides

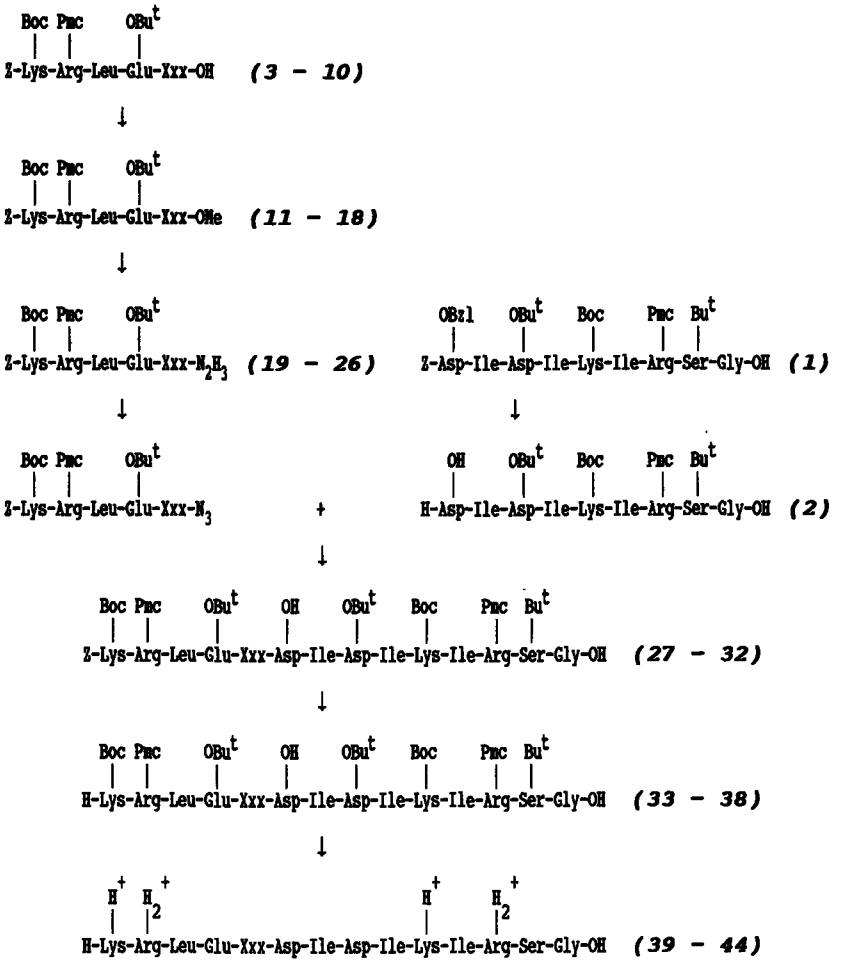
All peptide fragments to be condensed were synthesized using $N^{\alpha}$-Fmoc amino acid derivatives (ten Kortenaar *et al.* 1986) and with the aid of a half-automated peptide synthesizer (SP 640, Labortec, Bubendorf, Switzerland).

During solid phase peptide synthesis the amino acid side chains were protected with acid-labile protecting groups: the $\epsilon$-amino group of lysine with Boc, the $\delta$-guanidino group of arginine with Pmc, the $\gamma$-carboxyl group of glutamic acid with $OBu^t$, the ß-carboxyl group of aspartic acid with $OBu^t$ or OBzl, and the ß-hydroxyl group of serine with $Bu^t$. The cleavage of the Fmoc groups was carried out with 20% (vol/vol) piperidine in DMF, during three consecutive cycles of 6 minutes. Coupling of an amino acid derivative (3 eq. relative to the amount of first amino acid attached to the resin) was performed by *in situ* activation with TBTU (3 eq.) (Knorr *et al.* 1989), HOBt (3 eq.) and NMM (4.5 eq.). Following the performance of a coupling cycle, completion of the acylation reaction was monitored with ninhydrin to detect free amino groups (Kaiser *et al.* 1970). If the reaction indicated approximate completeness of the acylation the cyclus was terminated by acetylation with 10% (vol/vol) acetic acid anhydride in DMF.

Some physicochemical characteristics of the peptide fragments to be condensed are given in tables 1 and 2.

*Peptide derivative 1* (scheme 1) was synthesized on SASRIN resin, starting from Fmoc-Gly-SASRIN (0.65 mmol Fmoc-Gly/g resin, BACHEM, Bubendorf, Switzerland). The fully protected nonapeptide 1 was cleaved from the resin according to Mergler *et al.* (1988[b]), concentrated *in vacuo,* and precipitated from water.

*Peptide derivative 2* (650 mg, 0.375 mmole) was prepared by hydrogenolysis of compound 1 in 20 ml acetic acid in the presence of 10% palladium on charcoal. The catalyst was removed by filtration and the filtrate was evaporated to dryness *in vacuo* to give TLC-homogeneous 2 in quantitative yield.




| | | | |
|---|---|---|---|
| 3, 11, 19, 27, 33: | Xxx = Arg(Pmc) | 39: | Xxx = Arg($H_2^+$) |
| 4, 12, 20, 28, 34: | Glu(OBu$^t$) | 40: | Glu |
| 5, 13, 21, 29, 35, 41: | Gly | | |
| 6, 14, 22, 30, 36: | Lys(Boc) | 42: | Lys($H^+$) |
| 7, 15, 23: | Phe | | |
| 8, 16, 24, 31, 37, 43: | Pro | | |
| 9, 17, 25: | Ser(Bu$^t$) | | |
| 10, 18, 26, 32, 38, 44: | Val | | |

**Scheme 1** Schematic representation of the compounds synthesized in this study

*Peptide derivatives **3, 4, 5, 6, and 9*** (scheme 1) were synthesized on SASRIN resins, starting from Fmoc-Arg(Pmc)-SASRIN (0.55 mmol/g), Fmoc-Glu(OBu$^t$)-SASRIN (0.49), Fmoc-Gly-SASRIN (0.65 mmol/g), Fmoc-Lys(Boc)-SASRIN (0.65 mmol/g), and Fmoc-Ser(Bu$^t$)-SASRIN (0.69 mmol/g), respectively. The fully protected pentapeptides were cleaved from the resin and worked up as described for compound **1**.

*Peptide derivatives **7, 8, and 10*** (scheme 1) were synthesized on PepSyn KH resins, starting from Fmoc-Phe-, Fmoc-Pro-, or Fmoc-Val-PepSyn KH (0.1 mmol/g resin, MILLIGEN, Etten-Leur, the Netherlands). The fully protected pentapeptides were cleaved from the resin and worked up as described for compound **1**.

*Peptide derivatives **11 - 18***

Each peptide derivative **3 - 10** (0.1 mmol, 100-150 mg) was dissolved in acetone (10 ml). After cooling on melting ice, a solution of diazomethane in diethylether (0.3 mmol/ml) was added slowly until the colour of the reaction mixture turned yellow. The excess diazomethane was dispelled by purging a gentle stream of nitrogen through the solution. When the conversion was complete, the solution was concentrated *in vacuo* and the residue immediately used in the next reaction step.

*Peptide derivatives **19 - 26***

Each peptide ester **11 - 18** was dissolved in 2 ml DMF and $N_2H_4.H_2O$ (0.6 mmole, 30 $\mu$l) was added to the solution. After 24 hours the hydrazide was precipitated by the addition of 5 ml water. Filtration and washing with water yielded the pure compounds, reacting without exception with the reagent of Barton for hydrazides.

*Peptide derivatives **27 - 32***

Each peptide hydrazide **19 - 26** (0.05 mmol, 50-75 mg) was dissolved in 2 ml DMF and cooled to -15 °C. The cold solution was treated with 1.6 N HCl in ethyl acetate (0.15 mmol, 94 $\mu$l) and *tert*-butyl nitrite (0.06 mmol, 7.2 $\mu$l). After stirring for 20 minutes at -15 °C the reaction mixture (Barton negative on TLC) was neutralized (pH 7.5) by the addition of diisopropylethylamine (0.15 mmol, 26 $\mu$l) and added to a solution of **2** (0.05 mmol, 75 mg) in DMF (2 ml). After 3 days at 4 °C the product was precipitated with water (10 ml), filtered, washed with water

TABLE 1

Yields, melting points, $R_f$-, and $[\alpha]_D$-values of the compounds synthesized in this study.

| Compound | Yield (%) | m.p. (°C) | $R_f$ A | $R_f$ B | $R_f$ C | $[\alpha]_D$ (°) | c in DMF |
|---|---|---|---|---|---|---|---|
| ***1*** | 74 | dec. | 0.49 | 0.18 | 0.79 | -11.0 | 0.30 |
| ***2*** | 100 | dec. | 0 | 0 | 0.17 | – | – |
| ***3*** | 76 | 90-92 | 0.56 | 0.25 | 0.83 | - 8.3 | 0.48 |
| ***4*** | 75 | 114-117 | 0.57 | 0.12 | 0.86 | -13.9 | 0.38 |
| ***5*** | 72 | 100-102 | 0.40 | 0.12 | 0.77 | -11.7 | 1.12 |
| ***6*** | 74 | 102-104 | 0.56 | 0.25 | 0.93 | -11.9 | 0.32 |
| ***7*** | 58 | 114-117 | 0.57 | 0.28 | 0.85 | -11.2 | 0.34 |
| ***8*** | 50 | 114-116 | 0.58 | 0.29 | 0.86 | -11.8 | 0.14 |
| ***9*** | 80 | 98-100 | 0.58 | 0.29 | 0.86 | - 6.7 | 0.30 |
| ***10*** | 38 | 128-130 | 0.54 | 0.22 | 0.93 | - 8.3 | 0.06 |
| ***11*** | – | – | 0.75 | 0.77 | 0.88 | – | – |
| ***12*** | – | – | 0.72 | 0.77 | 0.88 | – | – |
| ***13*** | – | – | 0.65 | 0.71 | 0.84 | – | – |
| ***14*** | – | – | 0.74 | 0.76 | 0.88 | – | – |
| ***15*** | – | – | 0.80 | 0.81 | 0.88 | – | – |
| ***16*** | – | – | 0.73 | 0.79 | 0.88 | – | – |
| ***17*** | – | – | 0.78 | 0.78 | 0.88 | – | – |
| ***18*** | – | – | 0.69 | 0.77 | 0.88 | – | – |
| ***19*** | 82 | 87-89 | 0.61 | 0.71 | 0.93 | -11.0 | 0.20 |
| ***20*** | 90 | 96-99 | 0.62 | 0.71 | 0.91 | -12.3 | 0.30 |
| ***21*** | 80 | 90-92 | 0.52 | 0.63 | 0.88 | -12.8 | 0.60 |
| ***22*** | 76 | 94-96 | 0.62 | 0.69 | 0.90 | -24.4 | 0.09 |
| ***23*** | 70 | 88-90 | 0.65 | 0.72 | 0.90 | -10.0 | 0.06 |
| ***24*** | 75 | 100-102 | 0.57 | 0.69 | 0.92 | -14.5 | 0.06 |
| ***25*** | 80 | 94-96 | 0.63 | 0.70 | 0.93 | - 9.0 | 0.48 |
| ***26*** | 80 | 100-102 | 0.61 | 0.70 | 0.91 | -13.3 | 0.06 |
| ***27*** | 88 | dec. | 0.42 | – | 0.69 | – | – |
| ***28*** | 90 | dec. | 0.43 | – | 0.67 | – | – |
| ***29*** | 90 | dec. | 0.37 | – | 0.61 | – | – |
| ***30*** | 92 | dec. | 0.42 | – | 0.69 | – | – |
| ***31*** | 87 | dec. | 0.41 | – | 0.68 | – | – |
| ***32*** | 87 | dec. | 0.43 | – | 0.68 | – | – |
| ***33*** | 100 | dec. | 0.07 | – | 0.48 | – | – |
| ***34*** | 100 | dec. | 0.08 | – | 0.47 | – | – |
| ***35*** | 100 | dec. | 0.04 | – | 0.44 | – | – |
| ***36*** | 100 | dec. | 0.07 | – | 0.49 | – | – |
| ***37*** | 100 | dec. | 0.07 | – | 0.48 | – | – |
| ***38*** | 100 | dec. | 0.07 | – | 0.47 | – | – |
| ***39*** | 93 | dec. | – | – | – | -10.0 | 0.38 |
| ***40*** | 90 | dec. | – | – | – | -12.3 | 0.30 |
| ***41*** | 94 | dec. | – | – | – | -10.7 | 0.27 |
| ***42*** | 94 | dec. | – | – | – | -14.4 | 0.55 |
| ***43*** | 92 | dec. | – | – | – | -33.4 | 0.29 |
| ***44*** | 90 | dec. | – | – | – | -11.0 | 0.50 |

dec.: decomposition; solvent systems A, B, and C in Materials and Methods section

and dried. The reaction products of peptide hydrazides **23** and **25** did not precipitate in water but formed an oil that turned out to be a (Barton negative on TLC) mixture of the reactants: no reaction product (expected around $R_f$ 0.40 in system A or $R_f$ 0.70 in system C) could be detected.

*Peptide derivatives **33** - **38***

The Z-group of each of the derivatives **27** - **32** was quantitatively removed by hydrogenolysis in acetic acid in the presence of 10% palladium on charcoal. The catalyst was removed by filtration and the filtrate was lyophilized.

*Peptides **39** - **44***

Each of the derivatives **33** - **38** was dissolved in 2 ml of a mixture of TFA, ethanedithiol and water (95/2.5/2.5). After 4 hours at ambient temperature the peptide was precipitated in diethyl ether, washed several times with diethyl ether, dried at the air, and analyzed by HPLC, measurement of the specific rotation and amino acid composition (tables 1, 2, and 3).

## RESULTS AND DISCUSSION

Table 2 shows that the amino acid compositions are as expected. Table 3 summarizes the HPLC data and specific rotations for each of the peptides obtained via fragment condensations.

The overall yields of the tetradecapeptides obtained via fragment condensations are comparable to those obtained by sequential SPPS. More important is that the purity of the tetradecapeptides prepared in this study is significantly higher compared to the peptides that were synthesized sequentially on Wang's resin (shown in italics in table 3), notwithstanding the fact that none of the intermediate peptide derivatives had been recrystallized. Generally, most of the difficulties, with respect to the coupling efficiency, arising in a sequential SPPS from acylation cycle 10 until 15, can be avoided by the present procedure of fragment condensation. In this approach none of the peptide derivatives is larger than 9 residues.

Another advantage is that the deprotection of the target peptides is carried out in solution, allowing optimal mixing of the reactants.

TABLE 2

Amino acid compositions of the peptide derivatives prepared in this study. Expected values between parentheses.

| Compound | mol/mol peptide | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Arg | Asp | Glu | Gly | Ile | Leu | Lys | Pro | Ser* | Val |
| ***27*** | 3.06 (3) | 1.98 (2) | 1.02 (1) | 1.01 (1) | 3.03 (3) | 0.98 (1) | 1.00 (1) | — | 0.76 (1) | — |
| ***28*** | 2.02 (2) | 1.96 (2) | 2.00 (2) | 1.03 (1) | 3.17 (3) | 0.99 (1) | 2.02 (2) | — | 0.68 (1) | — |
| ***29*** | 1.98 (2) | 2.06 (2) | 0.96 (1) | 2.00 (2) | 2.87 (3) | 1.03 (1) | 2.06 (2) | — | 0.77 (1) | — |
| ***30*** | 2.07 (2) | 1.98 (2) | 1.00 (1) | 1.08 (1) | 3.09 (3) | 0.99 (1) | 2.90 (3) | — | 0.73 (1) | — |
| ***31*** | 2.13 (2) | 2.06 (2) | 0.98 (1) | 1.00 (1) | 3.03 (3) | 0.97 (1) | 1.99 (2) | 0.98 (1) | 0.79 (1) | — |
| ***32*** | 1.89 (2) | 2.00 (2) | 1.02 (1) | 0.98 (1) | 2.97 (3) | 1.00 (1) | 2.09 (2) | — | 0.81 (1) | 1.03 (1) |
| ***33*** | 3.01 (3) | 1.89 (2) | 1.01 (1) | 0.98 (1) | 3.11 (3) | 0.97 (1) | 2.00 (2) | — | 0.76 (1) | — |
| ***34*** | 1.96 (2) | 2.08 (2) | 2.09 (2) | 0.96 (1) | 3.00 (3) | 0.97 (1) | 2.13 (2) | — | 0.70 (1) | — |
| ***35*** | 2.00 (2) | 1.96 (2) | 1.01 (1) | 2.11 (2) | 3.18 (3) | 1.05 (1) | 2.03 (2) | — | 0.76 (1) | — |
| ***36*** | 2.14 (2) | 1.98 (2) | 1.00 (1) | 1.04 (1) | 3.15 (3) | 1.01 (1) | 2.86 (3) | — | 0.81 (1) | — |
| ***37*** | 2.00 (2) | 2.01 (2) | 1.08 (1) | 0.99 (1) | 2.88 (2) | 0.97 (1) | 2.01 (2) | 1.04 (1) | 0.75 (1) | — |
| ***38*** | 1.89 (2) | 2.06 (2) | 1.00 (1) | 1.02 (1) | 3.08 (3) | 0.93 (1) | 1.96 (2) | — | 0.76 (1) | 1.03 (1) |
| ***39*** | 2.98 (3) | 2.01 (2) | 0.99 (1) | 1.01 (1) | 2.99 (3) | 1.00 (1) | 2.00 (2) | — | 0.82 (1) | — |
| ***40*** | 2.00 (2) | 2.02 (2) | 2.00 (2) | 1.02 (1) | 3.05 (3) | 1.03 (1) | 2.00 (2) | — | 0.74 (1) | — |
| ***41*** | 2.02 (2) | 1.98 (2) | 1.00 (1) | 2.00 (2) | 3.06 (3) | 0.98 (1) | 2.01 (2) | — | 0.84 (1) | — |
| ***42*** | 2.01 (2) | 1.96 (2) | 1.00 (1) | 1.03 (1) | 3.06 (3) | 1.00 (1) | 3.03 (3) | — | 0.82 (1) | — |
| ***43*** | 2.00 (2) | 2.02 (2) | 1.02 (1) | 1.00 (1) | 2.97 (3) | 1.01 (1) | 2.02 (2) | 1.00 (1) | 0.80 (1) | — |
| ***44*** | 2.02 (2) | 2.00 (2) | 1.00 (1) | 0.99 (1) | 3.05 (3) | 1.02 (1) | 2.04 (2) | — | 0.81 (1) | 0.99 (1) |

*The value for Ser has not been corrected for losses during hydrolysis

TABLE 3

Reversed phase HPLC data and $[\alpha]_D$-values of the peptides prepared in this study. Values obtained by sequential syntheses are in italics.

| | [Xxx$^{152}$],[Gly$^{161}$] -Aα-(148-160) Xxx= | ret.time (mins) | % main peak | $[\alpha]_D$ |
|---|---|---|---|---|
| ***39*** | Arg | 19.55 | 97.8 | -10.0 (c=0.38, DMF) |
| | | *19.56* | *94.4* | *-10.8 (c=0.38, DMF)* |
| ***40*** | Glu | 20.26 | 94.6 | -12.3 (c=0.30, DMF) |
| | | *20.28* | *84.5* | *-10.7 (c=0.28, DMF)* |
| ***41*** | Gly | 19.40 | 96.0 | -10.7 (c=0.27, DMF) |
| | | *19.42* | *88.4* | *- 9.2 (c=0.26, DMF)* |
| ***42*** | Lys | 19.46 | 89.8 | -14.4 (c=0.55, DMF) |
| | | *19.41* | *86.2* | *-10.7 (c=0.59, DMF)* |
| ***43*** | Pro | 20.37 | 96.7 | -33.4 (c=0.29, DMF) |
| | | *20.37* | *77.1* | *-30.3 (c=0.31, DMF)* |
| ***44*** | Val | 21.96 | 95.4 | -11.0 (c=0.50, DMF) |
| | | *22.06* | *78.1* | *-10.4 (c=0.52, DMF)* |

Racemization of the $C_\alpha$-carbon of Val occurring during peptide azide formation of **26** would probably lead to a lowered biological activity of [Val$^{152}$],[Gly$^{161}$]-fibrinogen-Aα-(148-161). This was not observed. Table 3 also shows that the $[\alpha]_D$-values of the peptides prepared via both procedures are comparable, which is another indication for a low degree of racemization.

The method of acylation of a ß-unprotected amino-terminal aspartyl derivative includes the possibility of a secondary activation of free ß-carboxyl functions; this reaction is obviously slower, since no other products were obtained (*cf.* Ten Kortenaar *et al.* 1985).

We have no explanation for the fact that the peptide azides derived from **23** (Xxx=Phe) and **25** [Xxx=Ser($Bu^t$)] do not react with compound **2**.

In conclusion, we have shown that the coupling of a peptide azide to an amino-terminally positioned unprotected aspartyl peptide derivative, as proposed by ten Kortenaar *et al.* (1985), is quite generally applicable and proceeds in high yields and without any detectable by-products. The strategy of synthesizing small protected peptide fragments via SPPS, their subsequent coupling in solution to form target peptides which are deprotected in solution (*i.e.*, in high dilution), yields significantly more homogeneous peptides.

## Chapter 8

# THE USE OF Mpc-AMINO ACIDS IN SOLID PHASE PEPTIDE SYNTHESIS LEADS TO IMPROVED COUPLING EFFICIENCIES

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ABSTRACT

The Mpc-group has a somewhat better stability than the Fmoc-group, resists catalytic hydrogenolysis, is highly stable in acidic media and its elimination product does not polymerize spontaneously. In a direct comparison of coupling efficiencies obtained in solid phase peptide syntheses using Mpc- or Fmoc-amino acids it is shown that the use of Mpc-amino acids leads to better coupling efficiencies and, consequently, a more homogeneous peptide.

An improved synthesis of Mpc-ONSu and of Mpc-amino acid derivatives is presented.

## INTRODUCTION

The amino protective Mpc group (Verhart and Tesser 1988), derived from the Msc group (Tesser and Balvert-Geers), was designed to be of use both in solution and in solid phase peptide synthesis; to be comparable, in base-lability terms, with the Fmoc group (Carpino and Han 1970, 1972), and to expel an alkene with a lower tendency to polymerize than dibenzofulvene.

The frequently used Fmoc-group is suitable for solid phase syntheses, since the protection of amino groups is only required for a short period of time. Furthermore, it is relatively simple to wash away the small impurities which result from dibenzofulvene and which, during classical synthesis, cause the formation of insoluble, hard to remove polymers.

On several occasions we have observed incomplete coupling when using Fmoc-amino acids in SPPS, even when repeated coupling had been performed using the same Fmoc-amino acid. In contrast, the use of Mpc-amino acids has led, in some cases, to a complete acylation reaction.

Compared with the Fmoc-group, the Mpc-group is rather slim, and therefore it might be expected that there is improved coupling when using Mpc-amino acids in SPPS.

In order to investigate the suggested improvement of the coupling efficiency, a comparative study was set up.

Fibrinogen-Aα-(148-160) (H-Lys-Arg-Leu-Glu-Val-Asp-Ile-Asp-Ile-Lys-Ile-Arg-Ser-OH) is known to enhance the plasminogen activation mediated by t-PA with a

factor of about 12 (Voskuilen *et al.* 1987) whereas [Nle$^{157}$]-fibrinogen-Aα-(148-160) does not enhance this activation. The presence of Nle at position 157 causes an incomplete coupling of Fmoc-Arg(Pmc)-OH at position 149 (even after double coupling, coupling at higher temperatures and coupling in different solvents).

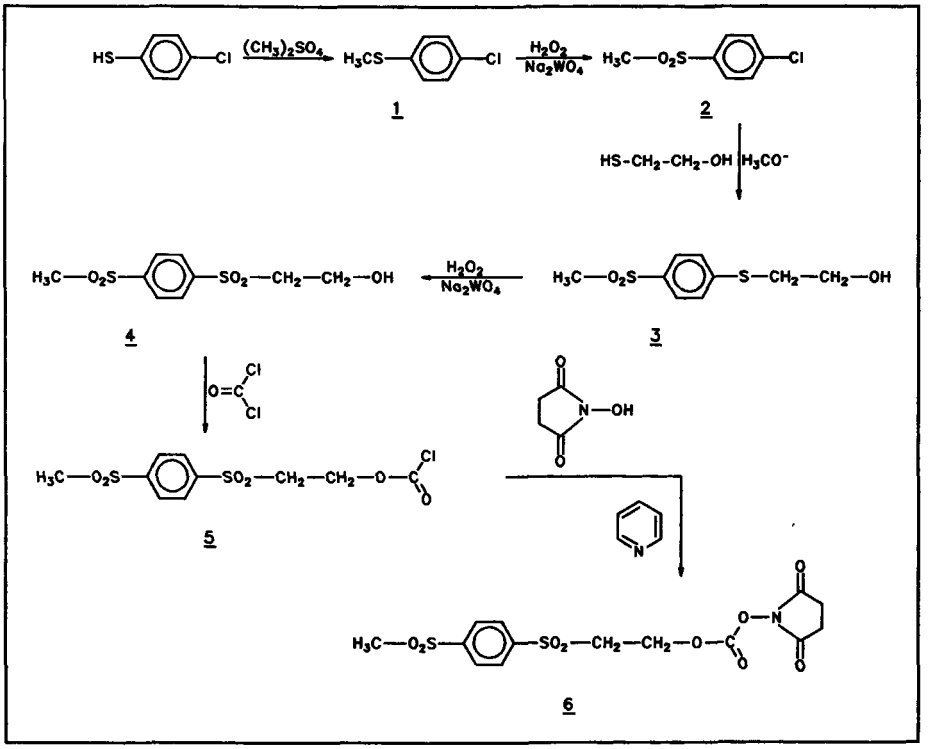
The tridecapeptides fibrinogen-Aα-(148-160) and [Nle$^{157}$]-fibrinogen-Aα-(148-160) were synthesized by SPPS twice; each with Mpc-amino acids and with Fmoc-amino acids. The results clearly indicate that the use of Mpc-amino acids leads to more constant coupling efficiencies and, obviously, to more homogeneous peptides.

In this article we also present an improved synthesis of Mpc-ONSu and of Mpc-amino acids.

## MATERIALS AND METHODS

### Methods

Amino acid analyses were performed on a Varian 9095 amino acid analyzer using the Fmoc protocol. Peptides were hydrolyzed in 5.7 N HCl (Merck Suprapur) in evacuated sealed glass tubes for 24 hours at 120 °C and concentrated *in vacuo*.
Elemental analyses were performed on a Carlo Erba Strumentazione EA MOD 1106.
HPLC analyses were performed on an Advanced Chromatography Systems apparatus. Chromatography was run in reversed phase mode on a Techsil 5C18 column at a flowrate of 1 ml/min using a 5 min isocratic elution with 7% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid in water, followed by a 30 min linear gradient of 7% (v/v) acetonitrile and 0.1% (v/v) trifluoroacetic acid in water to 70% (v/v) acetonitrile and 0.08% (v/v) trifluoroacetic acid in water. Peaks were detected by UV absorption at 215 nm. The UV data were analyzed using JCL 6000 Chromatography Data System software.
Infrared spectra were obtained from a Perkin-Elmer 298 IR Spectrophotometer.
Melting points were determined with a Büchi melting point apparatus and are uncorrected.
$^1$H-NMR spectra were obtained in appropriate solvents from a Bruker 90 Mhz Spectrophotometer.



**Scheme 1** Synthesis of Mpc-ONSu (6)

Optical rotations were obtained on a Perkin Elmer 241 polarimeter with a 1.0 dm cell in appropriate solvents and at ambient temperature.

Peptides were synthesized on a Labortec SP 640 semi-automated peptide synthesizer.

Thin-layer chromatography (t.l.c.) was performed on precoated t.l.c. plates (Merck, silica gel 60, F-254, 0.2 mm). Solvent systems used were: $CHCl_3$/MeOH/AcOH (95/20/3) and *n*-butanol/acetic acid/water (4/1/1). Spots were visualized using one or more of the following methods: UV-fluorescence quenching 254 nm, Barton's reagent spray (a mixture of equal volumes of $FeCl_3$ (30 g) in water (170 ml) and $K_3Fe(CN)_6$ (2 g) in water 200 ml), ninhydrine spray (240 mg in 400 ml n-butanol and 16 ml acetic acid).

Syntheses

*4-Chlorophenyl methyl sulphide* (1) Scheme 1.

The reaction was performed according to Schütz and Ciporin (1958), starting from 170 g 4-chlorothiophenol (1.175 mole). The distillation following the extraction by which Schütz and Ciporin obtained the sulfide was omitted, since the compound appeared to be chromatographically pure: one spot ($R_f$ 0.83) was found using the system $CHCl_3$/MeOH/AcOH (95/20/3, v/v/v); detection: UV-fluorescence quenching and Barton's reagent; pale yellow needles, yield 182.7 g (98%), m.p. 17 °C. $^1H$ NMR ($CDCl_3$): $CH_3$, s, (3H), $\delta$ 2.44 ppm; phenyl, m, (4H), $\delta$ 7.20 ppm.

*4-Chlorophenyl methyl sulphone* (2)

Compound 1 (182 g, 1.147 mole) was dissolved in 1 l ethanol and 200 ml water. An aqueous solution (150 ml) of $Na_2WO_4 \cdot 2H_2O$ (2%, g/v) was added as a catalyst. Hydrogen peroxide (105 ml of an aqueous 35% g/g solution) was then slowly added to the previously warmed (40 °C) solution. The temperature was kept at this value until the addition was complete and the sulfoxide had formed. The temperature was then increased to 80 °C and a further equivalent of hydrogen peroxide was added (105 ml) in the same way. After completion of the addition, the reaction mixture was refluxed for 1 h to complete the oxidation reaction and to decompose small amounts of residual hydrogen peroxide. The reaction mixture was allowed to cool down slowly to 4 °C. The sulphone 2 crystallized spontaneously to form colourless needles, 192.4 g (88%), m.p. 96-97 °C. TLC: $R_f$ 0.79 in $CHCl_3$/MeOH/AcOH (95/20/3, v/v/v); detection: UV-fluorescence quenching, the reaction with Barton's reagent (for reducing compounds) was negative. $^1H$ NMR ($CDCl_3$); $CH_3$, s, (3H), $\delta$ 3.07 ppm; phenyl, AA'BB', (4H), $\delta$ 7.71 ppm. Elemental analysis calcd.: C 44.10, H 3.70; found: C 44.02, H 3.71 %.

*2-[4-(Methylsulphonyl)phenylthio]ethanol* (3)

Sodium methoxide (prepared from 23 g clean sodium and water-free methanol) was dissolved in a solution of 77 ml (1.1 mole) 2-mercaptoethanol in 500 ml

dimethylformamide. The solution turned slightly green when 195.6 g (1 mole) of compound **2** was added, under a gentle stream of nitrogen. The mixture was heated for 1 hr at 60 °C (under nitrogen). A fine white precipitate (NaCl, 58 g) formed, which could be removed by filtration. The reaction mixture was allowed to stand at ambient temperature for 12 hours. After filtration the yellow filtrate was concentrated and the residue diluted with water (2.5 l). The resulting two-phase system was then heated until it was homogeneous, and was then slowly cooled to room temperature. The product **3** crystallizes spontaneously and 195.15 g (84%), m.p. 81-82 °C, was obtained. TLC: $R_f$ 0.68, in $CHCl_3$/MeOH/AcOH (95/20/3, v/v/v); detection UV-fluorescence quenching, positive reaction with Barton's reagent. $^1H$ NMR ($CDCl_3$): OH, t, (1H), $\delta$ 2.00 ppm; $CH_3$, s, (3H), $\delta$ 3.04 ppm; ß-$CH_2$, t, (2H), $\delta$ 3.22 ppm ($J$ 6 Hz); $\alpha$-$CH_2$, double t, (2H), $\delta$ 3.86 ppm ($J$ 6 Hz); phenyl, AA'BB', (4H), $\delta$ 7.62 ppm ($J_{AB}$ 8 Hz). Elemental analysis calcd.: C 46.53, H 5.21; found: C 46.40, H 5.20 %.

*2-[4-(Methylsulphonyl)phenylsulphonyl]ethanol* (Mpe-OH, **4**)
Compound **3** (195 g, 0.84 mole) was dissolved in 400 ml ethanol containing 150 ml water and 150 ml of a 2% (g/v) $Na_2WO_4$ solution in water. The oxidation was performed under a gentle stream of nitrogen, essentially as described for **2**, using two 75 ml portions of 35% $H_2O_2$. On slowly cooling the reaction mixture to room temperature, white needles were yielded, 197.6 g (89%), m.p. 165-167 °C. TLC: $R_f$ 0.63 in $CHCl_3$/MeOH/AcOH (95/20/3, v/v/v); detection: UV-fluorescence quenching; the reaction with Barton's reagent was negative. $^1H$ NMR ($CDCl_3$/CDOD 10/1): $CH_3$, s, (3H), $\delta$ 3.11 ppm; ß-$CH_2$, t, (2H), $\delta$ 3.40 ppm ($J$ 6 Hz); $\alpha$-$CH_2$, broadened t, (2H), $\delta$ 4.04 ppm ($J$ 6 Hz); phenyl, s, (4H), $\delta$ 8.16 ppm. Elemental analysis calcd.: C 40.90, H 4.58; found: C 40.77, H 4.58 %.

*2-[4-(Methylsulphonyl)phenylsulphonyl]ethyl chloroformate* (Mpc-Cl, **5**)
Phosgene (30 ml) was condensed in a 2 l three-necked flask and, with stirring, a solution of **4** (36.5 g, 138 mmoles) in 500 ml acetonitrile was slowly added to the cold (-78 °C) acid chloride. The reaction mixture was allowed to warm up slowly to room temperature overnight, contained in a reaction flask sealed with a

calcium chloride guard tube. Residual phosgene and hydrogen chloride were removed in a water trap by purging with $N_2$ for three hours. The solvent was evaporated *in vacuo* and the residue recrystallized from acetonitrile. M.p. of the clear colourless crystals (40.7 g, 90%) 159 °C. IR: C=O, 1700 $cm^{-1}$; $SO_2$ complicated peaks of two non-equivalent sulfone groups at 1140-1160 (symm) $cm^{-1}$ and 1290-1330 (ass) $cm^{-1}$. $^1H$ NMR ($CDCl_3$): $CH_3$, s, (3H), $\delta$ 3.08 ppm; β-$CH_2$, t, (2H), $\delta$ 3.58 ppm (*J* 6 Hz); α-$CH_2$, t, (2H), $\delta$ 4.67 ppm (*J* 6 Hz); phenyl, s, (4H), $\delta$ 8.16 ppm. Elemental analysis calcd.: C 36.76, H 3.39; found: C 36.95, H 3.53 %.

*2-[4-(Methylsulphonyl)phenylsulphonyl]ethyl succinimidyl carbonate* (Mpc-ONSu, 6) Compound 5 (27 g, 82.6 mmol) was slowly added to a solution of 9.55 g (83 mmol) of N-hydroxysuccinimide and 6.7 ml (83 mmol) of dry pyridine in 165 ml dry ($K_2CO_3$) acetonitrile. In 1 hr, a white compound precipitated, which, on filtration, and after washing with water and drying, proved to be pure 6 (31.2 g, 93%, m.p. 174-176 °C). Recrystallization from acetic acid/water (4/1, v/v) gave a product which was dried over solid sodium hydroxide and which melted at 174-175 °C. $^1H$ NMR (acetone-$d_6$): $CH_3$, s, (3H), $\delta$ 3.11 ppm; succinimide $CH_2$, s, (4H), $\delta$ 2.72 ppm; β-$CH_2$, t, (2H), $\delta$ 3.78 ppm; α-$CH_3$, t, (2H), $\delta$ 4.62 ppm; phenyl, s, (4H), $\delta$ 8.07 ppm.

**Mpc derivatives of Phe, Val, Ile, Leu, Nle, Glu(OBu$^t$), Asp(OBu$^t$), Ser(Bu$^t$), Arg(Pmc), Lys(Boc) via the Mpc-ONSu procedure**

Introduction of the Mpc function is performed using the route depicted by Ten Kortenaar *et al.* (1986).

To a suspension of 10 mmol amino acid in 40 ml acetonitrile/water (1/1) an amount of triethylamine was added to give an apparent pH of 9.0. The apparent pH was kept 8.5-9.0 after the addition of 9.9 mmole Mpc-ONSu (4.0 g) by adding triethylamine. The suspension turned clear. After 5-15 min, the uptake of base ceased. The mixture was stirred for 30 min. at room temperature. The solution was then acidified to an apparent pH of 5. After filtration of traces of unreacted amino acid the solution was concentrated *in vacuo* to about 20 ml and acidified

with 0.1 N HCl until a reading of pH 2 was recorded on a pH-meter. At this point a white solid precipitated. The precipitate was distributed between 100 ml ethylacetate and 100 ml water/HCl pH=2. The ethylacetate layer was washed with water until neutral, dried over $Na_2SO_4$, filtered, and concentrated. Recrystallization from ethylacetate/n-hexane gave homogeneous products and analytical results as shown in tables 1 and 2.

**Mpc derivative of Cys(Acm) via the Mpc-ONSu procedure**

The synthesis of Mpc-Cys(Acm)-OH is essentially the same as that described above. The precipitated crude Mpc-Cys(Acm)-OH, however, could not be distributed between ethylacetate and water/HCl pH=2. The precipitate was filtered, washed with water and dried to give a chromatographically pure product. Mpc-Cys(Acm)-OH can be recrystallized from acetic acid/water (4/1). Analytical results are shown in tables 1 and 2.

**Mpc derivatives of Ile and Lys(Boc) via the Tms-Cl procedure**

Introduction of the Mpc group is performed as described by Bolin *et al.* (1989). The finely ground amino acid (15 mmol) was suspended in 60 ml dry $CH_2Cl_2$. Under vigorous stirring 5.22 ml (30 mmol) diisopropylethylamine was added followed by careful addition of 3.78 ml (30 mmol) trimethylsilyl chloride. The mixture was allowed to reflux for 1.5 hr. After cooling in an ice bath, 3.68 g (11.25 mmol) Mpc-Cl was added in one portion. The solution was stirred in ice for 20 min., allowed to warm up to room temperature, left for 1 hour, supplied with 10 ml of methanol, and concentrated *in vacuo*. The residue was distributed between 100 ml diethyl ether and 125 ml 2.5% (g/l) $NaHCO_3$ solution in water. The aqueous layer was extracted with 2 x 50 ml diethyl ether. The ether layers were backwashed twice with 25 ml water. The combined aqueous layers were acidified to pH 2 with 1N HCl and extracted with 3 x 50 ml ethyl acetate. The combined ethyl acetate layers were dried over $Na_2SO_4$, filtered and concentrated *in vacuo*. Recrystallization from ethyl acetate/n-hexane gave homogeneous products and analytical data as shown in tables 1 and 2.

Solid Phase Peptide Syntheses

The peptides to synthesize were fibrinogen-Aα-(148-160)-tridecapeptide (H-Lys-Arg-Leu-Glu-Val-Asp-Ile-Asp-Ile-Lys-Ile-Arg-Ser-OH, **A**) and [Nle$^{157}$]-fibrinogen-Aα-(148-160)-tridecapeptide (H-Lys-Arg-Leu-Glu-Val-Asp-Ile-Asp-Ile-Nle-Ile-Arg-Ser-OH, **B**). Each peptide was synthesized twice; once with Mpc-amino acid derivatives (peptides **Mpc-A** and **Mpc-B**), and once with Fmoc-amino acid derivatives (peptides **Fmoc-A** and **Fmoc-B**).

During solid phase peptide synthesis the amino acid side chains were protected with acid-labile protecting groups: the ε-amino group of lysine with *tert*-butyloxycarbonyl (Boc), the δ-guanidino group of arginine with 2,2,5,7,8-pentamethylchroman-6-sulphonyl (Pmc), the γ-carboxyl group of glutamic acid and the ß-carboxyl group of aspartic acid with *tert*-butyl ester (OBu$^t$), and the ß-hydroxyl group of serine with *tert*-butyl (Bu$^t$). The carboxyl terminal amino acid [0.9 equivalents (eq.) Mpc-Ser(Bu$^t$)-OH or Fmoc-Ser(Bu$^t$)-OH] was coupled to Wang's resin (Wang 1973) (*p*-alkoxybenzyl alcohol resin, 1.07 mmol of alcohol groups/g) by *in situ* activation with DCC (0.9 equivalent [eq.]) and DMAP (0.9 eq.). To suppress racemization, HOBt (1.8 eq.) was added (Van Nispen *et al.* 1985). All reactants were dissolved in DMF. After 16 hours at 5 °C the resin was washed three times with successively each of DMF, dichloromethane, and isopropyl alcohol. Finally, the resin was washed with diisopropyl ether and dried *in vacuo*. The amount of Mpc- or Fmoc-amino acid attached to the resin was calculated from the amount of nitrogen in a sample of the resin. The cleavage of the Mpc or Fmoc groups was carried out with 20% (v/v) piperidine in DMF during three consecutive cycles of 6 minutes. Three samples of the pooled piperidine/DMF fractions were diluted each with DMF to an appropriate concentration (giving an optical density of about 0.5). The relative amount of 2-[4-(methylsulphonyl)phenylsulphonyl]ethyl-piperidine (Mpc-piperidine adduct) or 9-fluorenylmethyl-piperidine (Fmoc-piperidine adduct) was calculated using molar absorption coefficients of 4500 l/mol·cm at 279 nm and 17600 l/mol·cm at 267 nm, respectively. The relative amounts of measured Mpc-piperidine adduct or Fmoc-piperidine adduct served as a measure of the amount of Mpc-amino acid or Fmoc-amino acid that had been coupled.

TABLE 1

Yields, melting points and $R_f$-values of Mpc-amino acid derivatives

| Compound | Yield(%)[a] | Yield(%)[b] | m.p.(°C) | $R_f$[c] | $R_f$[d] |
|---|---|---|---|---|---|
| Mpc-Arg(Pmc)-OH | 76 | 60 | 100-3 | 0.27 | 0.60 |
| Mpc-Asp(OBu$^t$)-OH | 75 | 58 | 104-5 | 0.46 | 0.65 |
| Mpc-Cys(Acm)-OH | 80 | 59 | 117-8 | 0.13 | 0.35 |
| Mpc-Glu(OBu$^t$)-OH | 84 | 69 | 76-8 | 0.49 | 0.68 |
| Mpc-Ile-OH | 79 (95) | 71 (87) | 96-7 | 0.51 | 0.70 |
| Mpc-Leu-OH | 85 | 76 | 120-1 | 0.49 | 0.71 |
| Mpc-Lys(Boc)-OH | 74 (92) | 62 (83) | 110-2 | 0.48 | 0.69 |
| Mpc-Nle-OH | 87 | 79 | 140-1 | 0.49 | 0.70 |
| Mpc-Phe-OH | 85 | 78 | 72-4 | 0.46 | 0.66 |
| Mpc-Ser(Bu$^t$) | 87 | 77 | 97-9 | 0.49 | 0.66 |
| Mpc-Val-OH | 85 | 77 | 105-6 | 0.47 | 0.67 |

[a] Crude yield, values in parentheses are obtained by the Tms-Cl procedure

[b] Yield after recrystallization, values in parentheses are obtained by the Tms-Cl procedure

[c] In $CHCl_3$/MeOH/AcOH (95/20/3)

[d] In *n*-BuOH/AcOH/water (4/1/1)

The second Mpc-amino acid derivative, Mpc-Arg(Pmc)-OH (2 eq.), or Fmoc-amino acid derivative, Fmoc-Arg(Pmc)-OH (3 eq.), was coupled via *in situ* activation with TBTU (2 and 3 eq. respectively) (Knorr *et al.* 1989), HOBt (2 and 3 eq. respectively) and NMM (3 and 4.5 eq. respectively). The Mpc- or Fmoc-amino acid derivatives were allowed to couple for exactly 30 min, followed by acylation of any remaining free amino groups with 10% (v/v) acetic acid anhydride and 5% (v/v) diisopropylethylamine in DMF. Thus, no attempt was made to obtain optimal coupling of the Mpc- or Fmoc-amino acid derivatives. After each coupling the relative amount of coupled Mpc- or Fmoc-amino acid derivative was

calculated as described above. Finally, the fully protected peptides were deprotected and cleaved from the resin during a 4 h reaction with 2.5% (v/v) ethanedithiol and 2.5% (v/v) water in trifluoroacetic acid. The crude peptides were precipitated with diethyl ether, washed several times with diethyl ether, dried at the air, and analysed by HPLC and amino acid composition.

RESULTS

Table 1 lists the obtained yields, melting points, and $R_f$-values for Mpc-amino acid derivatives. Table 2 shows the measured optical rotations of Mpc-amino acid derivatives dissolved in DMF and dioxane.

Elemental analyses on the nitrogen content in samples of polystyrene bound Mpc-Ser($Bu^t$) and Fmoc-Ser($Bu^t$) showed that the amount of amino acid derivative attached to the resin is 0.21 mmol/g and 0.27 mmol/g respectively.

The change in the relative amount of coupled Mpc- or Fmoc-amino acid derivatives during the progress of the synthesis is shown in figure 1 [for the synthesis of fibrinogen-Aα-(148-160), peptides **Mpc-A** and **Fmoc-A**] and in figure 2 [for the synthesis of [$Nle^{157}$]-fibrinogen-Aα-(148-160), peptides **Mpc-B** and **Fmoc-B**].

Table 3 lists the data obtained from the amino acid analyses. Chromatograms prepared from the crude peptides are shown in figure 3. The chromatogram of the crude peptide **Mpc-A** (figure 3a) shows a major peak at 21.1 min of 96% of the total area and a small, tailing peak at 21.8 min of 4% of the total area, whereas the chromatogram of peptide **Fmoc-A** shows a major peak at 21.1 min of 87%, a small, tailing peak at 21.5 min of 4% and a minor peak at 21.8 min of 9% of the total area.

In figure 3b, the chromatogram of the crude peptide **Mpc-B** shows a major peak at 22.1 min of 91% of the total area and a minor tailing peak at 23.0 min of 9% of the total area, whereas the chromatogram of peptide **Fmoc-B** shows a major peak at 22.1 min of 79% and a peak at 22.8 min of 21% of the total area.

TABLE 2

Optical rotations obtained for some Mpc-amino acid derivatives

| Compound | $[\alpha]_D$ (°) in DMF | $[\alpha]_D$ (°) in dioxane |
|---|---|---|
| Mpc-Arg(Pmc)-OH | + 2.5, c=0.67 | + 1.5, c=1.10 |
| Mpc-Asp(OBu$^t$)-OH | - 1.7, c=0.42 | +10.1, c=1.09 |
| Mpc-Cys(Acm)-OH | -25.1, c=1.65 | -10.1, c=1.10 |
| Mpc-Glu(OBu$^t$)-OH | - 6.1, c=1.04 | + 2.9, c=1.16 |
| Mpc-Ile-OH | + 0.6, c=1.04 | + 1.8, c=1,21 |
| Mpc-Leu-OH | -12.5, c=0.72 | -10.2, c=1.22 |
| Mpc-Lys(Boc)-OH | - 4.1, c=0.78 | + 1.7, c=1.06 |
| Mpc-Nle-OH | - 5.5, c=0.66 | - 2.0, c=1.05 |
| Mpc-Phe-OH | -12.9, c=1.00 | +13.2, c=1.05 |
| Mpc-Ser(Bu$^t$) | + 8.5, c=0.65 | +13.8, c=1.10 |
| Mpc-Val-OH | + 0.4, c=0.48 | - 0.6, c=1.14 |

DISCUSSION

The synthesized Mpc-amino acid derivatives were obtained as easy to isolate solids in good yields. From table 1 it is clear that Mpc-amino acid derivatives synthesized via the Tms-Cl procedure give, in general, higher yields. Although the Tms-Cl procedure is more laborious than the Mpc-ONSu procedure, the crude derivatives are of higher purity, so in most cases recrystallization may not be necessary. Via the Mpc-ONSu procedure, however, most crude Mpc-amino acid derivatives can be obtained directly as solids (and not as oils, as is the case with most crude Fmoc-amino acid derivatives obtained via the Fmoc-ONSu procedure) that are easily recrystallized from ethylacetate/hexane. Since there is no trace of oligomerization (9), Mpc-ONSu is the method of choice for the introduction of the Mpc-group.

TABLE 3

Amino acid analyses of the synthesized peptides, expected values in parentheses

| | mole/mole of peptide | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| peptide | Arg | Asp | Glu | Ile | Leu | Lys | Nle | Ser* | Val |
| Mpc-A | 1.93 (2) | 1.95 (2) | 1.00 (1) | 2.90 (3) | 0.94 (1) | 1.93 (2) | — | 0.72 (1) | 0.99 (1) |
| Fmoc-A | 1.80 (2) | 1.97 (2) | 1.00 (1) | 2.95 (3) | 0.97 (1) | 1.75 (2) | — | 0.68 (1) | 0.96 (1) |
| Mpc-B | 2.06 (2) | 2.01 (2) | 1.00 (1) | 3.02 (3) | 0.99 (1) | 1.03 (1) | 1.02 (1) | 0.72 (1) | 1.02 (1) |
| Fmoc-B | 1.76 (2) | 2.11 (2) | 1.00 (1) | 3.13 (3) | 1.02 (1) | 0.85 (1) | 1.07 (1) | 0.71 (1) | 1.04 (1) |

*The value for Ser has not been corrected for losses during hydrolysis

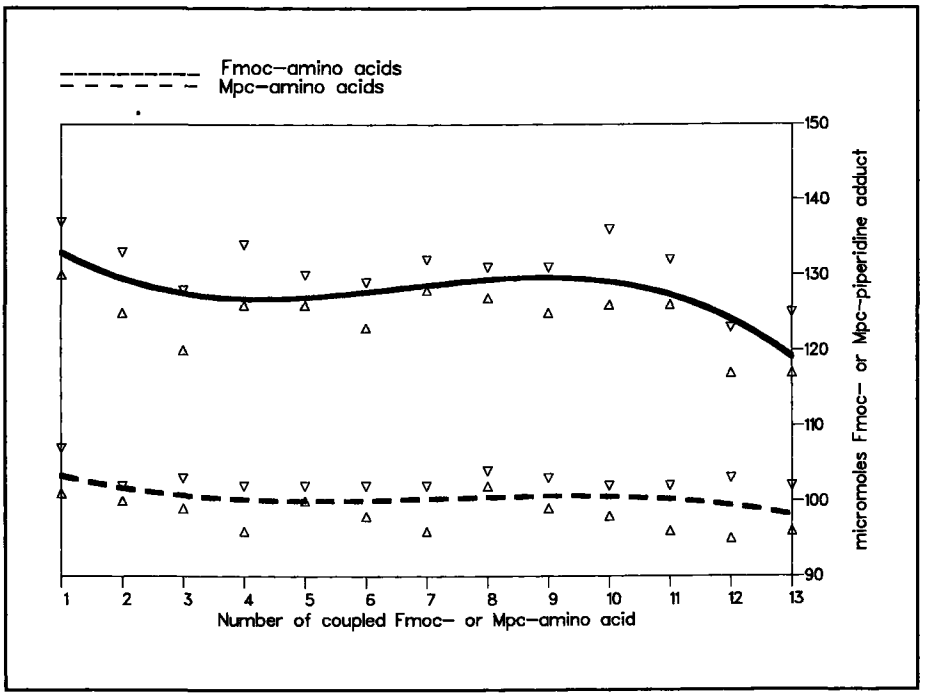



**Figure 1** Amount of obtained piperidine adduct following each coupling step in the synthesis of **Mpc-A** and **Fmoc-A**, 1: coupling of Mpc- or Fmoc-Ser(Bu$^t$)-OH to the resin, 2: coupling of Mpc- or Fmoc-Arg(Pmc)-OH to H-Ser(Bu$^t$)-resin, 3: coupling of Mpc- or Fmoc-Ile-OH to H-Arg(Pmc)-Ser(Bu$^t$)-resin, *etc.* Triangels reflect an estimated SD of the spectrophotometrical measurements (n=3).

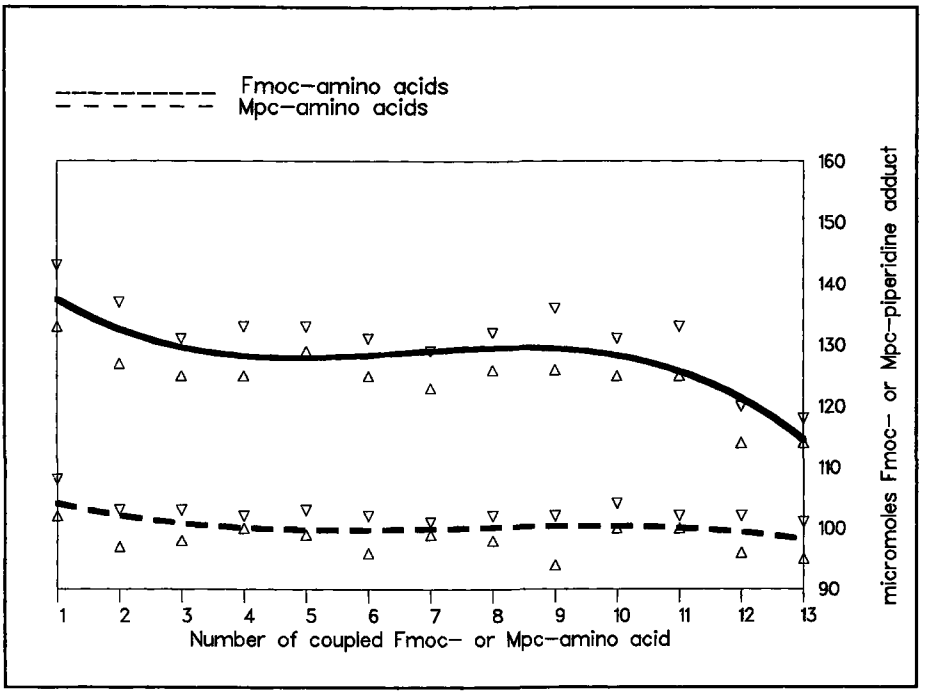



**Figure 2** Amount of obtained piperidine adduct per coupling step in the synthesis of **Mpc-B** and **Fmoc-B**, 1: coupling of Mpc- or Fmoc-Ser($Bu^t$)-OH to the resin, 2: coupling of Mpc- or Fmoc-Arg(Pmc)-OH to H-Ser($Bu^t$)-resin, 3: coupling of Mpc- or Fmoc-Ile-OH to H-Arg(Pmc)-Ser($Bu^t$)-resin, *etc.* Triangels reflect an estimated SD of the spectrophotometrical measurements (n=3).

Figures 1 and 2 show that the coupling of (3 equivalents) Fmoc-Arg(Pmc)-OH (cycle 12) is not complete within 30 minutes, whereas the coupling of (2 equivalents) Mpc-Arg(Pmc)-OH does reach completion within 30 minutes. As a result of this, the amino acid analyses (see table 3) of **Fmoc-A** and **Fmoc-B** show that the content of Arg is lower than expected. In these crude peptides the content of Lys is also lower than expected, since Fmoc-Lys(Boc)-OH is the last amino acid derivative to be coupled (cycle 13). The amino acid analysis data obtained for Lys confirm the low coupling efficiency of Fmoc-Arg(Pmc)-OH (cycle 12) to the growing chain.

Figures 1 and 2 (and table 3) also show much more consistent coupling efficiencies for the peptides synthesized with Mpc-amino acid derivatives; the

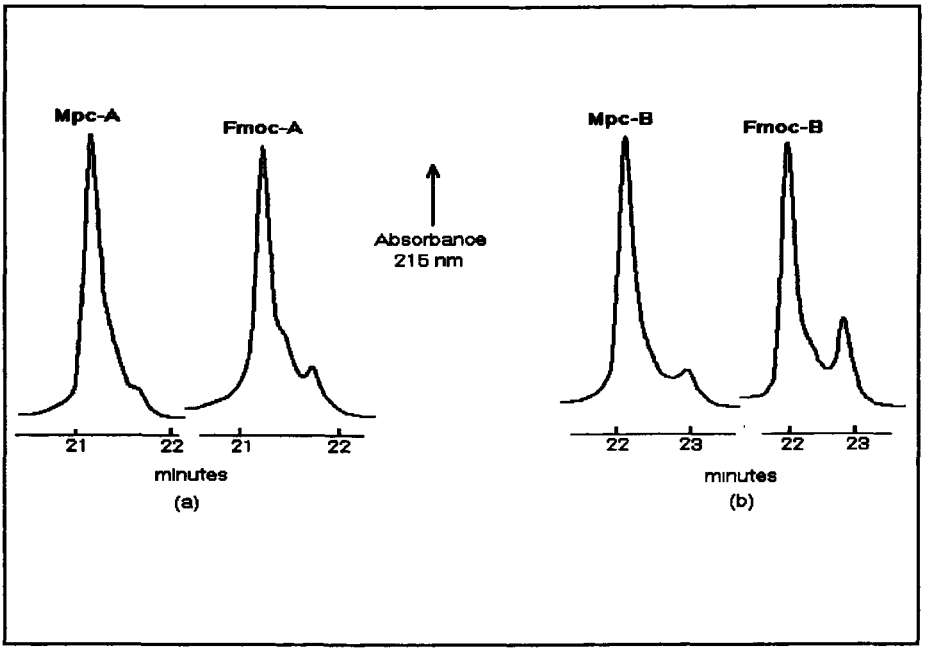



**Figure 3** Partial HPLC patterns of crude products of **Mpc-A** and **Fmoc-A** (a), and **Mpc-B** and **Fmoc-B** (b)

overall slope of the line connecting the obtained relative amounts of coupled Mpc-amino acid derivatives reaches almost zero. From the partial chromatograms in figure 3 it is clear that solid phase syntheses of peptides via Mpc-amino acid derivatives lead to more homogeneous products.

In conclusion, we have shown that when solid phase peptide syntheses are performed with Mpc-amino acids higher coupling efficiencies are obtained than with Fmoc-amino acids.

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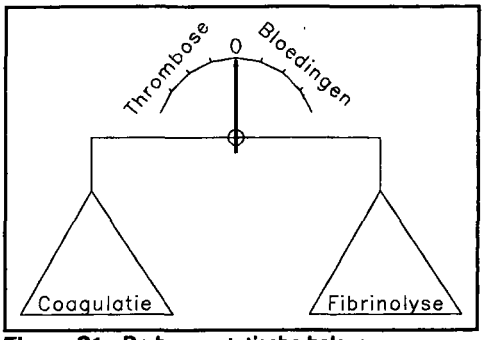
## Samenvatting

In een normale, gezonde bloedsomloop bestaat er evenwicht tussen bloedstolling (coagulatie) en het oplossen van een bloedstolsel (fibrinolyse). Dit evenwicht wordt de haemostatische balans genoemd.

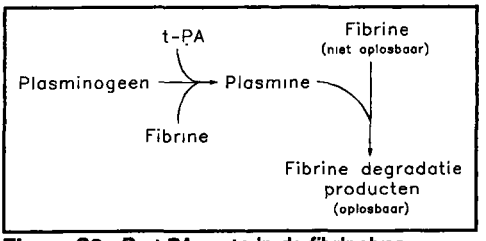
Zoals in figuur S1 is weergegeven zal bij een relatief (tov. fibrinolyse) verhoogde coagulatie thrombose optreden, terwijl bij een relatief (tov. coagulatie) verhoogde fibrinolyse bloedingen zullen optreden.



**Figuur S1** De haemostatische balans

Het bloedeiwit fibrinogeen, dat in bloedplasma een concentratie van *circa* 2.5 mg/ml heeft, speelt in beide processen een belangrijke rol. Wanneer het coagulatie-mechanisme wordt geactiveerd, bijvoorbeeld door een verwonding, zal thrombine worden gevormd, dat op zijn beurt fibrinogeen zal omzetten in fibrine. Het onoplosbare fibrine vormt het eiwitnetwerk dat aan een bloedstolsel stevigheid geeft. Wanneer een stolsel zijn rol heeft vervuld, bijvoorbeeld tijdens weefselherstel of wondheling, zal het fibrinolyse-mechanisme, relatief tov. het coagulatie-mechanisme, toenemen.

De hoofdroute in de fibrinolyse (de zogenaamde t-PA route) is weergegeven in figuur S2: Door het zeer specifieke enzym weefsel-type plasminogeen activator (t-PA) wordt plasminogeen omgezet in plasmine, hetwelk op zijn beurt fibrine afbreekt tot oplosbare degradatie producten van fibrine. De activering van plasminogeen



**Figuur S2** De t-PA route in de fibrinolyse

door t-PA blijkt nagenoeg alleen plaats te vinden in aanwezigheid van fibrine. De werking van t-PA (en dus van plasmine) blijft daardoor beperkt tot fibrine-oppervlakken (bv. bloedstolsels) en fibrine draagt dus de informatie voor z'n eigen afbraak met zich mee.

Tot nog toe zijn er twee plaatsen op het fibrine-molecuul bekend die in staat zijn de door t-PA gekatalyseerde omzetting van plasminogeen naar plasmine te versnellen: de een in Aα-(148-160) en de ander in een fragment dat FCB-5 genoemd wordt (en bestaat uit γ-(311-336) verbonden met γ-(337-379) via een disulfide brug). Het feit dat fibrinogeen (nagenoeg) geen versnellende werking heeft op de plasminogeen activering leidde tot de hypothese dat Aα-(148-160) is geëxposeerd in fibrine en "begraven" in fibrinogeen. Deze hypothese hebben we kunnen staven door monoclonale antilichamen (MoAbs) op te wekken tegen synthetisch Aα-(148-160); de verkregen MoAbs blijken specifiek te zijn voor fibrine. Door deze MoAbs wordt fibrinogeen niet herkend (Hoofdstuk 2).

Eenzelfde hypothese kan opgesteld worden voor het fragment FCB-5, waarvan waarschijnlijk (minstens) een gedeelte geëxposeerd is in fibrine. In dit fragment FCB-5 blijkt zich een sequentie te bevinden, nl. γ-(315-322), die, wat betreft de ladingsverdeling, sterk lijkt op Aα-(150-158):

```
Aα-(150-158):   Leu-Glu-Val-Asp-Ile-Asp-Ile-Lys-Ile
                 0   -   0   -   0   -   0   +   0

γ-(315-322):    Trp-Asp-Asn-Asp-Asn-Asp-Lys-Phe
                 0   -   0   -   0   -   +   0
```

MoAbs, opgewekt tegen synthetisch γ-(312-324) blijken fibrine-specifiek te zijn (Hoofdstuk 3), zodat de hypothese ook voor FCB-5 is bevestigd.

Aα-(148-160) dat, zoals reeds eerder vermeld, in staat is om de plasminogeen activering door t-PA te stimuleren, heeft de volgende aminozuurvolgorde en ladingverdeling:

```
Lys-Arg-Leu-Glu-Val[152]-Asp-Ile-Asp-Ile-Lys[157]-Ile-Arg-Ser
 +   +   0   -   0      -   0   -   0   +      0   +   0
```

Een sequentie van alternerend neutrale en negatieve aminozuurresten wordt ingesloten door twee paar positief geladen aminozuurresten. Het onderzoek heeft zich, wat betreft Aα-(148-160), geconcentreerd op een drietal vragen:

1. Is er een kleinere sequentie binnen Aα-(148-160) die in staat is de plasminogeen activering door t-PA te stimuleren?
2. Wat is de rol van de zijketen van het aminozuur in positie Aα 157?
3. Is de typische ladingsverdeling in Aα-(148-160) van belang voor de versnellende werking?

*ad* 1. In hoofdstuk 4 staat de synthese beschreven van opeenvolgende lineaire hexapeptiden uit Aα-(148-160). Deze aminoterminaal geacetyleerde peptiden zijn getest op hun stimulerende capaciteit. Daarbij bleek dat Aα-(154-159) het enige hexapeptide is dat in staat is de plasminogeen activering door t-PA te versnellen. Hoewel Aα-(152-157), Aα-(153-158), Aα-(154-159) en Aα-(155-159) alle (de volgens de literatuur belangrijke) $Lys^{157}$ bevatten, vertoont slechts Aα-(154-159) een significante stimulerende werking, hetgeen er sterk op duidt dat deze lysine op een speciale manier georiënteerd moet zijn.

*ad* 2. Het is algemeen geaccepteerd dat lysyl resten van belang zijn voor de stimulerende werking op de plasminogeen activering. In het verleden werd gevonden dat het lysine residu op positie Aα 157 van cruciaal belang is. Nader onderzoek toonde echter aan dat dit lysine residu zonder problemen vervangen kan worden door glutaminezuur. Dit verrassende resultaat was voor ons aanleiding om vast te stellen wat de minimum eisen zijn, die gesteld moeten worden aan het aminozuur op positie Aα 157, voor het vervullen van zijn functie in fibrine. Daartoe zijn een aantal analoga van het type [$Xxx^{157}$]-Aα-(148-160) gesynthetiseerd en getest op hun stimulerende werking op de plasminogeen activering. In hoofdstuk 5 wordt geconcludeerd dat het lysine residu op positie Aα 157 mag worden vervangen door de lysinederivaten Lys(Ac), Lys(Z) en Lys(Msc), en door een groot aantal aminozuurresten, waarbij aan de volgende criteria moet worden voldaan. Het residu op positie Aα 157 mag géén (zoals in Gly) of één C-atoom (zoals in Ala) in de zijketen hebben. Wanneer de zijketen twee of meer C-atomen atomen bezit, moet er ook een polaire groep aanwezig zijn, waaraan eventueel een nogal volumineuze groep mag zitten (bijv. Lys(Z)).

*ad* 3. Om de invloed van de typische ladingsverdeling in Aα-(148-160) op de stimulerende werking te onderzoeken, zijn er analoga van Aα-(148-160) gesynthetiseerd waarbij valine op positie Aα 152 vervangen is door geladen, neutraal polaire, neutraal apolaire aminozuurresten, of door glycine of proline. In hoofdstuk 6 wordt beschreven dat valine op positie Aα 152 slechts door een ander apolair neutraal aminozuur mag worden vervangen (zoals bijvoorbeeld norleucine of alanine).

De resultaten van deze experimenten (beschreven in de hoofdstukken 4, 5 en 6) duiden erop dat de ladingsverdeling in Aα-(148-160) belangrijk is, onder andere voor de juiste oriëntatie van het lysine residu op positie Aα 157. Het lijkt erop dat niet zozeer $Lys^{157}$ belangrijk is, of $Val^{152}$, maar veeleer de structuur die door hun zijketens gestabiliseerd wordt. Dit zou dan betekenen dat plasminogeen en/of t-PA een bepaalde, typische structuur op het fibrine-molecuul herkennen en binden, en dit leidt dan tot de versnellende werking.

Hoofdstuk 7 beschrijft de ontwikkeling van een nieuwe strategie voor fragmentcondensaties in de peptide chemie. De vaste drager synthese van peptiden groter dan 10 aminozuren leidt vaak tot een mengsel van het doelpeptide en, als verontreiniging, sequenties met inserties of deleties van aminozuurresten.

Een manier om dit probleem te ondervangen is het aan een vaste drager synthetiseren van korte fragmenten. De korte, volledig beschermde, fragmenten kunnen in oplossing gekoppeld worden tot het doelpeptide. De resultaten van experimenten, beschreven in hoofdstuk 7, tonen aan dat condensatie reacties van peptide aziden met onbeschermde aspartyl peptide derivaten als amino component vrij algemeen toepasbaar zijn en aflopen met hoge opbrengsten. De aldus vervaardigde peptiden, een selectie van de analoga uit hoofdstuk 6, blijken significant zuiverder (volgens HPLC analyse) dan de volledig sequentieel gesynthetiseerde peptiden.

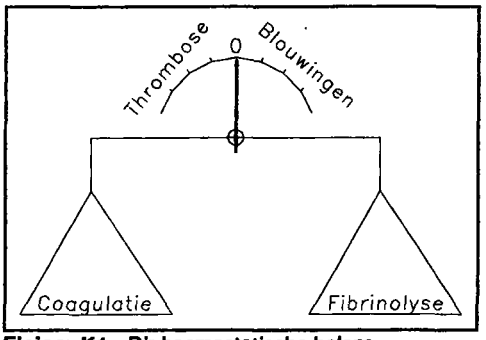
Hoofdstuk 8 tenslotte beschrijft een verbeterde synthese van Mpc-ONSu en van Mpc-aminozuren. Wanneer de koppelingsefficiënties in vaste drager syntheses van Aα-(148-160) en [$Nle^{157}$]-Aα-(148-160), van Mpc- en Fmoc-

aminozuren worden vergeleken, blijken de Mpc-aminozuren beter te koppelen dan Fmoc-aminozuren. Het gevolg hiervan is dat peptiden die gesynthetiseerd zijn aan een vaste drager met behulp van Mpc-aminozuren, zuiverder zijn (volgens HPLC analyses van de ruwe producten). De verklaring hiervoor is waarschijnlijk dat de Mpc-groep slanker (minder volumineus) en minder rigide is (en daardoor beter in de matrix van de vaste drager kan doordringen) dan de Fmoc-groep.

## Kód en jód

In inne jeweune, jezonge blódumloof jieët 't eëvewieët tusje blódjerinning (coagulatie) en 't luëze van e jerinsel (fibrinolyse). Dit eëvewieët weëd d'r haemostatische balans jeneumd.

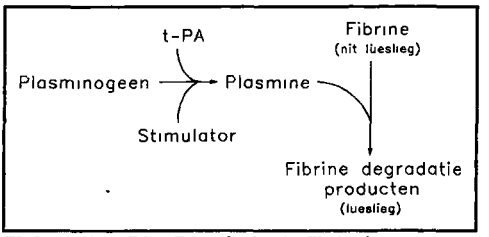
Zoeëwie i fiejoer K1 sjteet zal bij ing hoeëjere (tov. fibrinolyse) coagulatie thrombose optreëne, ongerwiels bij ing hoeëjere (tov. coagulatie) fibrinolyse blouwinge zalle optreëne.



**Fiejoer K1** D'r haemostatische balans

't Blódaiwies fibrinogeen, dat i blódplasma ing konsentrasie van ónjeveer 2.5 mg/ml hat, sjpilt i beide protsesse ing wiechtije rol. Wen 't coagulatie-mechanisme weëd aajesjwungeld, bijvuurbeeld bij ing wond, zal thrombine weëde aajemaad, dat óp zienne toer *fibrinogeen umsetst i fibrine. Oes 't nit-lüeslieje fibrine, weëd 't aiwiesnetswirk* jemaad dat an e blódjerinsel sjterkde jieëft. Wen 't jerinsel zie wirk jedoa hat, bijvuurbeeld bij wond- of weefselreparatoer, da zal 't fibrinolyse-mechanisme aajesjwungeld weëde (dat weëd da hoeëjer tov. 't coagulatie-mechanisme).

D'r hoofdwég in de fibrinolyse (d'r zoeëjeheesje t-PA wég) sjteet i fiejoer K2: Durch 't hoeëgjenauwe enzym weefsel-type plasminogeen activator weëd plasminogeen umjezatsd i plasmine, dat óp zienne toer fibrine aafbrikt bis lüeslieje degradatie producten va fibrine. De aktievieroeng va plasminogeen durch t-PA bliecht benoa alling tse



**Fiejoer K2** D'r t-PA wég in de fibrinolyse

jebeure wen d'r óch fibrine prezent is. De wirkoeng va t-PA (en dus va plasmine) bliet doadurch besjrenkt bis fibrine-oppervlakken (bijvuurbild jerinsels) en fibrine dreët dus de oeskónf vuur zienne eje aafbrooch mit zich mit.

Bis noe zunt d'r tswai plaatsje óp 't fibrine-molecuul bekank die in sjtaat zunt um de durch t-PA gekatalyseerde umzetsoeng tse versjnelle: de ing i Aα-(148-160) en de anger in e fragment dat FCB-5 jeneumd weëd (en besjteet oes γ-(311-336) verbónge mit γ-(337-379) via ing disulfide bruk).

't Feit dat fibrinogeen (benoa) jing versjnellende wirkoeng hat óp de plasminogeen aktievieroeng leiet noa de hypothese dat Aα-(148-160) is jeëksposeerd i fibrine en "bejrave" i fibrinogeen. Dis hypothese hant vuur kenne bewieze durch monoclonale antilichamen (MoAbs) óp tse wekke teëje synthetisch Aα-(148-160); de verkrèje MoAbs blieche sjpetsiefiek tse zieë vuur fibrine. Durch dizze MoAbs weëd fibrinogeen nit herkankd (Kapiettel 2).

Ing jelieche hypothese kan opjestald weëde vuur 't fragment FCB-5, woava waarsjainlieg (minnestens) e jedeelte jeëksposeerd is i fibrine. I dit fragment FCB-5 bliecht ziech ing tsekwèns tse bevinge, nl. γ-(315-322), die, wat betruft de ladingsverdeling, sjterk liecht op Aα-(150-158):

```
Aα-(150-158):   Leu-Glu-Val-Asp-Ile-Asp-Ile-Lys-Ile
                 0   -   0   -   0   -   0   +   0

γ-(315-322):    Trp-Asp-Asn-Asp-Asn-Asp-Lys-Phe
                 0   -   0   -   0   -   +   0
```

MoAbs, ópjewekt teëje synthetisch γ-(312-324) blieche fibrine-sjpetsiefiek tse zieë (Kapiettel 3), zoeëdat de hypothese óch vuur FCB-5 is vas-jelaad.

Aα-(148-160) dat, zoeëwie al ieëder vermeld, i sjtaat is um de plasminogeen aktievieroeng durch t-PA tse versjnelle, hat de volgende aminozuurvolgorde en ladingsverdeling:

```
Lys-Arg-Leu-Glu-Val[152]-Asp-Ile-Asp-Ile-Lys[157]-Ile-Arg-Ser
 +   +   0   -   0      -   0   -   0   +      0   +   0
```

Ing tsekwèns va alternerende neutrale en negatieve aminozuurresten weëd i-jesjloase durch tswai paar positief jelane aminozuurresten. 't Óngerzuk hat ziech, wat Aα-(148-160) aajeet, tsouwjesjpitst óp drei vroage:

1. Is d'r ing klinger tsekwèns binne Aα-(148-160) die i sjtaat is de plasminogeen aktievieroeng durch t-PA tse sjtiemulère?
2. Wat is de rol van d'r zijketen van 't aminozuur i positie Aα 157?
3. Is de tiepiesje ladingsverdeling i Aα-(148-160) va belang vuur de versjnellende wirkoeng?

*ad* 1. I kapiettel 4 sjteet de synthese besjreëve va ópeevolgende lineaire hexapeptiden oes Aα-(148-160). Dizze aminoterminaal geacetyleerde peptiden zunt jetést op hun sjtiemulèrende capaciteit. Doabij bliechet dat Aα-(154-159) 't intsiegste hexapeptide is dat i sjtaat is de plasminogeen aktievieroeng durch t-PA tse versjnelle. Hoewel Aα-(152-157), Aα-(153-158), Aα-(154-159) en Aα-(155-160) allenäu (de volgens de lieteratoer wiechtije) $Lys^{157}$ hant, tseegt alling Aα-(154-159) ing significante sjtiemulèrende wirkoeng, hetgeen d'r sjtirk op wiest dat dis lysine óp ing sjpetsiejaal maneer georiënteerd mós zieë.

*ad* 2. 't Weëd aljemeen aajenoame dat lysyl reste wiechtieg zunt vuur de sjtimulèrende wirkoeng óp de plasminogeen aktievieroeng. In 't verleie woeët jevonge dat 't lysine residu óp positie Aα 157 va cruciaal belang is. Noader óngerzuk hat oes-jewieëze dat evvel dis lysine residu oane probleme vervange kan weëde durch glutaminezuur. Dit uberrasjend resultaat woar vuur ós anlas um vas tse sjtelle wat de minimum eisen zunt, die jesjteld mósse weëde aan 't aminozuur óp positie Aα 157, vuur 't oesveure va zieng functie i fibrine. Doarum zunt e aantal analoga van 't type [$Xxx^{157}$]-Aα-(148-160) gesynthetiseerd en jetést óp hun sjtimulèrende wirkoeng óp de plasminogeen aktievieroeng. I kapiettel 5 weëd geconcludeerd dat 't lysine residu óp positie Aα 157 maag weëde vervange durch de lysinederivaten Lys(Ac), Lys(Z) en Lys(Msc), en durch e jroeës aantal aminozuurresten, woabij aan de noekómende criteria mós weëde voldoa. 't Residu óp positie Aα 157 maag jee (zoeëwie i Gly) of ee C-atoom (zoeëwie in Ala) in d'r zijketen han. Wen d'r zijketen tswai of mieë C-atomen hat, mós d'r óch inne polaire jroep doa zieë, woa-aa eventueel inge nogal volumineuze jroep maag zitte (bijv. Lys(Z)).

*ad* 3. Um d'r invloed van de tiepiesje ladingsverdeling in Aα-(148-160) óp de sjtiemulèrende wirkoeng tse óngerzukke, zunt d'r analoga va n Aα-(148-160) gesynthetiseerd, woabij valine óp positie Aα 152 vervange is durch jelane, neutraal polaire, neutraal apolaire aminozuurresten, of durch glycine of proline. I kapiettel 6 weëd besjreëve dat valine óp positie Aα 152 alling durch a anger apolair neutraal aminozuur maag weëde vervange (zoeëwie bijvuurbild norleucine of alanine).

De resultaten va dis experimenten (besjreëve in de kapiettele 4, 5 en 6) wieze dróp dat de ladingsverdeling i Aα-(148-160) wiechtieg is, onger angere vuur de riechtije oriëntatie van 't lysine residu op Aα 157. 't Liecht dróp dat nit zoeëzier $Lys^{157}$ wiechtieg is, of $Val^{152}$, mer vöalmieë de structuur die durch hun zijketens gestabiliseerd weëd. Dit zouw da betseechene dat plasminogeen en/of t-PA ing bepaalde tiepiesje structuur óp 't fibrine-molecuul herkenne en binge, en dit leit da bis de versjnellende wirkoeng.

Kapiettel 7 besjrieft de ontwikkeling van e nui kontsep vuur fragment condensaties in de peptide chemie. De vaste drager synthese va peptiden jroeëser wie 10 aminozuren leit duk bis ing misjoeng van 't doelpeptide en, als verontreiniging, tsekwénse mit inserties of deleties va aminozuurresten. Ing maneer um dit probleem tse óngervange is 't aan inge vaste drager synthetiseren va kótte fragmenten. De kótte, jans besjermde, fragmenten könne i luësoeng jekoppeld weëde bis 't doelpeptide. De resultaten va experimenten, besjreëve i kapiettel 7, jeëve aa dat condensatie reacties va peptide aziden mit ónbesjermde aspartyl peptide derivaten als amino component, tsemmelieg aljemeen tsouw tse passe zunt en aaflofe mit hoeëg ópbringste. De óp dis maneer jemaade peptiden, ing selectie van de analoga oes kapiettel 6, blieche significant zuuverder (volgens HPLC analyses) dan de jans sequentieel gesynthetiseerde peptiden.

Kapiettel 8 sjlieslieg besjrieft ing verbesserde synthese va Mpc-ONSu en va Mpc-aminozuren. Wen de kóppelingefficiënties i vaste drager syntheses va Aα-(148-160) en [$Nle^{157}$]-Aα-(148-160), va Mpc- en Fmoc-aminozuren weëde verjelèche, blieche de Mpc-aminozuren besser tse kóppele dan de Fmoc-

aminozuren. 't Jevolg heiva is dat peptiden die gesynthetiseerd zunt aan inge vaste drager mit behulp va Mpe-aminozuren, zuuverder zunt (volgens HPLC analyses van de rauw producten). De verkloaring heivuur is waarsjainlieg dat d'r Mpc-jroep sjlanker (winniejer volumineus) en winniejer sjtief is (en doadurg besser in d'r matrix van d'r vaste drager kan durchdringe) wie d'r Fmoc-jroep.

## Abbreviations

Abbreviations and nomenclature recommended by the IUPAC-IUB commission (1984) are used in most cases.

Special abbreviations:

| | |
|---|---|
| BSA | bovine serum albumin |
| CNBr | cyanogen bromide |
| DCC | dicyclohexylcarbodiimide |
| dec. | decomposes |
| $D_{EGTA}$ | plasmin-generated fragment of fibrinogen, formed in the presence of EGTA, consisting of fibrinogen chain fragments Aα 111-197, Bß 134-461, and γ 86-303, disulfide-bond linked |
| DMAP | 4-(dimethylamino)pyridine |
| DMF | dimethylformamide |
| EGTA | ethyleneglycol-bis-(2-aminoethyl)-*tetra*acetic acid |
| FCB-2 | CNBr fragment of fibrin(ogen) consisting of fibrin(ogen) chain fragments Aα 148-207, Bß 191-224, 225-242, 243-305 and γ 95-265, disulfide bond linked [also fibrin(ogen) cyanogen bromide fragment 2) |
| FCB-5 | CNBr fragment of fibrin(ogen) consisting of fibrin(ogen) chain fragments γ 311-336 and 337-379, disulfide bond linked [also fibrin(ogen) cyanogen bromide fragment 5) |
| FDM | fibrin(ogen) derived material |
| Fmoc | 9-fluorenylmethyloxycarbonyl |
| HOBt | 1-hydroxy-1H-benzotriazole |
| Msc | methylsulphonylethyloxycarbonyl |
| Mpc | 2-[4-(methylsulphonyl)phenylsulphonyl]ethyloxycarbonyl |
| NMM | 4-methylmorfoline |
| PBS | phosphate-buffered saline |

| | |
|---|---|
| Pmc | 2,2,5,7,8-pentamethylchroman-6-sulphonyl |
| Pms | pentamethylphenylsulphonyl |
| $R_f$ | relative mobility |
| TBTU | 2-(1H-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate |
| Tha | thioacetyl |
| TLC | thin layer chromatography |

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## Curriculum vitae

Wim Schielen werd geboren op 31-07-1959 te Ubach over Worms (nu Landgraaf) en is getogen in Simpelveld, alwaar, met succes, de lagere (jongens)school werd doorlopen.

Van 1971 tot 1978 werd het College Sancta Maria te Spekholzerheide, gemeente Kerkrade, bezocht. Deze periode werd afgesloten met het behalen van het diploma Atheneum-B.

In september 1978 begon hij met de studie scheikunde aan de Katholieke Universiteit Nijmegen, waar in januari 1983 het kandidaatsexamen ($S_2$) werd afgelegd. De doctoraalstudie omvatte het hoofdvak Biochemie (Prof. Dr. S.L. Bonting, Dr. W. de Grip), gedurende welke periode hij ook op de afdeling Organische Chemie (Prof. Dr. G.I. Tesser) een stage heeft volbracht, alsmede de bijvakken Biofysische Chemie (Prof. Dr. C.W. Hilbers, Dr. B.J.M. Harmsen) en Analytische Chemie (Prof. Drs. G. Kateman, Dr. B. Vandeginste). In februari 1987 werd het doctoraalexamen afgelegd.

Vanaf januari 1987 tot januari 1990 was hij werkzaam als junior wetenschappelijk medewerker op het laboratorium voor Organische Chemie van de Katholieke Universiteit Nijmegen aan een projekt dat volledig werd gefinancierd door het Gaubius Instituut TNO, Leiden. Het in dit proefschrift beschreven onderzoek werd verricht onder leiding van Prof. Dr. G.I. Tesser (Katholieke Universiteit Nijmegen) en Dr. W. Nieuwenhuizen (Gaubius Instituut TNO, Leiden).