Biochem. J. (1991) 276, 655–659 (Printed in Great Britain)

Structural requirements of position Aα-157 in fibrinogen for the fibrin-induced rate enhancement of the activation of plasminogen by tissue-type plasminogen activator

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The sequence fibrinogen-Aα-(148–160) can mimic part of the fibrin-induced rate enhancement of the activation of plasminogen by tissue-type plasminogen activator. Previously we have reported that the lysine residue at position Aα-157 is crucial. During our further investigations on Aα-157 we found that lysine at position Aα-157 may be replaced by glutamic acid. This unexpected finding prompted us to re-investigate the requirements of this position. We prepared analogues of Aα-(148–160) in which the lysine residue at position Aα-157 was replaced by lysine derivatives (acetyl-lysine, benzyloxycarbonyl-lysine and methanesulphonylethyloxycarbonyl-lysine), acidic residues (aspartic acid and glutamic acid), basic residues (arginine and ornithine), polar residues (glutamine and methanesulphonylethyloxycarbonylornithine), apolar residues (alanine, valine, norleucine and glutamic acid 4-nitrobenzyl ester) and glycine. These analogues were tested for their stimulatory activity. When aspartic acid, glutamic acid 4-nitrobenzyl ester or norleucine is present at position Aα-157 in Aα-(148–160) virtually all stimulatory capacity is lost. With valine at position Aα-157 the stimulatory activity is marginal. None of the other replacements at position 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 certain requirements: there must be one (as in alanine) or no (as in glycine) carbon atom in the side chain, or at least two carbon atoms and a polar group (charged or uncharged) to which a rather bulky group (such as the benzyloxycarbonyl group) or a polar group (such as the methanesulphonylethyloxycarbonyl group) may be attached. The highest activity [even higher than native Aα-(148–160)] was obtained with ornithine, methanesulphonylethyloxycarbonylornithine or methanesulphonylethyloxycarbonyl-lysine at position Aα-157.

INTRODUCTION

Activation of the coagulation system leads to the formation of thrombin, which converts circulating fibrinogen molecules into fibrin by cleaving off fibrinopeptides A and B from the *N*-terminal ends of the two Aα- and Bβ-chains of fibrinogen respectively. The new *N*-termini of the α- and β-chains contain sites that bind to complementary sites in the *C*-terminal domains (Doolittle, 1981; Laudano & Doolittle, 1981) of other fibrin(ogen) molecules. At low concentrations fibrin monomers are kept in solution by complex formation with 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 conversion of plasminogen into plasmin, as mediated by t-PA, is greatly enhanced by fibrin, but virtually not at all by fibrinogen (Wallén, 1977; Allan & Pepper, 1981; Hoylaerts *et al.*, 1982). Detailed studies (Nieuwenhuizen *et al.*, 1983*a*; Voskuilen *et al.*, 1987; Yonekawa *et al.*, 1989) 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 the CNBr-cleavage fragment FCB-5. The sequence Aα-(148–160) seems to be buried in fibrinogen and becomes exposed upon the conversion of fibrinogen into fibrin 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 conversion of fibrinogen into fibrin, since monoclonal antibodies raised against synthetic γ-(312–325) are fibrin-specific (Schielen *et al.*, 1991). Voskuilen *et al.* (1987) presented evidence that Lys-157 in Aα-(148–160) is essential for the rate-enhancing effect of fibrin, since 3-carboxypropionylation of synthetic Aα-(148–160) annihilated the accelerating capacity of this peptide completely. Moreover, replacing Lys-157 by Val in that study yielded a synthetic peptide that also showed no rate enhancement of the plasminogen activation mediated by t-PA. In our earlier study (Voskuilen *et al.*, 1987) we did not include [Glu^{157}]-Aα-(148–160). When we recently studied the stimulatory

Abbreviations and nomenclature recommended by the IUPAC–IUB Joint Commission on Biochemical Nomenclature (1984) for amino acids and substituents are used in most cases: Orn, ornithine; Nle, norleucine; Ac, acetyl; Z, benzyloxycarbonyl; Msc, methanesulphonylethyloxycarbonyl; Fmoc, fluoren-9-ylmethyloxycarbonyl; Boc, t-butyloxycarbonyl; Pmc, 2,2,5,7,8-pentamethylchroman-6-sulphonyl; Bu^t; t-butyl; ONb, 4-nitrobenzyl ester; OBu^t, t-butyl ester; NH-Np, 4-nitroanilide. Other abbreviations used: t-PA, tissue-type plasminogen activator; D_{EGTA}, plasmin-generated fragment of fibrinogen, formed in the presence of EGTA, consisting of fibrinogen chain fragments Aα-111–197, Bβ-134–461 and γ-86–303, disulphide-bond-linked; FCB-2, CNBr-cleavage fragment of fibrin(ogen) consisting of fibrin(ogen) chain fragments Aα-148–207, Bβ-191–224, Bβ-225–242, Bβ-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.

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capacity of this latter peptide (W. J. G. Schielen, H. P. H. M. Adams, M. Voskuilen, G. I. Tesser & W. Nieuwenhuizen, unpublished work), we observed that it had about 50% of the potency of native Aα-(148–160). This unexpected finding prompted us to re-investigate the requirements of the side chain of the residue in position Aα-157, since it indicates that lysine at position Aα-157 is not an absolute prerequisite for rate enhancement, in contrast with what we concluded before (Voskuilen *et al.*, 1987). We prepared analogues of Aα-(148–160) in which the lysine residue at position Aα-157 was replaced by lysine analogues [Lys(Ac), Lys(Z) and Lys(Msc)], acidic residues (Asp and Glu), basic residues (Arg and Orn), polar residues [Gln and Orn(Msc)], apolar residues [Ala, Val, Nle and 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 certain requirements: there must be one (as in Ala) or no (as in Gly) carbon atom in the side chain, or at least two carbon atoms and a polar group (charged or uncharged) to which a rather bulky group (such as the Z group) or a polar group (such as 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 by the procedure of 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 & Mertz, 1970) on lysine–Sepharose (Pharmacia Fine Chemicals, Uppsala, Sweden), precipitated with $(NH_4)_2SO_4$ and dialysed extensively against 0.1 M-Tris/HCl buffer, pH 7.5, containing 0.1% (v/v) Tween 80 (Baker Chemicals, Phillipsburg, NJ, U.S.A.).

Peptides

The peptide analogues [Xaa^{157}]-Aα-(148–160), where Xaa^{157} is 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) from N^{α}-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 groups of lysine with Boc, the δ-guanidino group of arginine with Pmc, the γ-carboxy group of glutamic acid and the β-carboxy group of aspartic acid with OBu^t, and the β-hydroxy group of serine with Bu^t. The *C*-terminal amino acid (0.9 equiv.) was coupled to the resin (1.07 mmol/g) by activation *in situ* with dicyclohexylcarbodi-imide (0.9 equiv.) and 4-(dimethylamino)-pyridine (0.9 equiv.). To suppress racemization, 1-hydroxy-1*H*-benzotriazole (1.8 equiv.) was added (van Nispen *et al.*, 1985). All reactants were dissolved in dimethylformamide. After 16 h at 5 °C the resin was washed three times with successively dimethylformamide, dichloromethane and propan-2-ol. Finally, the resin was washed with di-isopropyl 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% (v/v) piperidine in dimethylformamide, during three consecutive cycles of 6 min. Coupling of the second amino acid derivative (3 equiv. relative to the amount of the first amino acid attached to the resin) was performed by activation *in situ* with 2-(1*H*-benzotriazol-1-yl)-1,1,3,3-tetramethyluronium tetrafluoroborate (3 equiv.) (Knorr *et al.*, 1989), 1-hydroxy-1*H*-benzotriazole (3 equiv.) and *N*-methylmorpholine (4.5 equiv.). After coupling of each amino acid derivative, completion of the acylation reaction was monitored by a ninhydrin test for free amino groups (Kaiser *et al.*, 1970), followed by acylation of any remaining free amino groups with 10% (v/v) acetic anhydride in dimethylformamide. The fully protected peptides were cleaved from the resin during a 4 h reaction with 2.5% (v/v) ethanedithiol and 2.5% (v/v) water in trifluoroacetic acid followed by precipitation in diethyl ether. The crude peptides were washed several times with diethyl ether, dried in air and analysed by h.p.l.c., specific rotation and amino acid composition.

H.p.l.c. analyses

H.p.l.c. analyses were performed on an RP-18 column (Techsil 5 C_{18}; 250 mm × 4.6 mm; HPLC Technology, Macclesfield, Cheshire, U.K.) at a flow rate of 1.0 ml/min, with 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 from 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 u.v. measurement at 215 nm. The u.v. data were analysed by using JCL 6000 Chromatography Data System software (Jones Chromatography, Hengoed, Mid-Glamorgan, U.K.).

Amino acid analyses

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

Specific rotations

The specific rotations were measured with a Perkin–Elmer 241 polarimeter. All peptides were dissolved in dimethylformamide and centrifuged at 10000 *g* for 10 min before the measurement.

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

In total volumes of 0.250 ml of 0.1 M-Tris/HCl buffer, pH 7.5, containing 0.1% (v/v) Tween 80 were present 0.11 μM-Glu-plasminogen, 0.3 mM-D-Val-Leu-Lys-NH-Np, 300 m-i.u. of t-PA and various concentrations of the synthetic peptide (eight different concentrations from 0–500 μM) to be tested for stimulating activity. In this assay the concentration of 4-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 the stimulation factor.

RESULTS

The amino acid analyses, specific rotations and h.p.l.c. analyses showed that the purity of the peptides was such that no purification of any of the synthetic peptides was required.

Table 1 summarizes the stimulation factors found for the individual peptides. The concentrations of all peptides needed

Table 1. Maximum stimulation factors of the synthetic peptides

Maximum stimulation factors of the synthetic peptides were obtained with the assay system described in the Materials and methods section. The results are given as means ± S.D. for three experiments.

Peptide	Maximum 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
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

for half-maximal stimulation were around 50 μM; the maximum stimulation factor of all stimulatory peptides was reached between 150 and 300 μM.

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-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 disulphide rings that terminate the coiled-coil regions, 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) 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 conversion of fibrinogen into fibrin, e.g. by thrombin, or by chemical or enzymic digestion of fibrinogen.

Radcliffe (1983) presented evidence that lysine residues in fibrin are essential for the accelerating capacity of fibrin on the activation of plasminogen by t-PA. Plasmin is known to have lysine-binding sites that 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-aminohexanoic acid, which is another indication for the importance of the lysine-binding sites in the plasminogen molecule. The rate enhancement of the activation of plasminogen by t-PA has been ascribed mainly to *C*-terminal lysine residues (Christensen, 1984; Suenson *et al.*, 1984; Norrman *et al.*, 1985). The *C*-terminal lysine residues seem to fulfil the requirements for binding to the lysine-binding sites of plasminogen, i.e. an ε-amino- and an α-carboxy group separated by an aliphatic carbon chain. However, Voskuilen *et al.* (1987) showed that Lys-157 in the Aα-chain plays an essential role in the rate enhancement, since 3-carboxypropionylation of this lysine residue annihilates the stimulating effect of Aα-(148–160) completely. This lysine residue at Aα-157 is not a *C*-terminal residue, and therefore some non-*C*-terminal lysine residues may also contribute to the observed rate enhancement by fibrin.

In another study (Schielen, 1990), in which we prepared the *N*-terminally acylated hexapeptides Aα-(152–157), Aα-(153–158), Aα-(154–159) and Aα-(155–160), we showed that only the hexapeptide Aα-(154–159) is capable of stimulating the conversion of plasminogen into plasmin. Since all of these peptides contain lysine at position Aα-157, we concluded that the orientation of Lys-157 is important and we assumed that the stretch Aα-(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 belief is that lysine residues play a crucial role in the rate-enhancing properties of fibrin. This was corroborated by our study (Voskuilen *et al.*, 1987) in which we replaced Lys at position Aα-157 by several other amino acid residues. All replacements in that study led to peptides with virtually no stimulating capacity. That study did not include [Glu^{157}]-Aα-(148–160). Recently we prepared and tested the latter peptide (W. J. G. Schielen, H. P. H. M. Adams, M. Voskuilen, G. I. Tesser & W. Nieuwenhuizen, unpublished work) and surprisingly [Glu^{157}]-Aα-(148–160) appeared to be stimulatory. This was the reason for repeating and extending our previous work.

The results of the present re-investigation clearly show (in contrast with our earlier conclusions) that lysine is not the only residue that is allowed at position Aα-157 in the peptide Aα-(148–160) to maintain stimulatory capacity. In fact, from Table 1 it seems that many different residues are allowed in this position. These findings support the hypothesis of a certain typical (local) conformation, in which it is not the lysine residue at position Aα-157 as such that 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 to a lesser extent Val at position Aα-157 in the peptide Aα-(148–160) probably induces a conformation that 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 Fig. 1. The rate enhancement is not due to an effect on the plasmin activity, since at the concentrations used the peptides have no effect on the plasmin activity, as measured with D-Val-Leu-Lys-NH-Np as a substrate.

In a previous paper (Voskuilen *et al.*, 1987) we reported that [Arg^{157}]-Aα-(148–160) did not exert any stimulating capacity. We have now found (Table 1) that [Arg^{157}]-Aα-(148–160) in fact stimulates equally as 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 earlier rate-enhancement experiments. It now appears that (excessively) high concentrations of the peptide inhibit the activation of plasminogen by t-PA.

In Fig. 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 Fig. 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 certain requirements: there must be one (as in Ala) or no (as in Gly) carbon atom in the side chain, or at least two carbon atoms and a polar group (charged or uncharged) to which a rather bulky group (such as the Z group) or a polar group (such as the Msc group) may be attached. Val at position Aα-157 takes an intermediate position. The peptide [Val^{157}]-Aα-(148–160) has a low stimulatory

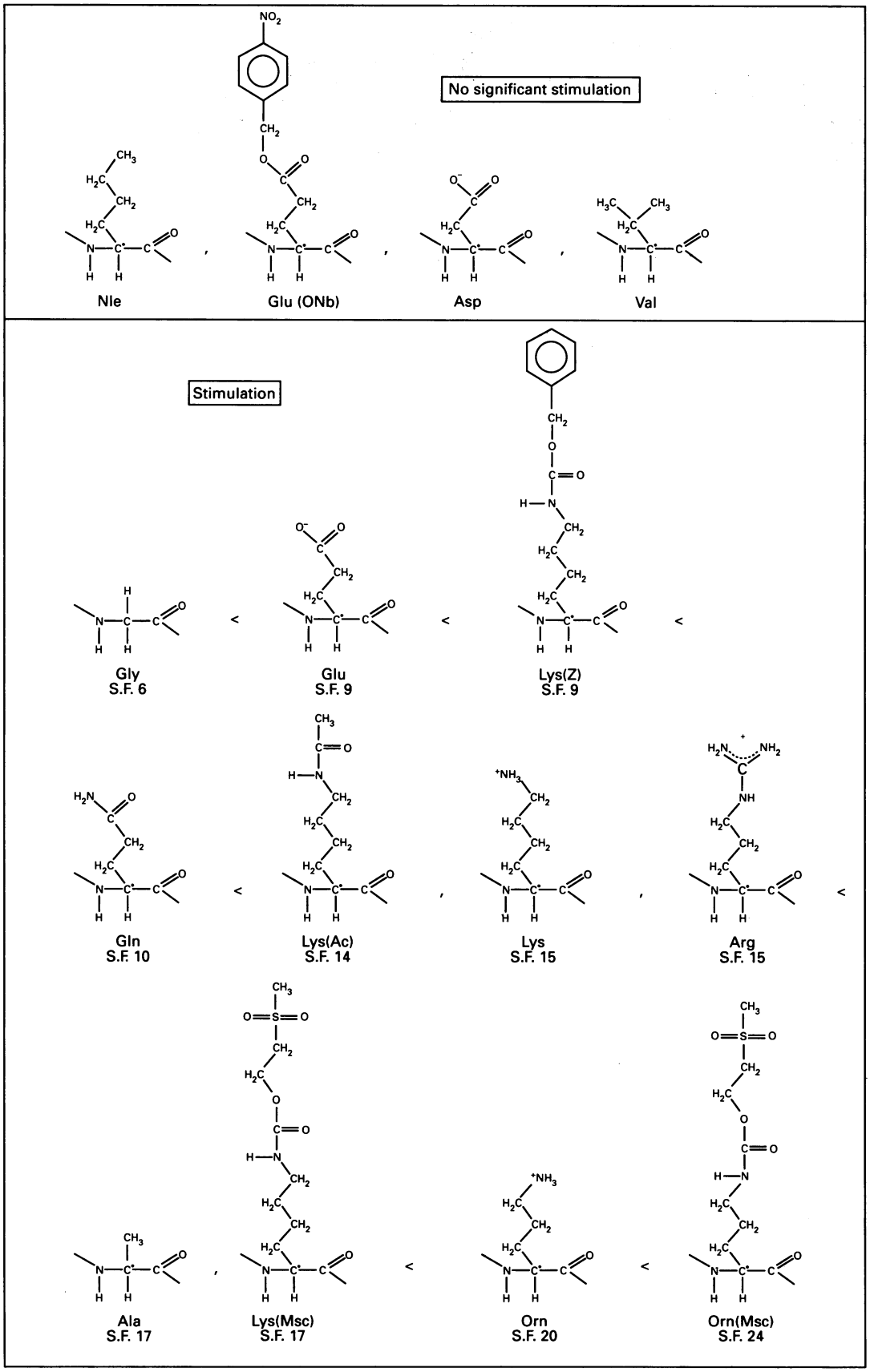

Fig. 1. Structures of Xaa in the peptide analogues [Xaa^{157}]-Aα-(148–160), listed in order of increasing stimulating capacity

Abbreviation: S.F., maximum stimulation factor.

activity on the borderline of significance. Introduction of the ONb group on the Glu side chain annihilates the rate enhancement completely, possibly because of the apolar nature of the resulting side chain, which may be similar to that of Nle. The peptides [Glu(ONb)^{157}]-, [Val^{157}]- and [Nle^{157}]-Aα-(148–160) tend to have a high affinity for the column material (C_{18}) in reverse-phase h.p.l.c., as evidenced by the long retention times of these peptides (results not shown). Introduction of a 3-carboxypropionyl group at the ϵ-amino group of Lys may also give rise to an apolar side chain, since the results obtained by Voskuilen *et al.* (1987) show that 3-carboxypropionylation of synthetic Aα-(148–160) annihilates the stimulating effect of Aα-(148–160) completely. On the other 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 owing to compensation by the polar ϵ-amino group.

From Table 1 and Fig. 1 it is clear that increases in the stimulating capacity of Aα-(148–160) can be obtained, the largest being obtained 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 lysine residue at position Aα-157 can be replaced by a variety of other residues or amino acid derivatives, indicating that it is not the residue at position Aα-157 that is important, but rather 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 by t-PA of the conversion of plasminogen into plasmin.

REFERENCES

Allan, R. A. & Pepper, D. S. (1981) Thromb. Haemostasis **45**, 43–50

Christensen, U. (1984) Biochem. J. **223**, 413–421

Collen, D. (1980) Thromb. Haemostasis **43**, 77–89

Deutsch, D. G. & Mertz, E. T. (1970) Science **170**, 1095–1096

Doolittle, R. F. (1981) Sci. Am. **254**, 92–101

Doolittle, R. F., Goldbaum, D. M. & Doolittle, L. R. (1978) J. Mol. Biol. **120**, 311–325

Drapier, J. C., Tenu, J. P., Lemaire, G. & Petit, J. F. (1979) Biochimie **61**, 163–171

Hoylaerts, M., Rijken, D. C., Lijnen, H. R. & Collen, D. (1982) J. Biol. Chem. **257**, 2912–2919

IUPAC–IUB Joint Commission on Biochemical Nomenclature (JCBN) (1984) Biochem. J. **219**, 345–373

Kaiser, E., Colescott, R. L., Bossinger, C. D. & Cook, P. I. (1970) Anal. Biochem. **34**, 595–598

Kluft, C., Van Wezel, A. L., Van der Velden, C. A. M., Emeis, J. J., Verheijen, J. H. & Wijngaards, G. (1983) Adv. Biotechnol. Processes **2**, 97–100

Knorr, R., Trzeciak, A., Bannwarth, W. & Gillessen, D. (1989) Tetrahedron Lett. **30**, 1927–1930

Laudano, A. P. & Doolittle, R. F. (1981) Science **212**, 457–459

Nieuwenhuizen, W., Vermond, A., Voskuilen, M., Traas, D. W. & Verheijen, J. H. (1983*a*) Biochim. Biophys. Acta **748**, 86–92

Nieuwenhuizen, W., Verheijen, J. H., Vermond, A. & Chang, G. T. G. (1983*b*) Biochim. Biophys. Acta **755**, 531–533

Nieuwenhuizen, W., Voskuilen, M., Traas, D. W., Hoegee-de Nobel, B. & Verheijen, J. H. (1985) in Fibrinogen: Structural Variants and Interactions (Henschen, A., Hessel, B., McDonagh, J. & Saldeen, T., eds.), vol. 3, pp. 331–342, Walter de Gruyter, Berlin

Norrman, B., Wallén, P. & Rånby, M. (1985) Eur. J. Biochem. **149**, 193–201

Radcliffe, R. (1983) Biochim. Biophys. Acta **743**, 442–430

Rijken, D. C., Wijngaards, G., Zaal-De Jong, M. & Welbergen, J. (1979) Biochim. Biophys. Acta **580**, 140–153

Schielen, W. J. G. (1990) Ph.D. Thesis, University of Nijmegen

Schielen, W. J. G., Voskuilen, M., Tesser, G. I. & Nieuwenhuizen, W. (1989) Proc. Natl. Acad. Sci. U.S.A. **86**, 8951–8954

Schielen, W. J. G., Adams, H. P. H. M., van Leuven, K., Voskuilen, M., Tesser, G. J. & Nieuwenhuizen, W. (1991) Blood **77**, in the press

Suenson, E., Lützen, O. & Thorsen, S. (1984) Eur. J. Biochem. **140**, 513–522

ten Kortenaar, P. B. W., van Dijk, B. G., Peeters, J. M., Raaben, B. J., Adams, P. J. H. M. & Tesser, G. I. (1986) Int. J. Pept. Protein Res. **27**, 398–400

van Nispen, J. W., Polderdijk, J. P. & Greven, H. M. (1985) Rec. Trav. Chim. Pays-Bas **104**, 99–100

Verheijen, J. H., Nieuwenhuizen, W. & Wijngaards, G. (1982) Thromb. Res. **27**, 377–385

Verheijen, J. H., Nieuwenhuizen, W., Mullaart, E., Chang, G. T. G. & Vermond, A. (1983*a*) in Fibrinogen: Structure, Functional Aspects, Metabolism (Haverkate, F., Henschen, A., Nieuwenhuizen, W. & Straub, P. W., eds.), vol. 2, pp. 305–314, Walter de Gruyter, Berlin

Verheijen, J. H., Nieuwenhuizen, W., Traas, D. W., Chang, G. T. G. & Hoegee-de Nobel, B. (1983*b*) Thromb. Res. **32**, 87–92

Verheijen, J. H., Nieuwenhuizen, W., Traas, D. W., Chang, G. T. G. & Hoegee-de Nobel, B. (1985) in Fibrinogen: Structural Variants and Interactions (Henschen, A., Hessel, B., McDonagh, J. & Saldeen, T., eds.), vol. 3, pp. 313–322, Walter de Gruyter, Berlin

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

Wang, S. S. (1973) J. Am. Chem. Soc. **95**, 1328–1333

Yonekawa, O., Vermond, A. & Nieuwenhuizen, W. (1989) Thromb. Haemostasis **62**, Abstr. 194

Received 18 September 1990/28 November 1990; accepted 10 December 1990