ELSEVIER

Biophysical Chemistry 110 (2004) 93–100

Biophysical Chemistry

www.elsevier.com/locate/bpc

Evidence that catalytically-inactivated thrombin forms non-covalently linked dimers that bridge between fibrin/fibrinogen fibers and enhance fibrin polymerization

M.W. Mosesson*, I. Hernandez, K.R. Siebenlist

The Blood Research Institute of the Blood Center of Southeastern Wisconsin, P.O. Box 2178, Milwaukee, WI 53201-2178, USA

Received 18 December 2003; received in revised form 22 January 2004; accepted 22 January 2004
Available online 5 May 2004

Abstract

Phe-pro-arg-chloromethyl ketone-inhibited α-thrombin [FPRα-thr] retains its fibrinogen recognition site (exosite 1), augments fibrin/fibrinogen [fibrin(ogen)] polymerization, and increases the incorporation of fibrin into clots. There are two 'low-affinity' thrombin-binding sites in each central E domain of fibrin, plus a non-substrate 'high affinity' γ′ chain thrombin-binding site on heterodimeric 'fibrin(ogen) 2' molecules ($\gamma_{A,}\gamma'$). 'Fibrin(ogen) 1' ($\gamma_{A,}\ \gamma_{A}$) containing only low-affinity thrombin-binding sites, showed concentration-dependent FPRα-thr enhancement of polymerization, thus indicating that low-affinity sites are sufficient for enhancing polymerization. FPRγ-thr, whose exosite 1 is non-functional, did not enhance polymerization of either fibrin(ogen)s 1 or 2 and DNA aptamer HD-1, which binds specifically to exosite 1, blocked FPRα-thr enhanced polymerization of both types of fibrin(ogen) (1>2). These results showed that exosite 1 is the critical element in thrombin that mediates enhanced fibrin polymerization. Des Bβ1-42 fibrin(ogen) 1, containing defective 'low-affinity' binding sites, was subdued in its FPRα-thr-mediated reactivity, whereas des Bβ1-42 fibrin(ogen) 2 ($\gamma_{A,}\gamma'$) was more reactive. Thus, the γ′ chain thrombin-binding site contributes to enhanced FPRα-thr mediated polymerization and acts through a site on thrombin that is different from exosite 1, possibly exosite 2. Overall, the results suggest that during fibrin clot formation, catalytically-inactivated FPRα-thr molecules form non-covalently linked thrombin dimers, which serve to enhance fibrin polymerization by bridging between fibrin(ogen) molecules, mainly through their low affinity sites.

Keywords: Thrombin; Fibrinogen; Fibrin; Exosite

Abbreviations: Fibrin(ogen), a solution containing both fibrin and fibrinogen; FPRck, phe-pro-arg-chloromethyl ketone; FPR-thr, FPRck-inactivated thrombin; FPR-α-thr, phe-pro-arg-α-thrombin; FPRγ-thr, phe pro arg γ-thrombin; α-profibrin, a fibrin intermediate lacking one fibrinopeptide A (des A-fibrin); α-fibrin, fibrin lacking both fibrinopeptides A (des AA-fibrin); FPA, fibrinopeptide A; E_A, complementary polymerization site on the α chain exposed by cleavage of FPA and beginning with the sequence gly-pro-arg.

* Corresponding author. Tel.: +1-414-937-3811; fax: +1-414-9376284.
E-mail address: mwmosesson@bcsew.edu (M.W. Mosesson).

0301-4622/$ - see front matter © 2004 Published by Elsevier B.V.
doi:10.1016/j.bpc.2004.01.007

1. Introduction

Kaminski and coworkers [1,2] showed that FPRck-inhibited thrombin, which retains the ability to bind to fibrin or fibrinogen through exosite 1 [3–6], accelerates fibrin polymerization and increases the incorporation of fibrin and fibrinogen into clots. In contrast, hirudin-inhibited thrombin, by virtue of bridge-binding of hirudin to both exosite 1 and the thrombin substrate pocket, does not bind to fibrin(ogen) [5,7], and consequently has no measurable effect on fibrin polymerization. Augmented polymerization depended either upon generation of fibrin by the addition of thrombin to fibrinogen or upon preparing a soluble mixture of fibrin and fibrinogen [2]. At the time those experiments were published we suggested that FPRck-inhibited thrombin enhanced polymerization through its ability to bridge between fibrin and fibrinogen, though not in a mechanistically defined way.

Since that report, a more complicated picture of thrombin binding to fibrin(ogen) has emerged, and this new information has caused us to expand the scope of our inquiry. First, investigation of non-substrate thrombin binding sites in fibrin revealed two low-affinity sites in the central E domain and in addition, a thrombin binding site of relatively high affinity in the C-terminal region of a variant γ chain termed γ′ [8]. Secondly, we have considerably more information about the specific sites and residues on the thrombin molecule that account for binding to fibrin(ogen) [9–12]. Furthermore, the reports by Lovely et al. [13] and Pospisil et al. [14] suggesting that the γ′ site on fibrin(ogen) binds to thrombin exosite 2, introduced the possibility that the acceleratory effect of FPR-thr on fibrin polymerization might be attributable to divalency of a single thrombin molecule. However, some data have suggested that thrombin itself may become dimeric. One of two recently published X-ray structures of two thrombin molecules bound to GpIbα indicated that the molecules had assumed a dimer configuration [15], whereas in the other study they did not [16]. Similar symmetry-related intermolecular contacts have been observed in crystals of FPR-thr [10]. The studies presented in this present report reflect renewed efforts to define the mechanism of the polymerization enhancing effect of thrombin on assembling fibrin, especially with regard to the possibility that thrombin might function as a dimer.

2. Experimental

Human fibrinogen 1 ('peak 1 fibrinogen'; γ_A, γ_A) or fibrinogen 2 ('peak 2 fibrinogen'; γ_A, γ') was prepared from plasma fraction I-2 fibrinogen by DEAE-cellulose ion exchange chromatography [17]. Des Bβ1-42 fibrinogen was prepared from fibrinogen 1 or fibrinogen 2 by digestion with *Crotalus atrox* protease [18]. Alpha-thrombin (specific activity, 3 units/μg) and γ-thrombin were purchased from Enzyme Research Laboratories (South Bend, IN). FPRck-inactivated thrombin (FPR-thr) was prepared by adding a five-fold molar excess of FPRck to the thrombin and monitoring residual amidolytic activity at 405 nm in the presence of S-2238 (Diapharma Group). There was no detectable amidolytic activity even after incubating the FPRck-thrombin mixture at 37 °C overnight. FPR-inactivated α- or γ-thrombin was dialyzed against 50 mM Tris, 100 mM NaCl, pH 7.4 buffer (TBS) prior to use. DNA aptamer GGTTGGTGTGGTTGG [19], termed HD-1, was synthesized in our peptide/oligonucletide synthesis core facility by standard methodology.

Turbidity measurements on thrombin-treated fibrinogen solutions were carried out at room temperature in TBS under the following general conditions: Thrombin (0.5 u/ml, 4.5 nM, final or 1.3 u/ml, 11.7 nM, final) was added to a fibrinogen solution (1.47 μM or 2.93 μM, final), rapidly mixed and incubated for 30 s prior to the addition of FPRck, 15 μM, final, or hirudin, 5 u/ml, final, plus additional reagents such as FPR-thr, as appropriate. Following this addition and rapid mixing, the cuvettes were transferred to a recording spectrophotometer and thereafter monitored at 340 nm. Typically, 45 s elapsed from the time of thrombin addition to the first absorbance measurement, and this is indicated as −0.75 min in graphic representations. The negative control for each experiment is the addition of FPRck (15 μM) or hirudin (5 units/ml) prior to thrombin addition. The amount of FPR-thr added is reported as a molar ratio relative to fibrin(ogen). Although representative single experimental sets are shown in most figures, all critical measurements were carried out at least in triplicate. Without exception all results were in close relative agreement with one another.

3. Results

3.1. Rationale

There are three thrombin binding sites on fibrin, two identical 'low-affinity sites in each central E domain [8,20] and a 'high affinity' site that is situated in a γ chain variant termed γ′. This chain amounts to 7–8% of the total γ chain population in fibrinogen and comprises 50% of the γ chain population in the heterodimeric fibrinogen species known as 'fibrinogen 2' [21,22]. The thrombin binding function of the γ′ site was not known at the time of our earlier study [2], and thus our interpretations were predicated upon the following: (1) the polymerization enhancing effect has an absolute requirement for the presence of fibrin; (2) the effect is recognizable by the turbidity differences between 'baseline' hirudin-inhibited thrombin, which binds to thrombin at both its catalytic site and at exosite 1, and FPRck-inhibited thrombin, which after catalytic inactivation still has a fully functional exosite 1.

We speculated that thrombin might function through thrombin-induced conformational changes in fibrin(ogen) that resulted in enhanced fibrin–fibrin or fibrin–fibrinogen interactions. These naïve speculations did not provide any mechanistic insights and ultimately proved to be inaccurate (this study). Since all prior experiments on enhancement of polymerization by FPR-thr had been performed on fibrinogen preparations containing both γ_A and γ′ chains (i.e. plasma fraction I-2), no information was available concerning the specific contribution of the γ′ site to augmented polymerization. Thus, our present report includes comparisons of: (1) FPR-thr enhanced polymerization of fibrinogens 1 and 2; (2) FPRα-thr vs. FPRγ-thr on fibrinogens 1 and 2; (3) the behavior of exosite 1-binding DNA aptamer, HD-1 as a modifier of FPRα-thr enhanced polymerization; (4) FPR-thr enhanced polymerization of des Bβ1-42 fibrinogens 1 and 2, both of which have defective low-affinity thrombin-binding sites.

3.2. Polymerization of fibrin(ogen)s 1 and 2 in the presence of FPRα-thrombin

Fibrinogen 1 contains the two thrombin substrate sites in the E domain, and displayed concentration-dependent FPRα-thr enhancement of turbidity (Fig. 1),

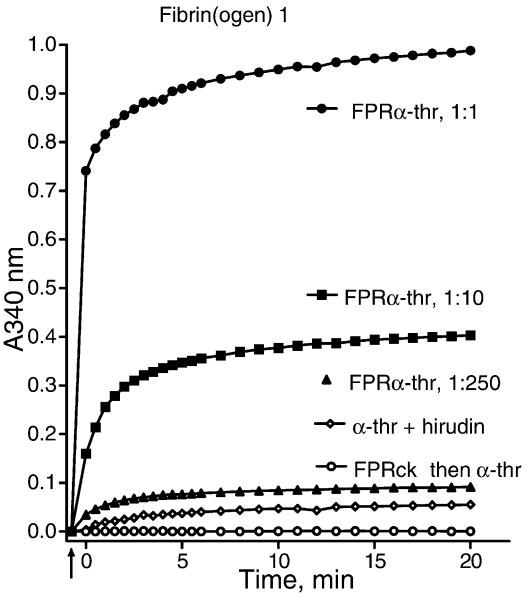

Fig. 1. Turbidity at 340 nm of fibrin(ogen) 1 after thrombin addition (11.7 nM) to fibrinogen (2.93 μM) for 30 s followed by the addition of FPRck (15 μM) plus additional amounts of FPRα-thr, as required. The molar ratios of thrombin to fibrinogen are indicated. Baseline controls included the addition of hirudin (5 u/ml, final) after thrombin treatment. A negative control included the addition of FPRck (this experiment) or hirudin (not shown) prior to thrombin treatment.

thus indicating that 'low-affinity' thrombin-binding sites in the fibrin(ogen) 1 E domain are sufficient for inducing the effect. However, these results did not eliminate the possibility that γ′ chains might also participate in this process, as indeed will be shown later in this experimental series. Like fibrin(ogen) 1, heterodimeric fibrin(ogen) 2 molecules, each containing one γ_A and one γ′ chain, developed concentration-dependent turbidity enhancement by FPRα-thr, but under almost all conditions the ultimate turbidity was lower than that formed from fibrin(ogen) 1 (Fig. 2).[1] These experiments were

[1] Fibrin 2 clots develop lower turbidity than those formed from fibrin 1, and the clot network is comprised of thinner, more branched fibers [23]. This effect is attributable to the fact that the evolution of divalent α-fibrin molecules from fibrinogen 2 is slower than it is from fibrinogen 1 (KR Siebenlist, MW Mosesson, I Hernandez, D Meh, JP DiOrio, JR Shainoff, C-E Dempfle, manuscript in preparation). This behavior of fibrin(ogen) 2 is peripheral to the present subject which primarily concerns FPR-thr dependent enhancement of fibrin(ogen) 1 or 2 polymerization.

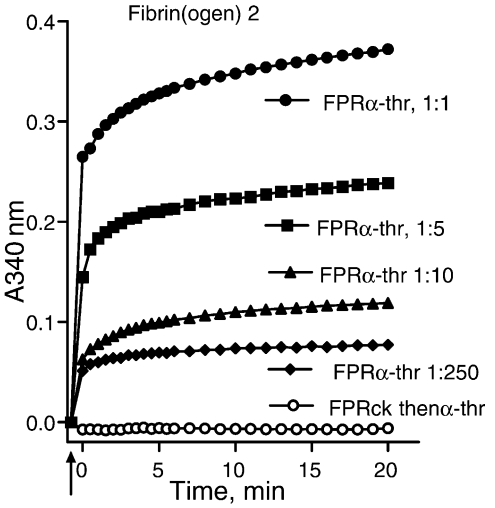

Fig. 2. Turbidity development at 340 nm of fibrin(ogen) 2 after thrombin addition to fibrinogen, followed by the addition of FPRck plus additional amounts of FPRα-thr. The general conditions for this experiment were the same as those described in the Fig. 1 legend.

carried out over a wider range of thrombin concentrations, and showed that maximum turbidity enhancement for fibrin(ogen) 1 occurred at a 2:1 molar ratio of thrombin to fibrin(ogen), a value corresponding to the number of low-affinity thrombin binding sites in the E domain (Fig. 3). In the case of fibrinogen 2, optimal turbidity enhancement occurred at a 1:1 molar ratio, and there were no increases at a 2:1 or 3:1 molar ratio. It seems likely that modest reductions in turbidity occurred at these ratios, and we can suggest some possible explanations for these differences. At any molar ratio of thrombin to fibrinogen, α-thr binding to fibrin(ogen) 2 is partitioned between the γ′ chain site and thrombin-fibrin(ogen) binding sites on E domains. Binding is probably skewed toward the γ′ chain site since its K_D is several fold lower than that for the low-affinity binding sites [8,14,24], and we now know that this value reflects divalent interactions of thrombin with a high- and low-affinity site [14,24]. FPRα-thr interactions at the γ′ chain site are probably less productive in terms of polymerization enhancement than is FPRα-thr binding at low-affinity sites (*vide infra*). It also seems possible that binding at the γ′ site might sterically hinder interactions at low-affinity sites in the E domain.

3.3. Comparison of FPRα-thrombin and FPRγ-thrombin, and the effect of HD-1

Our current understanding of fibrinogen-thrombin interactions stipulates that thrombin binding, and, therefore FPRα-thr enhanced fibrin polymerization, is mediated through thrombin exosite 1 [6,25,26]. Support for that notion was obtained in our present experiments by comparing the effect of FPRα-thr with that of FPRγ-thr. Exosite 1 of FPRγ-thr has been disrupted by proteolytic cleavage [6,27–29], resulting in virtually complete loss of its ability to bind to and cleave fibrinogen. FPRα-thr at a 1:1 molar ratio with respect to either fibrinogen 1 or 2 yielded the anticipated turbidity increment. In contrast, FPRγ-thr had a minimal augmenting effect with either fibrinogen (Fig. 4), corresponding to that observed at the thrombin/fibrinogen ratio of 1:327. At that low ratio the FPRα-thr effect is only slightly greater than that of hirudin-thrombin (cf. Fig. 1). These observations indicate that exosite 1 is the key player in thrombin-mediated enhancement of fibrin polymerization.

HD-1 binds exclusively to thrombin exosite 1 [11,19]. We used this site-specific ligand to further examine the role of exosite 1 in FPRα-thr enhanced polymerization of fibrin(ogen)s 1 and 2 (Fig. 5). At equimolar concentrations with respect to fibrinogen or thrombin, HD-1 treated FPRα-thr (1:1 molar ratio) reduced fibrin(ogen) 1 turbidity to the level observed with FPR-thr at a thrombin/fibrinogen ratio of 1:327.

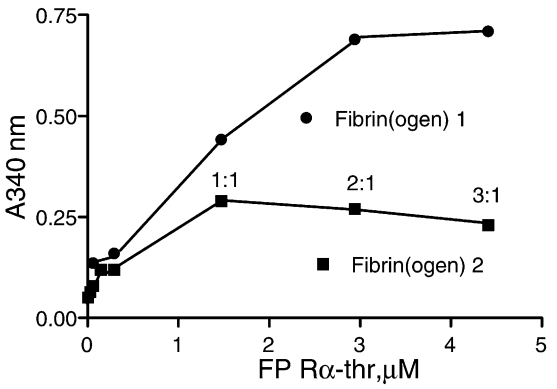

Fig. 3. Turbidity development at 340 nm 20 min after FPRα-thr addition vs. the concentration of FPRα-thr. Thrombin (4.5 nM) was added to fibrinogen (1.47 μM) for 30 s prior to addition of FPRck (15 μM) plus varying amounts of FPRα-thr. The molar ratios of FPRα-thr to fibrinogen are indicated for the higher FPRα-thr levels.

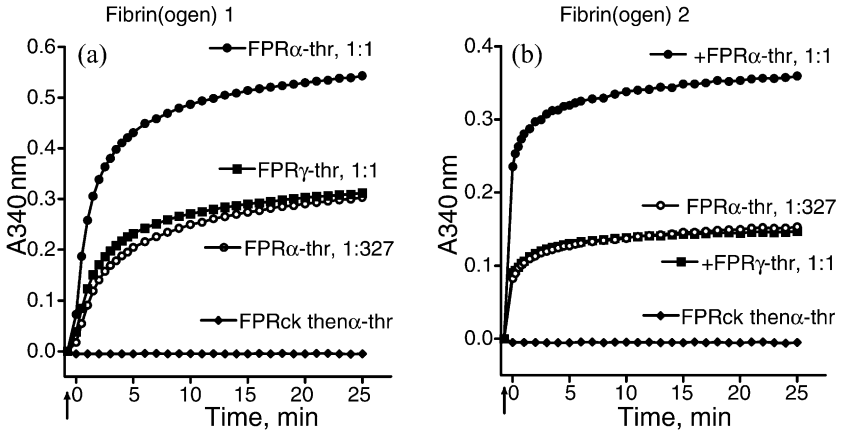

Fig. 4. Comparison of FPRα-thrombin and FPRγ-thrombin augmented turbidity at 340 nm in fibrin(ogen) 1 (panel A) or fibrin(ogen) 2 (panel B). The general conditions for this experiment were the same as those described in the Fig. 3 legend.

In the case of fibrin(ogen) 2, an equimolar ratio of HD-1 to FPRα-thr markedly reduced the level of turbidity, but it did not achieve the virtually 'baseline' value observed with fibrin(ogen 1). Increasing the HD-1/fibrinogen ratio to 2:1 caused only a minor increment in the inhibitory effect in both cases (data not shown). Thus, the findings with fibrin(ogen) 2 suggest that the γ′ chain plays an HD-1 independent role in the polymerization process. Studies of des B1-42 fibrin(ogen) polymerization reported in the next set of experiments support this contention.

3.4. Polymerization of fibrinogen with defective low-affinity thrombin binding in the presence of FPRα-thrombin

FPA is released from des Bβ1-42 fibrinogen at the same rate as it is from native fibrinogen [30], yet low-

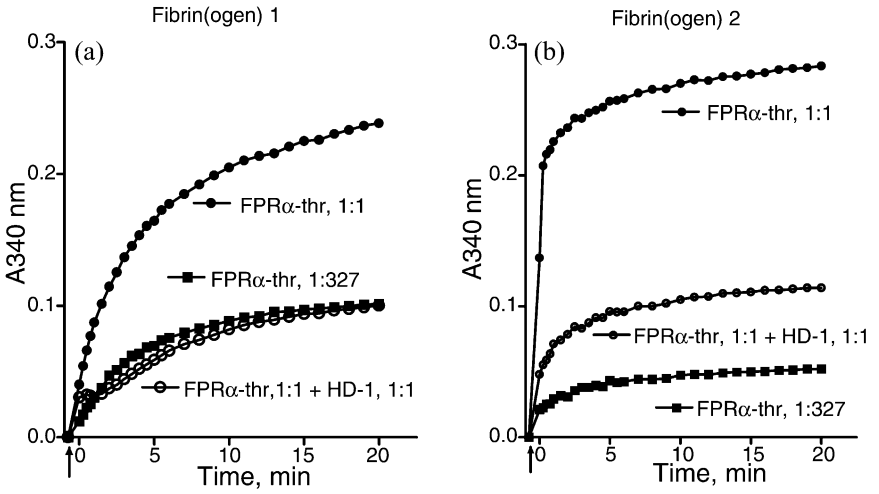

Fig. 5. The effect of HD-1 on FPRα-thrombin augmented polymerization of fibrin(ogen) 1 (panel A) or fibrin(ogen) 2 (panel B). The general conditions for this experiment were the same as those described in the Fig. 3 legend. The molar ratio of FPRα-thr to fibrinogen or HD-1 to fibrinogen is indicated.

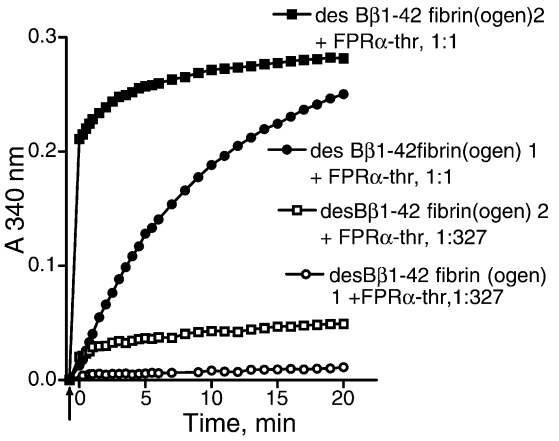

Fig. 6. Comparison of FPRα-thrombin-mediated polymerization enhancement of des Bβ 1-42 fibrin(ogen) 1 or fibrin(ogen) 2. The general conditions for this experiment were the same as those described in the Fig. 3 legend. The molar ratios of FPRα-thr to fibrinogen are indicated.

affinity thrombin binding in the E domain of des Bβ1-42 fibrin is markedly impaired [8,30]. Des Bβ1-42 fibrin also polymerizes slowly and incompletely due to a defective E_A polymerization site [30,31]. In the light of this information, we compared FPRα-thr enhanced polymerization of des Bβ1-42 fibrin(ogen) 1 with that of des Bβ1-42 fibrin(ogen) 2 (Fig. 6). The addition of FPRck to des Bβ1-42 fibrinogen 1 after the usual 30-s exposure to native α-thrombin, resulted in a negligible increment in turbidity (thrombin/fibrinogen ratio, 1:327). Following the addition of FPRα-thr at a 1:1 molar ratio, there was a gradual and progressive increment in turbidity, suggesting that the defective low-affinity thrombin binding potential in the E domain was still capable of enhancing polymerization. The polymerization rate of des Bβ1-42 fibrin(ogen) 2 at a 1:327 thrombin/fibrinogen ratio was higher than that observed with des Bβ1-42 fibrin(ogen) 1. At a 1:1 molar ratio of thrombin to fibrinogen, both the level of turbidity and the rate of polymerization of des Bβ1-42 fibrinogen 2 far exceeded those same parameters for des Bβ1-42 fibrin(ogen) 1. These observations clearly suggest that the γ′ chain thrombin-binding site can be utilized to enhance FPRα-thr mediated polymerization, and the effect is best observed in a system in which low-affinity thrombin binding sites are impaired. It seems likely that the thrombin interaction with γ′ chains takes place at a different location on thrombin than exosite 1, possibly exosite 2 [13,14].

4. Discussion

The dissociation constant (K_D) of thrombin for fibrin in the E domain is in the micromolar range [3,32–34] *inter alia*, and the K_D of the low-affinity binding component is 2–3 μM [8,14]. Chemical inactivation of the catalytic site of thrombin has made it possible to measure the K_D of thrombin binding to fibrinogen directly, and the determined value, 44 nM, is nearly two orders of magnitude lower than that for fibrin [35]. Thrombin bridge-binds to fibrinogen at the substrate cleavage site and at the fibrinogen recognition site via exosite 1, and it seems evident from these numbers that proteolytic conversion of fibrinogen to fibrin abrogates the high affinity divalent relationship between thrombin and fibrinogen, and concomitantly reduces its affinity for the residual fibrin molecule. The lowered thrombin affinity for fibrin should promote thrombin transfer from the newly formed fibrin to a new fibrinogen substrate site, thus continuing the fibrinogen conversion process. As shown in these present studies, non-catalytic enhancement of fibrin(ogen) polymerization is an integral part of this process and evidently occurs by bridging between assembling units of fibrin(ogen) in a concentration-dependent manner. Our results further indicate that the thrombin effect is mediated mainly through low-affinity fibrin or fibrinogen–substrate site intermolecular interactions. The thrombin-binding sequence in the γ′ chain plays a secondary but nevertheless demonstrable role in augmenting fibrin polymerization. Our present interpretation of these events is that thrombin functions as a dimer through each of two exosites 1 to bridge between adjoining fibrin(ogen) molecules. Although it is not clear how or why dimerization of thrombin molecules comes about, recent X-ray structural data support the idea that such interactions can occur. In one of two recently reported X-ray structures of thrombin-GpIbα crystals, two thrombin molecules bound to a platelet GpIbα fragment displayed intermolecular contacts, suggesting that binding to GpIbα might be cooperative [15]. This interaction was not observed in the other study [16]. Similar intermolec-

ular contacts have been reported between symmetry-related FPR-thr molecules [10].

Rocco and his colleagues have analyzed the early events in thrombin-mediated fibrin formation by using stopped-flow multi-angle laser light scattering [36,37]. They collected data on the radius of gyration and the apparent molecular weights during early phases of the fibrinogen to fibrin conversion. Their results did not conform to established models of fibrin assembly, and that discrepancy led them to propose alternative polymerization models. They modeled their data to a bifunctional polycondensation arrangement involving semiflexible worm-like, double-stranded, half-staggered polymers with persistent lengths between 200–600 nm, or to limited quantities of single-stranded fibrillar structures as transient intermediates. Either of these proposed forms eventually coalesced into classical double-stranded fibril structures. Since these investigators were not aware of the early and profound polymerization-enhancing effects brought about through non-catalytic thrombin interactions with fibrin(ogen), they did not consider the possibility that such interactions might explain their observations. Consideration of our present findings might lead them to include the effects of thrombin in their modeling.

5. Conclusions

Concentration-dependent FPRα-thr enhancement of polymerization occurred with 'fibrin(ogen) 1' or 'fibrin(ogen) 2'. FPRγ-thr, which has a non-functional exosite 1, did not enhance polymerization of either type of fibrin(ogen), and DNA aptamer HD-1, which binds specifically to thrombin exosite 1, blocked FPRα-thr enhanced polymerization of both types of fibrin(ogen). These results indicated that exosite 1 binding at low-affinity sites is sufficient for enhancing polymerization. Comparison of des Bβ1-42 fibrin(ogen)s 1 and 2, both of which have defective low-affinity thrombin binding sites, showed that the γ′ chain thrombin-binding site can contribute to enhanced FPRα-thr mediated polymerization through a site that is different from thrombin exosite 1, possibly exosite 2. The findings suggest that during clot formation, catalytically inactivated FPRα-thr becomes dimeric and enhances fibrin(ogen) polymerization mainly by bridging fibrin(ogen) molecules through non-covalently linked exosites 1.

Acknowledgements

We thank Enrico Di Cera for his constructive suggestions during the organization of this work. This work was submitted in abstract form and presented at the XVII Congress of the International Society for Fibrinolysis and Proteolysis in Melbourne, Australia, March, 2004. Our research was supported by NIH R01 HL70627.

References

[1] M. Kaminski, J. McDonagh, Enhancement of fibrin polymerization by active site-inhibited thrombin, in: A. Henschen, B. McDonagh, J. McDonagh, T.G. Saldeen (Eds.), Fibrinogen. Structural Variants and Interactions, Walter de Gruyter, Berlin, 1985, pp. 51–63.

[2] M. Kaminski, K.R. Siebenlist, M.W. Mosesson, Evidence for thrombin enhancement of fibrin polymerization that is independent of its catalytic activity, J. Lab. Clin. Med. 117 (1991) 218–225.

[3] C.Y. Liu, H.Y. Nossel, K.L. Kaplan, The binding of thrombin by fibrin, J. Biol. Chem. 254 (1979) 10 421–10 425.

[4] L.J. Berliner, Y. Sugawara, J.W.I. Fenton, Human α-thrombin binding to non-polymerized fibrin-sepharose: evidence for an anionic binding region, Biochemistry 24 (1985) 7005–7009.

[5] M. Kaminski, J. McDonagh, Inhibited thrombins. Interactions with fibrinogen and fibrin, Biochem. J. 242 (1987) 881–887.

[6] J.W. Fenton II, T.A. Olson, M.P. Zabinski, G.D. Wilner, Anion-binding exosite of human α-thrombin and fibrin(ogen) recognition, Biochemistry 27 (1988) 7106–7112.

[7] G.D. Wilner, M.P. Danitz, M.S. Mudd, Selective immobilization of α-thrombin by surface-bound fibrin, J. Lab. Clin. Med. (1981) 403–411.

[8] D.A. Meh, K.R. Siebenlist, M.W. Mosesson, Identification and characterization of the thrombin binding sites on fibrin, J. Biol. Chem. 271 (1996) 23121–23125.

[9] W. Bode, I. Mayr, U. Baumann, R. Huber, S.R. Stone, J. Hofsteenge, The refined 1.9 Å crystal structure of human α-thrombin: Interaction with D-Phe-Pro-Arg chloromethylketone and significance of the Tyr-Pro-Pro-Trp insertion segment, EMBO. J. 8 (1989) 3467–3475.

[10] W. Bode, D. Turk, A. Karshikov, The refined 1.9 Å X-ray crystal structure of D-Phe-Pro-Arg chloromethylketone-inhibited human α-thrombin, Protein. Sci. 1 (1992) 426–471.

[11] M. Tsiang, A.K. Jain, K.E. Dunn, M.E. Rojas, L.L. Leung, C.S. Gibbs, Functional mapping of the surface residues of human thrombin, J. Biol. Chem. 270 (1995) 16854–16863.

[12] S.W. Hall, C.S. Gibbs, L.L.K. Leung, Identification of critical residues on thrombin mediating its interaction with fibrin, Thromb. Haemost. 86 (2001) 1466–1474.
[13] R.S. Lovely, M. Moaddel, D.H. Farre II, Fibrinogen γ′ chain binds thrombin exosite II, J. Thromb. Haemostas. 1 (2003) 124–131.
[14] C.H. Pospisil, A.R. Stafford, J.C. Fredenburgh, J.I. Weitz, Evidence that both exosites on thrombin participate in its high affinity interaction with fibrin, J. Biol. Chem. 278 (2003) 21584–21591.
[15] R. Celikel, R.A. McClintock, J.R. Roberts, G.L. Mendolicchio, J. Ware, K.I. Varughese, Z.M. Ruggeri, Modulation of alpha-thrombin function by distinct interactions with platelet glycoprotein Ibalpha, Science 301 (2003) 218–221.
[16] J.J. Dumas, R. Kumar, J. Seehra, W.S. Somers, L. Mosyak, Crystal structure of the GpIb alpha-thrombin complex essential for platelet aggregation, Science 301 (2003) 222–226.
[17] K.R. Siebenlist, D.A. Meh, M.W. Mosesson, Plasma factor XIII binds specifically to fibrinogen molecules containing γ′ chains, Biochemistry 35 (1996) 10448–10453.
[18] B.V. Pandya, J.L. Gabriel, J. O'Brien, A.Z. Budzynski, Polymerization site in the γ chain of fibrin: mapping of the Bβ1-55 sequence, Biochemistry 30 (1991) 162–168.
[19] L.C. Bock, L.C. Griffin, J.A. Latham, E.H. Vermaas, J.J. Toole, Selection of single-stranded DNA molecules that bind and inhibit human thrombin, Nature 355 (1992) 564–566.
[20] I. Pechik, J. Madrazo, M.W. Mosesson, I. Hernandez, G.L. Gilliland, L. Medved, Crystal structure of the complex between thrombin and the central'E' region of fibrin, Proc. Natl. Acad. Sci. USA (in press) (2004).
[21] M.W. Mosesson, J.S. Finlayson, R.A. Umfleet, Human fibrinogen heterogeneities. III. Identification of γ chain variants, J. Biol. Chem. 247 (1972) 5223–5227.
[22] C. Wolfenstein-Todel, M.W. Mosesson, Human plasma fibrinogen heterogeneity: evidence for an extended carboxyl-terminal sequence in a normal gamma chain variant (γ′), Proc. Natl. Acad. Sci.USA. 77 (1980) 5069–5073.
[23] A.V. Cooper, K.F. Standeven, R.A. Ariens, Fibrinogen gamma-chain splice variant γ′ alters fibrin formation and structure, Blood 102 (2003) 535–540.
[24] D.A. Meh, M.W. Mosesson, K.R. Siebenlist, P.J. Simpson-Haidaris, S.O. Brennan, J.P. DiOrio, et al., Fibrinogen Naples I (Bβ A68T) non-substrate thrombin binding capacities, Thromb. Res. 103 (2001) 63–73.
[25] M.T. Stubbs, W. Bode, A player of many parts: the spotlight falls on thrombin's structure, Thromb. Res. 69 (1993) 1–58.
[26] C.G. Binnie, S.T. Lord, The fibrinogen sequences that interact with thrombin, Blood 81 (1993) 3186–3192.
[27] D.H. Bing, M. Cory, J.W. Fenton II, Exosite affinity labeling of human thrombins; similar labeling on the A chain and B chain fragments of clotting α- and non-clotting γ/β thrombins, J. Biol. Chem. 252 (1977) 8027–8034.
[28] J.P. Boissel, B. LeBonniec, M.-J. Rabiet, D. Labie, J. Elion, Covalent structure of β and γ autolytic derivatives of human α-thrombin, J. Biol. Chem. 259 (1984) 5691–5697.
[29] T.J. Rydel, M. Yin, K.P. Padmanabhan, D.T. Blankenship, A.D. Cardin, P.E. Correa, et al., Crystallographic structure of human γ-thrombin, J. Biol. Chem. 269 (1994) 22000–22006.
[30] K.R. Siebenlist, J.P. DiOrio, A.Z. Budzynski, M.W. Mosesson, The polymerization and thrombin-binding properties of des-(B beta 1-42)- fibrin, J. Biol. Chem. 265 (1990) 18650–18655.
[31] B.V. Pandya, C.S. Cierniewski, A.Z. Budzynski, Conservation of human fibrinogen conformation after cleavage of the Bβ chain NH2-terminus, J. Biol. Chem. 260 (1985) 2994–3000.
[32] M. Kaminski, J. McDonagh, Studies on the mechanism of thrombin interaction with fibrin, J. Biol. Chem. 258 (1983) 10530–10535.
[33] M.C. Naski, J.A. Shafer, thrombin-catalyzed hydrolysis of fibrin I, J. Biol. Chem. 265 (1990) 1401–1407.
[34] K.P. Hopfner, E. Di Cera, Energetics of thrombin-fibrinogen interaction, Biochemistry 31 (1992) 11567–11571.
[35] K. Hosokawa, T. Ohnishi, M. Shima, M. Nagata, T. Koide, Preparation of anhydrothrombin and characterization of its interaction with natural thrombin substrates, Biochem. J. 354 (2001) 309–313.
[36] S. Bernocco, F. Ferri, A. Profumo, C. Cuniberti, M. Rocco, Polymerization of rod-like macromolecular monomers studied by stopped- flow, multiangle light scattering: set-up, data processing, and application to fibrin formation, Biophys. J. 79 (2000) 561–583.
[37] M. Rocco, S. Bernocco, M. Turci, A. Profumo, C. Cuniberti, F. Ferri, Early events in the polymerization of fibrin, Ann. NY. Acad. Sci. 936 (2001) 167–184.