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

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In previous publications [e.g. Voskuilen, Vermond, Veenenman, Van Boom, Klasing, Zegers & Nieuwenhuizen (1987) J. Biol. Chem. 262, 5944–5946] we have shown that fibrinogen chain fragment Az-(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 Az-(148–160) are present in fibrin. In the present investigation we have localized a possible second type of stimulatory site in the fibrinogen 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 Az-(148–160)]; the latter had not been observed before and was characterized further. The stimulating material in the low-M₆ fraction of the Bio-Gel P-2 column was precipitated at pH 8.3 in a virtually pure form. It has a high trypothan content, and an M₆ of 6500 as assessed by SDS/PAGE. On reduction, a main band of M₆ 2500 is seen, plus a weakly staining band of M₆ 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 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; Hoyalerts et al., 1982).

We have demonstrated that not only fibrin but also plasmin-generated fragments of both fibrin (D-dimer) and fibrinogen (D₄₅₀ₐ₅₄) 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 Az-(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 Az-(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 Az-(148–160), does not enhance the activation of mini-plasminogen (Verheijen et al., 1982b; Nieuwenhuizen et al., 1988).

In the present study we have identified 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 (= Hol-DSK), CNBr-cleavage fragment of fibrinogen consisting of fibrinogen chain fragments Az-(148–207), Bβ-(191–234), Bβ-(225–242), Bβ-(243–305) and γ-(95–265) disulphide-bond linked; FCB-5, CNBr-cleavage fragment of fibrinogen consisting of fibrinogen chain fragments γ-(311–336) and γ-(337–379), disulphide-bond linked; D₄₅₀ₐ₅₄, Plasmin-generated fragment of fibrinogen consisting of fibrinogen chain fragments Az-(111–197), Bβ-(134–461) and γ-(86–303), disulphide-bond linked; D₄₅₀ₐ₅₄, Plasmin-generated fragment of fibrinogen (formed in the presence of Ca²⁺) consisting of fibrinogen chain fragments Az-(111–197), Bβ-(134–461) and γ-(86–411); D-dimer, plasmin-generated fragment of cross-linked fibrin, with the formula (D₄₅₀ₐ₅₄); PBST, 0.05 mm-NaHPO₄/1.6 mm-NaH₂PO₄/0.15 mm-NaCl, pH 7.4, containing 0.05% (v/v) Tween 20.

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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, 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 Kluit 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 was a Mr of 95000. End-group analysis by the dansyl method (Gray, 1967) yielded glutamic acid as the N-terminal amino acid. Mini-plasminogen (Val442-plasminogen) was prepared by limited elastase digestion of Glu-plasminogen and purified as described by Sottrup-Jensen et al. (1978). Micro-plasminogen (lacking all y-krings) 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 A1οm 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, cut-off of 1000 and freeze-dried; the digest of the γ-chain was dialysed 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 0.1 μM-Glu-plasminogen, mini-plasminogen or micro-plasminogen, 0.3 mM–S-2251, 300 μIU 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 microtitre plates were coated with FCB-5 by overnight incubation with an FCB-5 solution (10 μg/ml) in 0.05 M-Tris/HCl buffer, pH 8.4. After three washes with 10 mM-Na2HPO4/1.6 mm-NaH2PO4/0.15 M-NaCl, pH 7.4, containing 0.05% (w/v) Tween 20, pH 7.4 (PBST) 100 μl portions of serial 2-fold dilutions of a t-PA solution (5 μg/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 5.0, containing 0.1% (v/v) Tween 80, pH 5.0. 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 Schägger & 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 Schägger & 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 × 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 Mr 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](image)

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

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-Mr, 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-Mr stimulating fragment

<table>
<thead>
<tr>
<th>Cycle</th>
<th>Amino acid</th>
<th>Amount (pmol)</th>
</tr>
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<tbody>
<tr>
<td>1</td>
<td>Gln</td>
<td>19</td>
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<tr>
<td>2</td>
<td>Asn</td>
<td>37</td>
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<tr>
<td>3</td>
<td>Phe</td>
<td>22</td>
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<td>Thr</td>
<td>10</td>
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<td>5</td>
<td>His</td>
<td>15</td>
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<tr>
<td>6</td>
<td>Trp</td>
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<tr>
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<td>Asn</td>
<td>7</td>
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<tr>
<td></td>
<td>His</td>
<td>7</td>
</tr>
</tbody>
</table>

terminated by SDS/PAGE. The results of SDS/PAGE are shown in Fig. 2. The void-volume stimulatory peak contained a broad band around Mr 43000, which corresponds to the previously described stimulating fragment FCB-2 (Nieuwenhuizen et al., 1983b). The low-Mr, rate-enhancing fractions showed a main band of apparent Mr 6500. The fractions of this latter peak were pooled. The 6500-Mr 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-Mr 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 Mr 2700 and a weakly staining band with Mr 4000 are observed on reduction (Fig. 2). The sequence and the Mr of the 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)

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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., 1982a,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 Aα-(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, Aα-(148–160) and FCB-2 do not enhance the activation of mini-plasminogen. These facts indicate that more types of stimulatory sites than Aα-(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 (Henschel, 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 pyroglyutamic acid under the conditions of the CNBr cleavage.

Some anomalous properties of FCB-5 should be noted. First, SDS/PAGE yields an apparent Mr of 6500, whereas the calculated Mr is 7900. The reason for this discrepancy is not clear. Secondly, SDS/PAGE of reduced FCB-5 shows one strongly staining band with an Mr of 2700 and a much more weakly staining band at Mr 4000. This corresponds to the Mr 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 Schägger & 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-Cel P-2 column is not in agreement with the Mr 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., 1983b) 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 (Mf, cut-off 10000). FCB-5, which has an apparent Mr 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-Mr band was indeed virtually absent from that preparation.

Our previous finding, that FCB-2 and Aα-(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 Aα-(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 Aα-(150–158) in Aα-(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 (Aα-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 Aα-(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 Aα-chain and γ-chain predicts that both Aα-(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 Aα-(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 Aα-(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 Aα-(148–160) does not react with fibrinogen, but reacts with fibrin (Schielen et al., 1989). Our results indicate that, like Aα-(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 fibrinogen-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 & Heinez, 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 Aα-(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 Aα-(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.

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