Localization in the fibrinogen γ -chain of a new site that is involved in the acceleration of the tissue-type plasminogen activator-catalysed activation of plasminogen

Osamu YONEKAWA,* Marijke VOSKUILEN and Willem NIEUWENHUIZEN†

Gaubius Laboratory IVVO–TNO, P.O. Box 430, 2300 AK Leiden, The Netherlands

In previous publications [e.g. Voskuilen, Vermond, Veenman, Van Boom, Klasen, Zegers & Nieuwenhuizen (1987) *J. Biol. Chem.* **262**, 5944–5946] we have shown that fibrin(ogen) chain fragment A α -(148–160) contains a site that contributes to the acceleration of Glu-plasminogen activation by tissue-type plasminogen activator (t-PA). In contrast with fibrin, this peptide, however, does not enhance the rate of mini-plasminogen activation. Therefore, possibly more stimulatory sites than A α -(148–160) are present in fibrin. In the present investigation we have localized a possible second type of stimulatory site in the fibrin(ogen) molecule. A whole CNBr digest of fibrinogen was applied to a Bio-Gel P-2 column run in water, pH 4. Two peaks with stimulatory activity were observed, one at the void volume and one between the void volume and the total volume. The former contained the previously described stimulating fragment FCB-2 [which comprises A α -(148–160)]; the latter had not been observed before and was characterized further. The stimulating material in the low- M_r fraction of the Bio-Gel P-2 column was precipitated at pH 8.3 in a virtually pure form. It has a high tryptophan content, and an M_r of 6500 as assessed by SDS/PAGE. On reduction, a main band of M_r 2500 is seen, plus a weakly staining band of M_r 4000. These properties plus the amino acid sequence data identify the fragment as FCB-5. FCB-5 consists of two chains, i.e. γ -(311–336) and γ -(337–379), linked by a single disulphide bond between Cys- γ -326 and Cys- γ -339. Both these chains and the disulphide bond appear to be essential for rate enhancement. FCB-5 enhances the activation rates of Glu-, mini- and micro-plasminogen, with all five kringle, only kringle V and without kringle V respectively. FCB-5 binds t-PA, but none of the plasminogen forms binds to FCB-5. This indicates that the rate enhancements induced by FCB-5 are due to an effect on t-PA.

INTRODUCTION

Fibrin is known to play an important role as the protein matrix of a blood clot, and in tissue repair. After fibrin has fulfilled these roles, it is removed by the process known as fibrinolysis. The crucial step in the fibrinolytic process is the activation of plasminogen to plasmin by tissue-type plasminogen activator (t-PA). The plasmin formed converts fibrin into soluble degradation products.

Fibrin, however, is not merely the substrate of fibrinolysis, but also acts as a cofactor in the fibrinolytic system by enhancing 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, the soluble precursor of fibrin, has been reported to have virtually no rate-enhancing capacity (Wallén, 1977; Allan & Pepper, 1981; Hoylaerts *et al.*, 1982).

We have demonstrated that not only fibrin but also plasmin-generated fragments of both fibrin (D-dimer) and fibrinogen (D_{EGTA}) can accelerate the t-PA-catalysed plasminogen activation (Verheijen *et al.*, 1982b). Also, CNBr digests of both fibrin and fibrinogen accelerate the plasminogen activation and the effect is

at least partly recovered in CNBr-cleavage fragment 2 (Nieuwenhuizen *et al.*, 1983b), also known as FCB-2 (Henschen, 1981) or Hol-DSK (Gårdlund *et al.*, 1977). We have previously presented evidence that the fibrinogen stretch A α -(148–160) plays a crucial role in the accelerating effect on the plasminogen activation by t-PA (Voskuilen *et al.*, 1987).

However, several observations indicate that rate-enhancing sites other than A α -(148–160) are present in fibrin. The strongest indication is that fibrin accelerates the t-PA-catalysed activation of both Glu-plasminogen and mini-plasminogen (442-Val-plasminogen) (Verheijen *et al.*, 1983) whereas FCB-2, which comprises A α -(148–160), does not enhance the activation of mini-plasminogen (Verheijen *et al.*, 1982b; Nieuwenhuizen *et al.*, 1988).

In the present study we have indentified an additional, hitherto unknown, stimulatory site. It appears to be located near the C-terminus of the γ -chain of fibrinogen, i.e. in the CNBr-cleavage fragment γ -(311–379), known as FCB-5 (Henschen, 1981). FCB-5 is composed of two γ -chain remnants, i.e. γ -(311–336) and γ -(337–379), kept together by a single disulphide bond between cysteines γ -326 and -339. A mixture of the S-carboxymethylated chains of FCB-5 does not enhance the rate of plasminogen activation. FCB-5 binds t-PA but none of the plasminogen forms (Glu-, mini-, or micro-).

Abbreviations used: t-PA, tissue-type plasminogen activator; FCB-2 (= HolDSK), CNBr-cleavage fragment of fibrin(ogen) consisting of fibrin(ogen) chain fragments A α -(148–207), B β -(191–224), B β -(225–242), B β -(243–305) and γ -(95–265) disulphide-bond linked; FCB-5, CNBr-cleavage fragment of fibrin(ogen) consisting of fibrin(ogen) chain fragments γ -(311–336) and γ -(337–379), disulphide-bond linked; D_{EGTA}, plasmin-generated fragment of fibrinogen consisting of fibrinogen chain fragments A α -(111–197), B β -(134–461) and γ -(86–303), disulphide-bond linked; D_{cate}, plasmin-generated fragment of fibrin(ogen) formed in the presence of Ca²⁺, consisting of fibrin(ogen) chain fragments A α -(111–197), B β -(134–461) and γ -(86–411); D-dimer, plasmin-generated fragment of cross-linked fibrin, with the formula (D_{cate})₂; PBST, 10 mM-Na₂HPO₄/1.6 mM-NaH₂PO₄/0.15 M-NaCl, pH 7.4, containing 0.05% (v/v) Tween 20.

* On leave from the Department of Laboratory Medicine, Hamamatsu University School of Medicine, Hamamatsu 431–31, Japan.

† To whom correspondence should be addressed.

MATERIALS AND METHODS

Materials

Formic acid, acetic acid, Tris and SDS were obtained from E. Merck, Darmstadt, Germany; CNBr was from Aldrich Chemical Co., Milwaukee, WI, U.S.A.; Tween 80 was from Baker, Deventer, The Netherlands; Coomassie Brilliant Blue G-250 was from Serva, Heidelberg, Germany; lysine-Sepharose was from Pharmacia, Uppsala, Sweden; Bio-Gel P-2 was from Bio-Rad Laboratories, Richmond, CA, U.S.A.; fibrinogen (human, grade L, 80–90% clottable) and D-Val-L-Leu-L-Lys-p-nitroanilide (S-2251) was from Kabi, Stockholm, Sweden.

Dialysis tubing (Spectrapor 6), M_r cut-off 1000, was obtained from Spectrum Medical Industries, Los Angeles, CA, U.S.A.

Two-chain t-PA was purified from melanoma cell culture medium according to the method of Rijken *et al.* (1979) as modified by Kluft *et al.* (1983). Glu-plasminogen was prepared as described by Deutsch & Mertz (1970) from fresh human plasma by affinity chromatography on lysine-Sepharose. SDS/PAGE revealed that Glu-plasminogen has an M_r of 95000. End-group analysis by the dansyl method (Gray, 1967) yielded glutamic acid as the N-terminal amino acid. Mini-plasminogen (Val 442-plasminogen) was prepared by limited elastase digestion of Glu-plasminogen and purified as described by Sottrup-Jensen *et al.* (1978). Micro-plasminogen (lacking all kringles) was prepared by digestion of Glu-plasminogen with plasmin as described by Shi & Wu (1988). Measurement of the concentration of all types of plasminogen was based on $A_{1\text{cm}}^{280}$ 16.1 at 280 nm (Wallén & Wiman, 1970).

Purification of γ -chains of fibrinogen was carried out according to the method of Doolittle *et al.* (1977).

CNBr digests of fibrinogen and fibrinogen γ -chain were made with CNBr in 70% (v/v) formic acid as described previously (Nieuwenhuizen *et al.*, 1983b). The fibrinogen digest was dialysed against distilled water in dialysis tubing with an M_r cut-off of 1000 and freeze-dried; the digest of the γ -chain was diluted 10-fold with distilled water, dialysed and freeze-dried. Fragment FCB-2 was isolated from a digest of fibrinogen as described previously (Nieuwenhuizen *et al.*, 1983b).

Methods

Rate enhancement of the t-PA-catalysed plasminogen activation was assessed as described by Verheijen *et al.* (1982a). In brief, in a total volume of 0.25 ml of 0.1 M-Tris/HCl buffer, pH 7.5, containing 0.1% (v/v) Tween 80 were present 0.11 μ M-Glu-plasminogen, mini-plasminogen or micro-plasminogen, 0.3 mM-S-2251, 300 mIU of t-PA and various concentrations of fibrin(ogen) fragments to be tested for rate enhancement. In this assay, 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 rate enhancers to that in the absence of the enhancers is designated the stimulation factor.

Binding of t-PA and plasminogen to FCB-5 was studied in a system similar to that described previously (Bosma *et al.*, 1988). Wells of polystyrene micro-titre plates were coated with FCB-5 by overnight incubation with an FCB-5 solution (10 μ g/ml) in 0.05 M-Tris/HCl buffer, pH 8.4. After three washes with 10 mM- Na_2HPO_4 /1.6 mM- NaH_2PO_4 /0.15 M-NaCl, pH 7.4, containing 0.05% (w/v) Tween 20, pH 7.4 (PBST) 100 μ l portions of serial 2-fold dilutions of a t-PA solution (5 μ g/ml) in PBST were added to the FCB-5-coated wells, and incubated for 75 min at 37 °C. The wells were washed three times with PBST and once with 0.1 M-Tris/HCl buffer, pH 8.0, containing 0.1% (v/v) Tween 80, pH 8.0. In control experiments, wells not coated with FCB-5 were used.

To demonstrate binding of t-PA, the assay described by Verheijen *et al.* (1982a) was performed based on the activation of plasminogen. Wells not coated with FCB-5 did not show any t-PA binding.

Plasminogen binding to FCB-5 was assessed similarly. In this case, however, possible binding of plasminogen to adsorbed FCB-5 was demonstrated by addition of t-PA (2.5 i.u./ml), to activate bound plasminogen if any.

SDS/PAGE was performed according to the method of Schagger & von Jagow (1987). Sample solutions were mixed with an equal volume of incubation buffer. The composition of the incubation buffer was 4% (w/v) SDS/12% (v/v) glycerol/50 mM-Tris/2% (v/v) 2-mercaptoethanol/0.01% (w/v) Coomassie Brilliant Blue G 250 adjusted with HCl to pH 6.8. Non-reducing buffer had the same composition, but contained no 2-mercaptoethanol. Staining was carried out as described by Schagger & von Jagow (1987). The N-terminal amino acid sequences were determined with a gas-phase Sequenator (Applied Biosystems model 490A protein sequencer, on-line equipped with a model 120A phenylthiohydantoin analyser).

Isolation of rate-enhancing fragments from a whole CNBr digest of fibrinogen. A whole CNBr digest of 1 g of fibrinogen was dialysed extensively against distilled water and freeze-dried. The dry protein was dissolved in a minimum amount of distilled water and acidified with formic acid until a clear solution was obtained. This solution was applied to a Bio-Gel P-2 column (2.5 cm $\times$ 80 cm) run in distilled water acidified to pH 4.0 with acetic acid. Two peaks with rate-enhancing activity were observed: one at the void volume and one eluted much later between the void volume and the total volume of the column. The void-volume peak contained the previously described stimulatory FCB-2 fragment (Nieuwenhuizen *et al.*, 1983b). The material in the peak eluted later was further purified by adjustment of the pH to 8.3 with NaOH solution. The stimulatory material precipitated quantitatively in a virtually pure form. Remaining impurities were removed by rechromatography on Bio-Gel P-2. The yield was approximately 25 mg (i.e. approx. 70% of the theoretical maximum).

RESULTS

Bio-Gel P-2 chromatography of a dialysed CNBr digest of fibrinogen yielded the elution pattern shown in Fig. 1. Two peaks with rate-enhancing capacity were observed: one at the void volume and one eluted much later. The M_r and purity of the material in these fractions with stimulatory activity were de-

Fig. 1. Elution pattern of a fibrinogen CNBr digest on a Bio-Gel P-2 column

Hatched bars indicate the position of fractions with plasminogen-activation-rate-enhancing material, which were pooled.

Fig. 2. SDS/PAGE of rate-enhancing fibrinogen CNBr fragment isolated by Bio-Gel P-2 chromatography

Lane 1, void volume material; lane 2, low- M_r protein markers (Pharmacia); lane 3, peptide markers (Pharmacia); lanes 4 and 5, near total-volume material, reduced; lane 6, same as lanes 4 and 5 but not reduced; lane 7, peptide markers.

Table 1. Amino acid sequences of 6500- M_r stimulating fragment

Cycle	Amino acid	Amount (pmol)
1	Gln	19
	Asn	37
2	Phe	22
	Lys	84
3	Ser	22
	—	—
4	Thr	10
	His	15
5	Trp	11
	Ala	41
6	Asp	22
	Gly	32
7	Asn	7
	His	7

terminated by SDS/PAGE. The results of SDS/PAGE are shown in Fig. 2. The void-volume stimulatory peak contained a broad band around M_r 43 000, which corresponds to the previously described stimulating fragment FCB-2 (Nieuwenhuizen *et al.*, 1983b). The low- M_r rate-enhancing fractions showed a main band of apparent M_r 6500. The fractions of this latter peak were pooled. The 6500- M_r material precipitated in a virtually pure form on adjustment of the pH to 8.3 with NaOH. Remaining traces of contaminants were removed by rechromatography on the same Bio-Gel P-2 column used in the first step. The amino acid sequence of the 6500- M_r material is given in Table 1. It appears from the sequence that the material is composed of two polypeptide chains. This is confirmed by SDS/PAGE, i.e. a band of M_r 2700 and a weakly staining band with M_r 4000 are observed on reduction (Fig. 2). The sequence and the M_r of the

Fig. 4. Rate enhancement of the t-PA-catalysed activation of different forms of plasminogen

(a) Rate enhancement of the t-PA-catalysed activation of Glu-plasminogen (—) and mini-plasminogen (----) by FCB-2. (b) Rate enhancement of the t-PA-catalysed activation of Glu-plasminogen (—), mini-plasminogen (----) and micro-plasminogen (····) by FCB-5.

Fig. 5. Binding of t-PA to FCB-5 adsorbed to microtitre plates

Experimental details are described in the Materials and methods section.

bands before and after reduction show that the fragment is FCB-5 (Henschen, 1981). This fragment consists of the γ -chain stretches γ -(311–336) and γ -(337–379) held together by Cys- γ -326 and Cys- γ -339 (Fig. 3). As a next step we investigated whether this disulphide bond is essential for the expression of the rate-enhancing activity. We digested isolated *S*-carboxymethylated γ -chain with CNBr and checked the digests for rate-enhancing capacity. The whole γ -chain digests did not enhance the rate of plasminogen activation. Since both FCB-5 chains are present in these digests, this indicates that the disulphide bridge in FCB-5 is a prerequisite for stimulatory activity, and that both FCB-5 chains are involved in the rate-

Fig. 3. Partial sequence of fragment FCB-5 (Henschen, 1981)

enhancing capacity. However, a possible deleterious effect of the carboxymethylation on the activity of one or both FCB-5 chains cannot be ruled out. The concentration-dependent effects of FCB-5 on the activation rates of Glu-plasminogen, mini-plasminogen and micro-plasminogen were assessed and compared with FCB-2. The results are shown in Fig. 4.

The most remarkable difference between FCB-5 and FCB-2 is that FCB-5 enhances the rates of Glu-, mini- and micro-plasminogen activation (albeit to a different extent), whereas FCB-2 does not accelerate the mini-plasminogen [in agreement with the results obtained by Verheijen *et al.* (1983) and Nieuwenhuizen *et al.* (1988)] and micro-plasminogen activation.

We could not show any binding of Glu-, mini- or micro-plasminogen to FCB-5. However, t-PA binds to FCB-5 (Fig. 5).

DISCUSSION

Plasminogen activation, catalysed by t-PA, is known to be accelerated in the presence of fibrin. This acceleration cannot be induced by fibrinogen (Wallén, 1977) or only to a limited extent. Some years ago, we demonstrated that fragments derived from fibrin and also from fibrinogen can exert the same effect as fibrin (Verheijen *et al.*, 1982*a,b*). Activation rate-enhancing fragments can be generated from fibrin(ogen) by digestion with plasmin or chemically with CNBr. We concluded that stimulatory sites pre-exist in fibrinogen, which are exposed on conversion of fibrinogen to fibrin and on fragmentation of fibrinogen by plasmin or CNBr.

From our previous work (Voskuilen *et al.*, 1987), we know that the peptide A α -(148–160), which is a part of fragment FCB-2, plays an important role in the acceleration of Glu-plasminogen activation by t-PA. The enhancement induced by this peptide, however, is less than that induced by fibrin. In contrast with fibrin, A α -(148–160) and FCB-2 do not enhance the activation of mini-plasminogen. These facts indicate that more types of stimulatory sites than A α -(148–160) may be present in fibrin.

In the initial phase of the present study we have localized such a site in the C-terminal region of the γ -chain of fibrinogen, known as FCB-5 (Henschen, 1981), as is evident from the sequence data in Table 1. FCB-5 consists of two γ -chain fragments, i.e. γ -(311–336) and γ -(337–379), which are connected by a single disulphide bond. It should be noted that the yields of the cycles of the sequence analysis of the stretch γ -(311–336) are only about 30 % of those of γ -(337–379). This is probably due to a partial conversion of Gln- γ -311 into pyroglutamic acid under the conditions of the CNBr cleavage.

Some anomalous properties of FCB-5 should be noted. First, SDS/PAGE yields an apparent M_r of 6500, whereas the calculated M_r is 7900. The reason for this discrepancy is not clear. Secondly, SDS/PAGE of reduced FCB-5 shows one strongly staining band with an M_r of 2700 and a much more weakly staining band at M_r 4000. This corresponds to the M_r values for γ -(311–336) and γ -(337–379) respectively. It is not clear why the larger peptide γ -(337–379) stains so weakly. This may be due to the electrophoresis system used (Doolittle *et al.*, 1977), since Schagger & von Jagow (1987) state that not all small proteins are detected equally well in their system, since some do not bind the used dye. Thirdly, the late elution position on the Bio-Gel P-2 column is not in agreement with the M_r of FCB-5. This may be due to the unusually high tryptophan and tyrosine content of FCB-5, i.e. six and five residues respectively. Such peptides are known to be retarded on size-exclusion chromatography on columns run in water.

In the second phase of our study we investigated whether one of the two FCB-5 γ -chain remnants contained the constituents essential for the rate enhancement of the Glu-plasminogen

activation by t-PA. We made a CNBr digest of purified S-carboxymethylated γ -chain. The digest (which contains both FCB-5 chains), however, did not show any rate-enhancing properties. Furthermore, we found that FCB-5 lost its rate-enhancing properties on reduction and carboxymethylation (results not shown). This strongly suggests that the disulphide bridge between Cys- γ -326 and Cys- γ -339 is also essential, possibly by keeping (parts of) γ -(311–336) and γ -(337–379) in position. However, the possibility that either γ -(311–336) or γ -(337–379) is sufficient and that the carboxymethylation, which introduces a negative charge on positions 326 and 339, destroys the rate-enhancing capacity of γ -(311–336) and/or γ -(337–379) cannot be excluded.

In our recent studies, we consistently found FCB-5, whereas in our previous studies (Nieuwenhuizen *et al.*, 1983*b*) we did not find FCB-5, despite the fact that the same starting material was used as was chromatographed on Sephadex G-100. One possible explanation is that a different type of dialysis tubing was used (M_r cut-off 10000). FCB-5, which has an apparent M_r of 6500 (calculated 7900), may have passed through the tubing in the previous experiments and hence remained unobserved. To check this possibility we ran gels of a sample of a CNBr digest of fibrinogen that had been prepared and dialysed in the 'old' dialysis tubing about 5 years ago and had been kept at -20°C . The 6500- M_r band was indeed virtually absent from that preparation.

Our previous finding, that FCB-2 and A α -(148–160) accelerate the activation of Glu-plasminogen but not that of mini-plasminogen, strongly suggests that kringle I–IV of plasminogen play an important role in the accelerating effect of FCB-2 and A α -(148–160) on the activation of Glu-plasminogen (Verheijen *et al.*, 1983).

It is interesting that FCB-5 accelerates the activation of Glu-, mini- and micro-plasminogen (Fig. 4) by t-PA. Since micro-plasminogen lacks all kringle domains, this excludes the possibility of an interaction between kringle V and FCB-5 causing a stimulatory activity. FCB-5 has no effect on the amidolytic activity of single-chain t-PA on the tripeptide substrate S-2288 (results not shown).

Our binding experiments show that FCB-5 adsorbed to microtitre plates binds t-PA and none of the plasminogen forms. In this respect FCB-5 differs from FCB-2, which binds, in a purified system, both Glu-plasminogen and t-PA (Bosma *et al.*, 1988). These data strongly suggest that FCB-5 exerts its rate-enhancing action by interaction with t-PA.

As we have previously described, the sequence A α -(150–158) in A α -(148–160) has an interesting charge distribution, i.e.

Sequence: Leu-Glu-Val-Asp-Ile-Asp-Ile-Lys-Ile

Charge: 0 – 0 – 0 – 0 + 0

Negative and neutral amino acids alternate and the sequence ends with a lysine residue followed by a neutral amino acid (isoleucine). This lysine (A α -157) is essential for the rate enhancement (Voskuilen *et al.*, 1987). The sequence γ -(315–322) in γ -(311–336) has a similar charge distribution:

Sequence: Leu-Glu-Val-Asp-Ile-Asp-Ile-Lys-Ile

Charge: 0 – 0 – 0 – 0 + 0

The major differences are that the neutral amino acid residues in γ -(315–322) are more polar (asparagine instead of isoleucine) than in A α -(150–158), and that the neutral amino acid residue preceding the lysine residue is missing. This may be related to the different specificity of FCB-5 compared with FCB-2 for the binding of t-PA and for plasminogen. Application of the Chou–Fasman algorithm (Chou & Fasman, 1978) to the sequences of

the A α -chain and γ -chain predicts that both A α -(150–158) and γ -(315–322) have a random-coil conformation. Use was made of the University of Wisconsin Genetics Computer Group sequence analysis software package (Devereux *et al.*, 1984). This may allow these sequences to adopt easily a special conformation on interaction with t-PA and/or plasminogen.

Both A α -(148–160) and FCB-5 are present in fibrinogen. Yet fibrinogen only very slightly enhances the activation of plasminogen by t-PA (Verheijen *et al.*, 1982b). We have hypothesized previously (Nieuwenhuizen *et al.*, 1983a) that A α -(148–160) is hidden in fibrinogen, and is exposed on fibrin formation or on fibrinogen fragmentation. This was supported by the fact that a monoclonal antibody to A α -(148–160) does not react with fibrinogen, but reacts with fibrin (Schielen *et al.*, 1989). Our results indicate that, like A α -(148–160), at least parts of FCB-5 are also buried in fibrinogen, and become accessible on fibrin formation. To assess this, we have prepared a monoclonal antibody against synthetic γ -(312–324) (Schielen *et al.*, 1991). In a sandwich enzyme immunoassay in which this antibody is used as a capture antibody, in combination with horseradish-peroxidase-labelled polyclonal antibodies against fibrin(ogen)-related material, purified FCB-5 shows a strong immune reactivity. The monoclonal antibody is fibrin-specific (Schielen *et al.*, 1991). This finding not only supports our hypothesis, but the antibody is also a valuable tool to detect fibrin in plasma, which is a molecular marker for an impending thrombotic event. We also tried to block the stimulatory action of FCB-5 with the anti- γ -(312–324) antibody. Although inhibition was observed, some stimulatory activity remained. This appeared to be due to rate enhancement exerted by the monoclonal antibody preparation, which may contain some denatured monoclonal antibody. It is known (Radcliffe & Heinze, 1981; Radcliffe, 1983) that denatured immunoglobulins can enhance the t-PA-catalysed plasminogen activation rate. Furthermore, the existence of two types of rate-enhancing sites which are both exposed in fibrin may help to explain why polymerized fibrin is such a good stimulator. In fibrin, the A α -(148–160) site and the FCB-5 site have a special orientation with respect to each other. It is conceivable that in that situation the A α -(148–160) site binds plasminogen and the FCB-5 site t-PA. This not only concentrates the two reaction partners t-PA and plasminogen on the fibrin surface but also assures a correct mutual orientation. Moreover, as a result of the binding to the stimulatory sites, t-PA appears to become a more effective enzyme, and plasminogen a better substrate.

Thanks are due to Dr. R. Amons, who performed the N-terminal amino acid analysis at the Gas Phase Sequencer Facility (Department of Medical Biochemistry, State University of Leiden, Leiden, The Netherlands), which is supported by the Netherlands Organization for the Advancement of Pure Research (NWO) through the Netherlands Foundation for Chemical Research (SON). Thanks are also due to Dr. W. J. G. Schielen (Catholic University of Nijmegen, The Netherlands)

for performing the Chou–Fasman algorithm calculations, to Mr. K. van Leuven for preparing and characterizing the anti- γ -(312–324) monoclonal antibody and to Mrs. C. and M. Horsting for typing the manuscript.

REFERENCES

- Allan, R. A. & Pepper, D. S. (1981) *Thromb. Haemostasis* **45**, 43–50
- Bosma, P. J., Rijken, D. C. & Nieuwenhuizen, W. (1988) *Eur. J. Biochem.* **172**, 399–404
- Chou, P. Y. & Fasman, G. D. (1978) *Adv. Enzymol. Relat. Areas Mol. Biol.* **47**, 45–147
- Deutsch, D. G. & Mertz, E. T. (1970) *Science* **170**, 1095–1096
- Devereux, J. R., Haeberli, P. & Smithies, O. (1984) *Nucleic Acids Res.* **12**, 387–395
- Doolittle, R. F., Cassman, K. G., Cottrell, B. A., Friezner, S. J., Hucko, J. T. & Takagi, T. (1977) *Biochemistry* **16**, 1703–1709
- Gårdlund, B., Hessel, B., Marguerie, G., Murano, G. & Blombäck, B. (1977) *Eur. J. Biochem.* **77**, 595–610
- Gray, W. R. (1967) *Methods Enzymol.* **11**, 139–151
- Henschen, A. (1981) *Haemostaseologie* **1**, 49–61
- Hoylaerts, M., Rijken, D. C., Lijnen, H. R. & Collen, D. (1982) *J. Biol. Chem.* **257**, 2812–2919
- Kluft, C., Van Wezel, A. L., Van der Velden, C. A. M., Emeis, J. J., Verheijen, J. H. & Wijngaards, G. (1983) in *Advances in Biotechnological Processes* (Mizrahi, A. & Van Wezel, A. L., eds.), vol. 2, pp. 97–110, Alan R. Liss, New York
- Nieuwenhuizen, W. (1988) in *Tissue-type Plasminogen Activator (t-PA): Physiological and Clinical Aspects* (Kluft, C., ed.), vol. 1, pp. 171–187, CRC Press, Boca Raton
- Nieuwenhuizen, W., Vermond, A., Voskuilen, M., Traas, D. W. & Verheijen, J. H. (1983a) *Biochim. Biophys. Acta* **748**, 86–92
- Nieuwenhuizen, W., Verheijen, J. H., Vermond, A. & Chang, G. T. G. (1983b) *Biochim. Biophys. Acta* **755**, 531–533
- Nieuwenhuizen, W., Voskuilen, M., Vermond, A., Hoegee-de Nobel, B. & Traas, D. W. (1988) *Eur. J. Biochem.* **174**, 163–169
- Radcliffe, R. (1983) *Biochim. Biophys. Acta* **743**, 422–430
- Radcliffe, R. & Heinze, T. (1981) *Arch. Biochem. Biophys.* **211**, 750–761
- Rijken, D. C., Wijngaards, G., Zaal-de Jong, M. & Welbergen, J. (1979) *Biochim. Biophys. Acta* **580**, 140–153
- Schägger, H. & von Jagow, G. (1987) *Anal. Biochem.* **166**, 368–379
- Schielen, W. J. G., Voskuilen, M., Tesser, G. I. & Nieuwenhuizen, W. (1989) *Proc. Natl. Acad. Sci.* **86**, 8951–8954
- Schielen, W. J. G., Adams, H. P. H. M., van Leuven, K., Voskuilen, M., Tesser, G. I. & Nieuwenhuizen, W. (1991) *Blood* **77**, 2169–2173
- Shi, G.-Y. & Wu, H.-L. (1988) *J. Biol. Chem.* **263**, 17071–17075
- Sottrup-Jensen, L., Claeys, H., Zajdel, H., Petersen, T. E. & Magnusson, S. (1978) *Prog. Chem. Fibrinolysis Thrombolysis* **3**, 191–209
- Verheijen, J. H., Mullaart, E., Chang, G. T. G., Kluft, C. & Wijngaards, G. (1982a) *Thromb. Haemostasis* **48**, 266–269
- Verheijen, J. H., Nieuwenhuizen, W. & Wijngaards, G. (1982b) *Thromb. Res.* **27**, 377–385
- Verheijen, J. H., Nieuwenhuizen, W., Traas, D. W., Chang, G. T. G. & Hoegee, E. (1983) *Thromb. Res.* **32**, 87–92
- Voskuilen, M., Vermond, A., Veeneman, G. H., Van Boom, J. H., Klasen, E. A., Zegers, N. D. & Nieuwenhuizen, W. (1987) *J. Biol. Chem.* **262**, 5944–5946
- Wallén, P. (1977) in *Thrombosis and Urokinase* (Paoletti, R. & Sherry, S., eds.), vol. 9, pp. 91–102, Academic Press, New York
- Wallén, P. & Wiman, B. (1970) *Biochim. Biophys. Acta* **221**, 20–30

Received 20 June 1991/19 August 1991; accepted 25 September 1991