Localization of an Effective Fibrin β-Chain Polymerization Site: Implications for the Polymerization Mechanism†

Kun-hwa Hsieh

Department of Veterinary and Comparative Anatomy, Pharmacology and Physiology, Washington State University, Pullman, Washington 99164-6520

Received November 1, 1996; Revised Manuscript Received May 22, 1997⊗

ABSTRACT: To examine whether fibrin N-terminal Aα 17−23 and Bβ 15−25 may contain high-affinity polymerization sites, GPRVVER and GHRPLDKKREE analogs were prepared, and their abilities to inhibit fibrin monomers from repolymerizing were compared in turbidity and clottability assays. Within Aα 17−23, GPR is the most active site (IC_{30} of 0.95−1.36 mM). Its extension into GPRVVER (IC_{30} of 1.75−2.3 mM) reduced activity. Within Bβ 15−25, acyl-DKKREE (IC_{30} of 0.30−0.53 mM) can account for GHRPLDKKREE activity (IC_{30} of 0.33−0.44 mM). Comparison of the assays showed that calcium, whose presence induces thick fibrin fibers, elicited a higher turbidity than clottability inhibition. Similarly, the lateral-association-promoting GHRP (IC_{30} of 1.25−1.43 mM) gave a high turbidity vs clottability inhibition ratio (137%). In contrast, low ratios were found for the linear-association-initiating GPR (73%) and for acyl-DKKREE (34%). Structure−activity correlation showed that fibrinogen-like acyl-GPRP and acyl-GHRP could inhibit D·E association at the millimolar range, but in a manner different from fibrin-related GPR peptides did, which required the NH_2 as well as Arg presence. To explain Bβ 20−25 masking, it is proposed that DKKREE in fibrinogen may engage in ionic and hydrogen bonds with KDSDW, the Aα 29−33 sequence implicated in thrombin binding. To explain acyl-GPRP and acyl-GHRP inhibition of D·E association, it is proposed that fibrinogen packing may be mediated by E domain association with αC (Aα 220−609) fragments of adjacent molecules, and by αC−αC association. A modified polymerization mechanism is deduced by taking into account fibrinogen N-terminal conformation as well as E domain binding to thrombin vs αC fragments. This model proposes the following. (1) Upon thrombin binding to fibrinogen KDSDW, DKKREE may become exposed. (2) Fibrinopeptide A cleavage further unmasks the NH_2 and Arg group of GPR, leading to DKKREE and GPR initiation of polymerization. (3) The micromolar-effective thrombin−fibrin(ogen) binding may initiate a partial αC repulsion. Subsequent DKKREE and GPR binding to D domains of other fibrin(ogen) will lead to the formation of the trimer and bring additional molecules to fibrin N-terminal region, and the combined steric congestion may lead to a complete αC repulsion from the overcrowded E domain. (4) Repulsion of the large Aα 220−609 fragments may unmask multiple polymerization sites beyond the fibrin N-terminal region.

Fibrinogen is a soluble plasma protein composed of two identical units in a six-chain $(A\alpha,B\beta,\gamma)_2$ structure (Blomback et al., 1976; Doolittle, 1984; Henschen et al., 1983). At the site of injury, thrombin is generated to sequentially cleave two pairs of fibrinopeptides (FpA[1] and FpB) from circulating fibrinogen (Davie et al., 1991; Blomback et al., 1978; Mann et al., 1992; Shafer & Higgins, 1988). The resulting desAABB-fibrin spontaneously polymerizes into the insoluble three-dimensional clot (Drake et al., 1989; Weisel, 1986; Mosesson et al., 1989). Concurrently, polymerizing fibrin selectively adsorbs platelets (Niewiarowski et al., 1972; Jen & Hsu, 1987) and thrombin (Liu et al., 1979; Wilner et al., 1981), thereby reinforcing the hemostatic plug and preserving thrombin from heparin−antithrombin inactivation (Ruggeri, 1995; Hogg & Jackson, 1989; Weitz et al., 1990). Fibrin-bound thrombin is thrombogenic and could proteolyze FpA as well as accelerate factor V- and VIII-dependent coagulation (Hogg & Jackson, 1990; Weitz et al., 1990; Kumar et al., 1994). In addition, fibrin clots markedly enhanced the procoagulant activity of platelets (Kumar et al., 1995), which bound more fibrin than fibrinogen [520 vs 363 ng/(10^9 platelets); Harfenist et al., 1985]. Thus, some fibrin sites exposed during polymerization appear to propagate thrombosis. Identification of these sites is of interest for thrombus detection with fibrin-specific antibodies and for targeting fibrinolytics to the clot (Procyk et al., 1991; Koblik et al., 1989; Runge et al., 1987).

Studies of the polymerization mechanism indicate that this process is initiated upon the exposure of desAA-fibrin (Blomback et al., 1978). In contrast, thrombin exposure of desBB-fibrin from fibrinogens Ledyard and Metz, whose mutated Aα 16 (Arg to Cys) did not release FpA, produced defective clots with a low A_{350} turbidity (Lee et al., 1991; Mosesson et al., 1987). As fibrinogen Detroit with the mutated GPR (Aα 19 Arg to Ser) exhibited a low A_{450}

† This work was supported by the American Heart Association, Washington Affiliate (89WA519 and 90WA522R).

⊗ Abstract published in *Advance ACS Abstracts,* July 15, 1997.

[1] Abbreviations: Abu, γ-aminobutyric acid; Ahx, ϵ-aminohexanoic acid; βA, β-alanine; dA, D-Ala; dF, D-Phe; 2Ind, 2-indan amino acid; AcOH, 20 mM acetic acid; Ac-GPRP, acetyl-Gly-Pro-Arg-Pro; Boc, *tert*-butoxycarbonyl; Boc-DKKREE(OMe), γ-methylated Bβ 20−25; desAA-fibrin, $(\alpha,B\beta,\gamma)_2$; desAABB-fibrin, $(\alpha,\beta,\gamma)_2$; desBB-fibrin, $(A\alpha,\beta,\gamma)_2$; FpA, fibrinopeptide A; FpB, fibrinopeptide B; GPR(Ac), guanidine-acetylated Aα 17−19; IC_{30}, concentration needed to effect 30% inhibition; NMR, nuclear magnetic resonance; Pr-PRP, propionyl-Pro-Arg-Pro; TLC, thin-layer chromatography.

S0006-2960(96)02741-9 CCC: $14.00

turbidity for its desAABB-fibrin polymers (Blomback et al., 1978), fibrin polymerization appears to be mediated by the GPR (Aα 17−19) and GHRP (Bβ 15−18) sites exposed upon FpA and FpB cleavage. Taken together with the affinity labeling of D domain γ-363 and γ-373 by GPR peptides (Yamazumi & Doolittle, 1992; Cierniewski & Budzynski, 1993), and the impaired GHRP binding to fibrinogen lacking a portion of its C-terminal Aα-chain (Hasegawa & Sasaki, 1990), it is thought that FpA or FpB cleavage exposes N-terminal polymerization sites for interaction with their respective complementary sites in D domains, leading to fibrin linear or lateral association.

In accord with this concept, atomic-force-microscopy visualization of the polymerization process showed that addition of thrombin to fibrinogen initially generated fibrin trimers, which subsequently formed linear protofibrils, whose lateral packing and branching led to the mature clot (Drake et al., 1989). However, the N-terminal linear and lateral association sites of GPR and GHRP bound to the fibrinogen D domain in the 10^4 M^{-1} range (Laudano et al., 1983), whereas desAA-fibrin associated with an equilibrium constant of 10^7 M^{-1} (Lewis et al., 1985). The events leading to the over 100-fold improved affinity are not understood. For example, it is not known whether the small FpA and FpB (16 and 14 residues) may directly mask high-affinity polymerization sites. If so, how might fibrinogen at high concentrations (3×10^{-4} vs 10^{-5} M in plasma) pack into fibrin-like polymers (Voter et al., 1986) and undergo cross-linking by factor XIIIa (Kanaide & Shainoff, 1975)? If not, where are the additional sites, and how are they masked in fibrinogen?

Because fibrin N-terminal Aα 17−23 (GPRVVER) and Bβ 15−25 (GHRPLDKKREE) are unmasked by FpA and FpB cleavage, this study examined the polymerization inhibitory activities (IC_{30}) of their homologs in functional (turbidity and clottability) assays. The IC_{30} values of 10^{-3}, 10^{-3}, and 10^{-4} M for, respectively, GPR and acylated GPRP, GHRP and acyl-GHRP, and acyl-DKKREE indicate that FpA or FpB unmasking alone does not directly generate micromolar-effective sites, for which additional mechanisms may be involved. The millimolar-effective acyl-GPRP analogs and acyl-GHRP show that fibrinogen-like N-acylated peptides can inhibit D•E packing, but in a manner different from that of fibrin-related GPR peptides. A modified polymerization mechanism is proposed to explain the results. This mechanism takes into account the fibrinogen N-terminal folding inferred from NMR and recombinant studies (Marsh et al., 1985; Lord et al., 1990), the E domain association with its own C-terminal Aα 220−609 observed in morphology studies (Veklich et al., 1993; Gorkun et al., 1994), and thrombin interaction with fibrin GPRVVER, GHRP, acyl-DKKREE (Hsieh, 1997), and Aα 27−50 (Binnie & Lord, 1993) during clotting.

MATERIALS AND METHODS

All chemicals were reagent grade. Boc-amino acids, including that of Abu (γ-aminobutyric acid), βAla (β-alanine), and Ahx (ε-aminohexanoic acid), were from Bachem (Torrance, CA). The 2-indan amino acid [2Ind, $C_6H_4(CH_2)_2C(NH_2)COOH$] and Boc-2Ind were prepared as described (Hsieh et al., 1989). Sephadex G-25 and SP-Sephadex C-25 were from Pharmacia (Piscataway, NJ). Human fibrinogen was from Kabi (grade L), Helena Lab (Beaumont, TX), and human thrombin (3000 units/mg) was from Sigma (St. Louis, MO).

Peptide Synthesis. The following peptides were prepared by solid-phase synthesis (Marshall & Merrifield, 1965; Hsieh et al., 1996). These included the NH_2-terminus-containing GPRP (Gly-Pro-Arg-Pro), GHRP (Gly-His-Arg-Pro), PRP (Pro-Arg-Pro), AbuRP (γAbu-Arg-Pro), AhxRP (εAhx-Arg-Pro), βAPRP (βAla-Pro-Arg-Pro), dAPRP (D-Ala-Pro-Arg-Pro), FPRP (Phe-Pro-Arg-Pro), 2IndPRP (2Ind-Pro-Arg-Pro), dFPRP (D-Phe-Pro-Arg-Pro), and GPR2Ind (Gly-Pro-Arg-2Ind), in addition to the NH_2-acylated Pr-PRP (propionyl-Pro-Arg-Pro), Boc-PRP, Boc-GPRP, Boc-dAPRP, Boc-2IndPRP, Boc-dFPRP, Boc-GPR2Ind, and Boc-GHRP. Synthesis of fibrin Aα 17−23 and Bβ 15−25 homologs, including GPR and its guanidine-acetylated GPR(Ac), Boc-DKKREE and its γ-methylated Boc-DKKREE(OMe), and Ac-GPRP has been reported (Hsieh et al., 1981, 1996).

The first Boc-amino acid was attached to the resin by the cesium procedure, and stepwise amino acid coupling utilized the side chain protection of Arg(NO_2) and His(Bzl). Boc-peptides were removed from the resin and the side chain protecting groups by catalytic hydrogenation. This approach provided N-protected peptides without contamination by the NH_2-terminal free species. Subsequent deprotection (trifluoroacetic acid) of the former gave the latter. The peptides were purified by ion-exchange and gel filtration chromatography until homogeneous to TLC and with the correct amino acid composition (Supporting Information).

Polymerization Inhibition Assays. Fibrin monomers (Brosstad et al., 1977) were prepared by clotting human fibrinogen (10 mg/mL saline) with human thrombin (1 unit) at 37 °C for 30 min. The clot was wound tightly around a glass rod, washed with water, blotted dry, and dissolved in 20 mM acetic acid (AcOH) for 30 min. This was diluted with 100 mM NaBr/5 mM NaOAc (bromide-acetate buffer, pH 5.3) and centrifuged (2000 rpm, 20 min) to give a fibrin (2 mg/mL) solution in 3:2 (v/v) AcOH/bromide-acetate. Freshly prepared fibrin monomers were >99% clottable in clottability assays and remained >95% clottable for 3 months when stored at 4 °C (storage of this saturated solution at −20 °C will lead to fibrin precipitation).

In turbidity assays (Hasegawa & Sasaki, 1990; Belister et al., 1968), fibrin monomers (40 μL) were repolymerized by mixing with 760 μL of Tris buffer (60 mM, pH 6.7) containing the peptide, and the A_{280} of the mixture was assessed after 40 min. The control A_{280} increase due to polymerization alone was A_{280} of polymerized fibrin in Tris buffer − 0.15 for A_{280} of the fibrin added. The level of polymerization in the presence of peptide was (A_{280} of polymerized fibrin in the peptide solution − 0.15 − peptide blank)/(the control A_{280} increase). Turbidity inhibition was 100% minus the level of polymerization. At least three experiments were performed for each peptide dose, and the IC_{30} values were determined from dose−response curves.

Clottability assays were performed for the inactive GPR-(Ac), βAPRP, PRP, AbuRP, and AhxRP, and for fibrin Aα 17−23 and Bβ 15−25 homologs, in order to verify their results in turbidity assays. In these studies, fibrin monomers (40 μL) were repolymerized in 760 μL of Tris (60 mM, pH 6.7) or Tris-$CaCl_2$ (50:10 mM, pH 6.7) containing the peptide and centrifuged (2000 rpm, 10 min) after 40 min. Unpolymerized fibrin in the supernatant was assessed by A_{280}. In

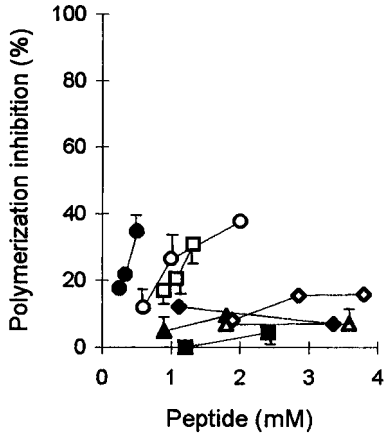


FIGURE 1: Inhibition of polymerization by fibrin-related peptides in turbidity assays. From left to right are the dose−response curves for GPRP (●), GPR (○), GHRP (□), AbuRP (▲), GPR(Ac) (◆), βAPRP (■), AhxRP (△), and PRP (◇). Fibrin monomers (80 μg) were repolymerized for 40 min in 760 μL of Tris buffer (60 mM, pH 6.7) containing the peptide. Polymerization inhibition was 100% − [(A_{280} of polymerized fibrin in peptide solution − 0.15 for the A_{280} fibrin added − peptide blank)/(A_{280} of polymerized fibrin in buffer control − 0.15)]. At least three determinations were made for each dose, and results were mean ± SD.

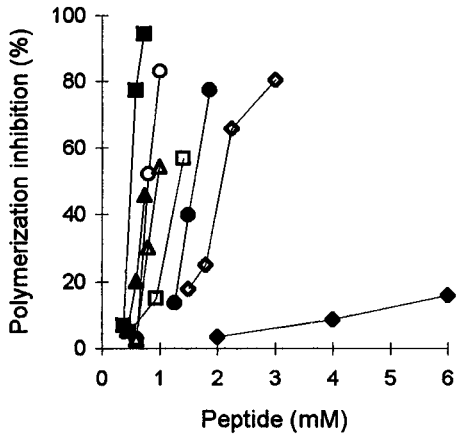


FIGURE 2: Inhibition of polymerization by NH_2-containing and N-acylated peptides in turbidity assays. From left to right are dAPRP (■), FPRP (▲), 2IndPRP (○), dFPRP (△), Boc-GHRP (□), PrPRP (●), Boc-GPRP (◇), and Ac-GPRP (◆). Fibrin polymerization and data analysis were conducted as in Figure 1. Results ($n \geq 3$) were mean values, and SD values (not shown) were similar to those of Figure 1.

Tris, Tris-Ca^{2+} control, or inactive peptide solution, a zero A_{280} was found for the supernatant. The level of unpolymerized fibrin was (A_{280} of supernatant − peptide blank)/0.15, the A_{280} of fibrin added. Clottability was 100% minus unpolymerized fibrin, and clottability inhibition was 100% minus clottability. Therefore, clottability inhibition equaled (A_{280} of supernatant − peptide blank)/0.15. At least three experiments were performed for each peptide dose, and the IC_{30} values were determined from dose−response curves.

The ratios of turbidity vs clottability inhibition for fibrin homologs were calculated for each peptide dose, and mean values were reported.

Table 1: Polymerization Inhibition IC_{30} of Fibrin-Related Peptides and Their N-Acylated Analogs in Turbidity Assays

NH_2-containing peptide	IC_{30} (mM, no Ca^{2+})	N-acylated peptide	IC_{30} (mM, no Ca^{2+})
PRP	inactive	Pr-PRP	1.41
		Boc-PRP	2.67
GPRP	0.44	Boc-GPRP	1.85
dAPRP	0.44	Boc-dAPRP	2.02
FPRP	0.64		
2IndPRP	0.71	Boc-2IndPRP	2.20
dFPRP	0.79	Boc-dFPRP	1.16
GPR2Ind	0.80	Boc-GPR2Ind	1.21
fibrin homologs			
GPR (Aα 17−19)	1.36		
GPRVVER	1.88		
GHRP (Bβ 15−18)	1.30	Boc-GHRP	1.10
GHRPLDKKREE	0.44	Boc-DKKREE	0.53

RESULTS

The Concurrent Presence of the Fibrin α-Chain NH_2 Terminus and the Arg Side Chain of GPR (Aα 17−19) Is Important for D·E Polymerization. To identify the critical fibrin N-terminal features involved in polymerization, we modified an Aα 17−20 analog, GPRP (Laudano & Doolittle, 1978; Hsieh et al., 1981). Figure 1 shows that an about 1500-fold excess of GPRP (0.44 mM) over fibrin (0.29 μM) was needed to inhibit polymerization by 30%. Truncation of the C-terminal Pro gave an active GPR, whose side chain acetylation gave an inactive GPR(Ac), indicating a critical role for Arg. Since N-terminal truncation to PRP, or extension of the NH_2 group by one atom in βAPRP and AhxRP gave inactive analogs, the spatial distance between the NH_2 and the Arg side chain appears to be crucial. Not surprisingly, shortening the distance between the NH_2 and Arg also gave an inactive AbuRP.

The importance of properly spaced NH_2 and Arg is evident in the highly active dAPRP (Figure 2), in contrast to the inactive βAPRP shown in Figure 1. Since Gly is the smallest amino acid and also optically inactive due to the lack of a side chain, we examined how increasing the size of the side chain and changing its spatial orientation might affect GPRP inhibition of polymerization. For this purpose, Gly was replaced by L-Phe, D-Phe, and their optically inactive homolog, 2Ind. As the resulting FPRP, 2IndPRP, and dFPRP were similarly active (Figure 2), the steric orientation of the N-terminal residue does not seem important. Instead, the rank order (GPRP and dAPRP ≪ FPRP < 2IndPRP < dFPRP) of their IC_{30} values (Table 1) indicates that the small GPRP and dAPRP were more effective than the bulky analogs of FPRP, 2IndPRP, and dFPRP.

In accord with a required fibrin NH_2 and Arg presence for activity, the C-terminal modified GPR2Ind was active (Table 1). Unexpectedly, Boc-GPRP and Ac-GPRP, whose NH_2 termini were acylated to resemble a fibrinogen-like sequence, could also inhibit polymerization (Figure 2). Since Pr-PRP, Boc-PRP, and Boc-GHRP were more active than their NH_2-containing fibrin-like counterparts, whose rank order differed from that for N-acylated peptides (Table 1), fibrinogen-like peptides appear to participate in D·E packing in a manner different from that of fibrin-like peptides.

Fibrin β-Chain DKKREE (Bβ 20−25) Contains an Effective Polymerization Site. Although the turbidity assay has been useful for identifying small peptide polymerization sites (Laudano & Doolittle, 1978; Hasegawa & Sasaki, 1990), a markedly lower A_{350} turbidity was observed for desBB- than for desAA-fibrin polymers (Mosesson et al., 1987; Lee et al., 1991), in spite of the indistinguishable desBB-, desAA-, and desAABB-fibrin fibers found in electron microscopy and X-ray studies (Weisel, 1986; Voter et al., 1986). Paradoxically, the A_{350} turbidity of fibrin clots was decreased by 40%

Table 2: Polymerization Inhibition IC_{30} of Fibrin Homologs in Clottability Assays

	IC_{30} (mM, no Ca^{2+})	IC_{30} (mM, with Ca^{2+})
GPRP	0.51	0.51
α-chain homologs		
GPR	0.95	1.23
VVER	>2.1	>2.1
GPRVVER	about 2.3	1.75
β-chain homologs		
GHRP	1.43	1.25
Boc-DKKREE	0.38	0.30
Boc-DKKREE(OMe)	0.93	0.85
GHRPLDKKREE	0.35	0.33

when fibrin monomers were repolymerized in the presence of calcium (from 1.066 to 0.641; Siebenlist et al., 1990; Maekawa et al., 1992) but increased when fibrinogen was clotted by thrombin, reptilase, or copperhead venom in the presence of calcium (Siebenlist et al., 1989; Dang et al., 1989b; Lee et al., 1991).

To unambiguously determine the level of polymerization, we centrifugally removed the polymerized clot and assessed the amount of unpolymerized fibrin monomers at A_{280}. In these clottability assays, GPR was the most active site within Aα 17−23 (Table 2). Extension of GPR into GPRVVER (Aα 17−23) reduced activity, indicating the lack of GPR and VVER synergism, which would have increased activity. Extension of GHRP into GHRPLDKKREE (Bβ 15−25) led to a 4-fold improved activity, accounted for by the presence of acyl-DKKREE. Masking of the carboxylate side chain in acyl-DKKREE(OMe) decreased the ability to participate in ionic and H bonds and reduced polymerization inhibitory activity.

Comparison of A_{280} turbidity vs clottability results gave slightly different IC_{30} values for fibrin homologs (Tables 1 and 2). Calcium, whose presence increased the thickness of fibrin fibers (Weisel, 1986; Siebenlist et al., 1990) and improved the IC_{30} values of GPRVVER and Bβ homologs (Table 2), reduced fibrin A_{280} turbidity (by 32%) but not clottability (data not shown). The greater inhibition of turbidity than clottability appears to be characteristic of fibrin lateral association, and Figure 3 shows that the lateral GHRP site also elicited a higher ratio (137%) of turbidity inhibition (broken lines) than clottability inhibition (solid lines). In contrast, a lower ratio (73 and 34%, respectively) of turbidity than clottability inhibition was found for the linear association-initiating GPR and for acyl-DKKREE. Side-by-side comparison by simultaneous monitoring at A_{280} and A_{350} gave similar IC_{30} values at both wavelengths for GPRP and acyl-DKKREE in turbidity assays (data not shown), indicating a similar A_{280} and A_{350} detection of fibrin polymerization.

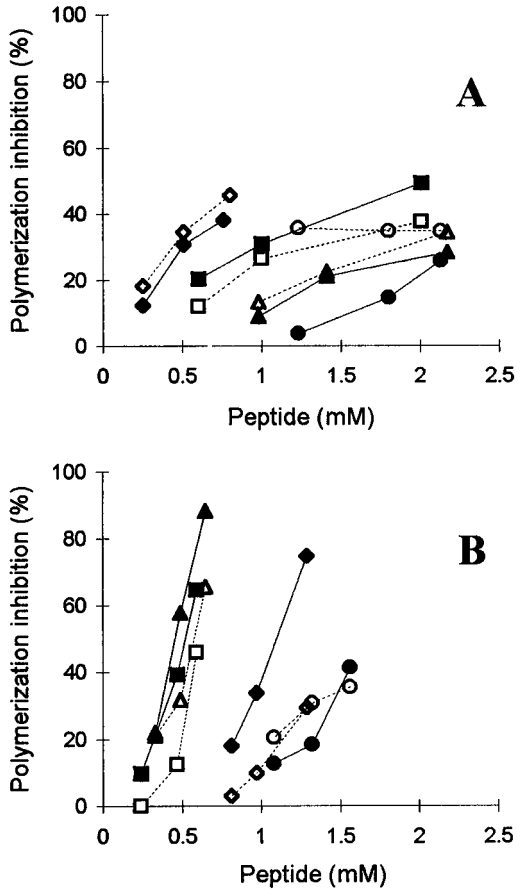


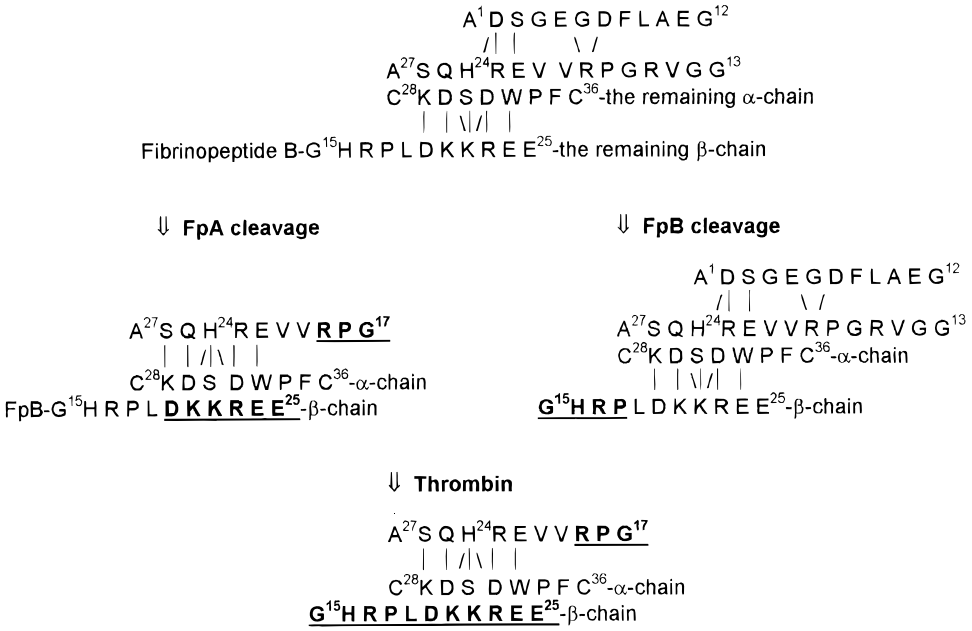
FIGURE 3: Inhibition of polymerization by fibrin homologs in turbidity (broken lines and open symbols) vs clottability (solid lines and solid symbols) assays. From left to right are (A) GPRP (◆ and ◇), GPR (■ and □), GPRVVER (▲ and △), and VVER (● and ○) and (B) GHRPLDKKREE (▲ and △), Boc-DKKREE (■ and □), Boc-DKKREE(OMe) (◆ and ◇), and GHRP (● and ○). In clottability assays, fibrin monomers were repolymerized in Tris buffer for 40 min as described for Figure 1. Polymerized fibrin was removed (2000 rpm, 10 min), and unpolymerized monomers in the supernatant were assessed by A_{280}. Clottability inhibition was (A_{280} of fibrin monomers in supernatant − peptide blank)/0.15, the A_{280} of fibrin added. Turbidity inhibition was analyzed as in Figure 1. Results ($n \geq 3$) were mean values, and SD values (not shown) were simliar to those of Figure 1.

DISCUSSION

Three questions are central to the understanding of the polymerization mechanism. Where are the polymerization sites? How may these sites be masked in fibrinogen? Why can different enzymes like thrombin, reptilase, or copperhead venom elicit morphologically similar three-dimensional clots?

The results show that, in addition to the N-terminal GPR (Blomback et al., 1978; Laudano & Doolittle, 1978), fibrin β-chain also contains an effective DKKREE polymerization site. For GPR-mediated polymerization, the NH_2 terminus and the Arg side chain are important. Both features may be masked by FpA (ADSGEGDFLAEGGGVR), via the amide bond and by the FpA−GPR salt bridge. The latter is inferred from NMR observations of FpA binding with GPRP (an Aα 17−20 analog) at pH 7 in the absence of salt, but the lack of such interaction in Aα 1−23 under conditions which interfered with salt bridge formation, i.e., high-ionic strength buffer at pH 5.3 (Root-Bernstein & Westall, 1984; Marsh et al., 1985; Ni et al., 1988). Mutagenesis of a fibrinogen Aα 1−51-containing fusion protein showed that its optimal proteolysis by thrombin was observed with Val substitution for Aα Gly-14 but not for Gly-12 or Gly-13 (Lord et al., 1990), indicating a biologically important Gly-Gly β-bend at Aα 12−13. Taken together, it is likely that salt bridges and H bonds exist between fibrinogen Asp-2/Arg-23 and His-24, Ser-3 or Ser(PO_4)-3/Glu-22, and Glu-5 and Asp-7/Arg-19 (Scheme 1, top). The millimolar IC_{30} value found for GPRVVER suggests that FpA may not directly mask a micromolar-effective site. Whether cleavage of a pair of FpA may unmask a high-affinity polymerization site, with two GPR groups linked via the Aα 28−Aα′ 28 disulfide, is not presently known.

Scheme 1: A Proposed Folding of Fibrinogen Aα 1−36 and Bβ 15−25[a]



[a] Selective FpA cleavage (left) may expose a linear GPR (Aα 17−19) site and DKKREE (Bβ 20−25). Selective FpB cleavage (right) may expose the lateral GHRP (Bβ 15−18) site. Thrombin cleavage of FpA and FpB may expose the GPR and GHRPLDKKREE sites, capable of linear and lateral associations. The exposed sites are shown in bold. Lines and slashes indicate potential ionic and H bonds.

How DKKREE (Bβ 20−25) is masked in fibrinogen has not been examined. Morphology studies showed that Bβ 15−42 deletion impaired fibrin linear as well as lateral associations (Siebenlist et al., 1990). Polymerization studies showed that high-ionic strength (0.1 M NaCl) conditions reduced the A_{350} for normal fibrin polymer but abolished the A_{350} for the fibrin polymer lacking Bβ 15−42 (Kamura et al., 1995). These results suggest Bβ 15−42 participation in fibrin associations via reinforced ionic bonds, thus withstanding highly ionic conditions. Interestingly, all DKKREE side chains are capable of ionic bonds, which may reinforce one another, and masking of the Glu-25 side chain reduced activity (Table 2). Taken together, DKKREE may be masked in fibrinogen, as well as exert its effect in fibrin, via ionic bonds. A search of the N-terminal disulfide knot sequence of (Aα 1−51, Bβ 1−118, γ 1−78)$_2$ shows that only KDSDW (Aα 29−33) is fully complementary to DKKRE via ionic and H bonds (Scheme 1, top). In comparison, FpB (ZGVNDNEEGFFSAR) is neither linked with nor complementary to DKKREE.

To initiate clotting, thrombin exosite-1 binds to the fibrinogen KDSDW-containing Aα 30−41 (Blomback et al., 1977; Vali & Scheraga, 1988; Binnie & Lord, 1991; Rasmussen et al., 1991), and the enzyme catalytic site binds to the FpA−GPRVVER region (Martin et al., 1992; Hsieh, 1997). If DKKRE is indeed masked by KDSDW as proposed, thrombin binding to the fibrinogen KDSDW region alone should expose the DKKREE site, and subsequent cleavage of FpA will further unmask the NH_2 and Arg group of GPR (Scheme 1, left). Since the ERHQS (Aα 22−26) region is also complementary to KDSDW, it is tempting to speculate that reptilase cleavage of FpA may free ERHQS to engage in ionic and H bonds with KDSDW, leading to a similar expression of the GPR and DKKREE sites. In contrast, copperhead venom cleavage of FpB may expose the lateral GHRP site (Scheme 1, right). Finally, thrombin cleavage of both FpA and FpB may expose GPR and GHRPLDKKREE, capable of linear and lateral associations. The proposed DKKREE and GHRP roles are in agreement with the finding that Bβ 15−42 deletion impaired linear as well as lateral associations. The functional importance of the GPR and DKKREE sites is evident in the identical GPR expression in human, bovine, and lamprey desAA-fibrins (Cottrell & Doolittle, 1976), and in the highly conserved DKKREE sequence among human, bovine, canine, ovine, and porcine fibrinogens (Blomback et al., 1976; Chung et al., 1981; Birken et al., 1975; Matsueda & Margolies, 1986). Interestingly, the Aα Gly-12 and Aα 29−32 KDSD important for, respectively, the proposed β-bend and DKKREE masking are also conserved in mammalian fibrinogens (Henschen et al., 1983).

Although FpA and FpB cleavage may initially expose, respectively, a linear and a lateral polymerization site (Blomback et al., 1978), the resulting desAA-, desBB-, and desAABB-fibrin eventually formed similar three-dimensional clots regardless of whether they were generated by reptilase, copperhead venom, or thrombin (Weisel, 1986; Voter et al., 1986). Hence, a common mechanism is probably involved in exposing additional linear, lateral, and branching sites on fibrin. Because most (93%) fibrinogen molecules contain an E domain associated with their own αC fragments (M_r of 40 000; Veklich et al., 1993; Gorkun et al., 1994), it is likely that E domain binding with procoagulant enzymes like thrombin (M_r of 36 500), which is $^1/_{10}$ of the size of fibrinogen (M_r of 340 000), will lead to steric repulsion of αC, thus exposing multiple sites initially bound to this large Aα 220−609 fragment. The defective polymerization displayed by fibrinogen Detroit with mutated GPR sites (Blomback et al., 1978) further suggests that N-terminal binding to the D domain also contributes to this process. Taken together with the higher affinity of fibrin for thrombin (K_d of 10^{-6} M) than for GPR and DKKREE (IC_{30} of 10^{-3}−10^{-4} M), it is proposed that thrombin binding to the E domain initiates a partial αC repulsion which, in turn, promotes GPR

binding to the D domain of other fibrin(ogen). The resulting D·E association may allow a complete αC repulsion, thus exposing polymerization sites beyond fibrin N-terminal region. In this manner, two GPR groups linked via an Aα 28−Aα′ 28 disulfide bridge will bring two D domains plus thrombin to a congested fibrin N-terminal region, and the collective steric crowding should markedly accelerate αC repulsion. In contrast, GHRP or other β-chain sites in desBB-fibrin are distantly linked via Bβ 65−Aα′ 36, with the latter being several residues from Aα 28−Aα′ 28. As a result of less steric crowding, desBB-fibrin may induce a less complete αC repulsion, leading to a lower turbidity for desBB- than desAA-fibrin polymers. Since most (83%) αC fragments in fibrin monomers are already repelled from the E domain at pH 3.5 (Veklich et al., 1993), thrombin binding is not needed for polymerization. The proposed αC role is consistent with the findings that αC was not required for polymerization but effectively inhibited this process (Gorkun et al., 1994; Lau, 1993) presumably by blockading multiple sites.

The proposed polymerization mechanism may be relevant to fibrinogen packing (Cohen et al., 1983; Voter et al., 1986) and clot desorption of thrombin (Liu et al., 1979). At a high fibrinogen concentration, αC fragments of different molecules will be crowded into close contact. Since αC−αC association and αC binding with the N-terminal region have been observed (Veklich et al., 1993; Hasegawa & Sasaki, 1990), it is proposed that αC crowding leads to its association with the αC and E domain of adjacent molecules, thus unmasking multiple sites for fibrinogen packing. Table 1 shows that unlike fibrin GPR, whose NH_2 group is required for a complementary site preferring a small N-terminal residue, fibrinogen-like N-acylated peptides inhibited D·E packing with a different structural requirement and rank order. Consistent with the nonidentical interaction, crystallographic and computer modeling studies suggest a one-third stagger in fibrinogen at low ionic strengths, but a one-half stagger in fibrin and fibrinogen under high-ionic strength conditions like 0.3 M KF (Cohen et al., 1983). Jointly, the αC fragment and the millimolar-effective N-acylated GPR and GHRP may allow concentrated fibrinogen ($>10^{-4}$ M) to pack in a manner similar, but not necessarily identical, to that of fibrin, thus permitting fibrinogen cross-linking by factor XIIIa.

As 5-fold more thrombin was adsorbed by polymerizing fibrin than preformed clot (Wilner et al., 1981), yet a significant amount (60% in 2 days at 37 °C) of fibrin-bound enzyme could diffuse out of the clot (Liu et al., 1979), thrombin appears to initially bind fibrin polymerization sites but become dislodged upon D·E polymerization. Our results show that GPRVVER, GHRP, and acyl-DKKREE inhibited polymerization at 10^{-3}−10^{-4} M and interacted with thrombin at a similar range (Hsieh, 1997). These findings indicate that thrombin binding and D·E association involve similar fibrin sites and that GPR alone is insufficient to initiate a micromolar-affinity D·E association. To surmount the micromolar fibrin−thrombin binding (Liu et al., 1979; Binnie & Lord, 1993), the concerted action of GPR with DKKREE, the second GPR/DKKREE, and/or sites exposed by the partial αC repulsion may be needed to synergistically enhance D·E binding to the micromolar range. Subsequent fibrin trimer formation may lead to a complete αC repulsion and high-affinity (10^7 M^{-1}) polymerization, whose consumption of thrombin-binding sites may dislodge the enzyme from fibrin clot, thus re-exposing thrombin exosite-1 and the catalytic site for fibrinolysis and wound healing.

In summary, fibrin E domain DKKREE, GPR, and GHRPLDKKREE polymerization sites may be directly exposed by thrombin binding, and by FpA and FpB cleavage. Indirectly, fibrin E domain binding to thrombin and to D domains of other fibrin(ogen) may promote αC repulsion, thus exposing other sites beyond the N-terminal region. As FpA cleavage will disrupt salt bridges and H bonds within Aα 1−24, the newly unmasked sites may drive the fibrin N-terminal region to form alternative ionic and H bonds, thereby sequentially exposing additional sequences (Scheme 1). Such a conformation-driven mechanism may also contribute to D-site formation. By a combination of mechanisms, discrete fibrin sequences on different chains may be brought together to form high-affinity sites. The sequential nature of the proposed polymerization model may explain two seemingly contradictory observations. On one hand, the clotting defects displayed by various single-site-mutated fibrinogens (Dang et al., 1989a; Bantia et al., 1990) may be attributed to their interrupted polymerization-site formation. On the other hand, abnormal fibrinogens still formed defective gels (Mosesson et al., 1987; Siebenlist et al., 1990; Kamura et al., 1995), as would be expected from the weak D·E interaction of their incompletely formed polymerization sites.

SUPPORTING INFORMATION AVAILABLE

Analytical data of peptide analogs (1 page). Ordering information is given on any current masthead page.

REFERENCES

Bantia, S., Bell, W. R., & Dang, C. V. (1990) *Blood 75*, 1659−1663.

Belister, V. A., Varetskaja, T. V., & Malneva, G. V. (1968) *Biochim. Biophys. Acta 154*, 367−375.

Binnie, C. G., & Lord, S. T. (1991) *Thromb. Haemostasis 65*, 165−168.

Binnie, C. G., & Lord, S. T. (1993) *Blood 81*, 3186−3192.

Birken, S., Wilner, G. D., & Canfield, R. E. (1975) *Thromb. Res. 7*, 599−610.

Blomback, B., Hogg, D. H., Gardlund, B., Hessel, B., & Kudryk, B. (1976) *Thromb. Res. 8* (Suppl. II), 329−346.

Blomback, B., Hessel, B., Hogg, D., & Claesson, G. (1977) in *Chemistry & Biology of Thrombin* (Lundblad, R. L., Fenton, J. W., II, & Mann, K. G., Eds.) pp 275−290, Ann Arbor Science, Ann Arbor, MI.

Blomback, B., Hessel, B., Hogg, D., & Therkildsen, L. (1978) *Nature 275*, 501−505.

Brosstad, F., Godal, H. C., & Kierulf, P. (1977) *Haemostasis 6*, 213−214.

Chung, D. W., Rixon, M. W., MacGillivray, R. T. A., & Davie, E. W. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 1466−1470.

Cierniewski, C. S., & Budzynski, A. Z. (1993) *Eur. J. Biochem. 218*, 321−325.

Cohen, C., Weisel, J. W., Phillips, G. N., Jr., Stauffacher, C. V., Fillers, J. P., & Daub, E. (1983) *Ann. N.Y. Acad. Sci. 408*, 194−213.

Cottrell, B. A., & Doolittle, R. F. (1976) *Biochim. Biophys. Acta 453*, 426−438.

Dang, C. V., Bell, W. R., & Shuman, M. (1989a) *Am. J. Med. 87*, 567−576.

Dang, C. V., Shin, C. K., Bell, W. R., Nagaswami, C., & Weisel, J. W. (1989b) *J. Biol. Chem. 264*, 15104−15108.

Davie, E. W., Fujikawa, K., & Kisiel, W. (1991) *Biochemistry 30*, 10363−10370.

Doolittle, R. F. (1984) *Annu. Rev. Biochem. 53*, 195−229.

Drake, B., Prater, C. B., Weisenhorn, A. L., Gould, S. A. C., Albrecht, T. R., Quate, C. F., Cannell, D. S., Hansma, H. G., & Hansma, P. K. (1989) *Science 243*, 1586−1589.
Gorkun, O. V., Veklich, Y. I., Medved, L. V., Henschen, A. H., & Weisel, J. W. (1994) *Biochemistry 33*, 6986−6997.
Harfenist, E. J., Packham, M. A., & Mustard, J. F. (1985) *Thromb. Haemostasis 58*, 183−187.
Hasegawa, N., & Sasaki, S. (1990) *Thromb. Res. 57*, 183−195.
Henschen, A., Lottspeich, F., Kehl, M., & Southan, C. (1983) *Ann. N.Y. Acad. Sci. 408*, 28−43.
Hogg, P. J., & Jackson, C. M. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 3619−3623.
Hogg, P. J., & Jackson, C. M. (1990) *J. Biol. Chem. 265*, 248−255.
Hsieh, K. H. (1997) *Thromb. Res. 86*, 301−316.
Hsieh, K. H., Mudd, S. M., & Wilner, G. D. (1981) *J. Med. Chem. 24*, 322−327.
Hsieh, K. H., LaHann, T. R., & Speth, R. C. (1989) *J. Med. Chem. 32*, 898−903.
Hsieh, K. H., DeMaine, M. M., & Gurusiddaiah, S (1996) *Int. J. Pept. Protein Res. 48*, 292−298.
Jen, C. J., & Hu, S. J. (1987) *Am. J. Physiol. 253*, H745−H750.
Kamura, T., Tsuda, H., Yae, Y., Hattori, S., Ohga, S., Shibata, Y., Kawabata, S. I., & Hamsaki, N. (1995) *J. Biol. Chem. 270*, 29392−29399.
Kanaide, H., & Shainoff, J. R. (1975) *J. Lab. Clin. Med. 85*, 574−597.
Koblik, P. D., De Nardo, G. L., & Berger, H. J. (1989) *Semin. Nucl. Med. 19*, 221−237.
Kumar, R., Begun, S., & Hemker, H. C. (1994) *Thromb. Haemostasis 72*, 713−721.
Kumar, R., Begun, S., & Hemker, H. C. (1995) *Thromb. Haemostasis 74*, 962−968.
Lau, H. K. F. (1993) *Blood 81*, 3277−3284.
Laudano, A. P., & Doolittle, R. F. (1978) *Proc. Natl. Acad. Sci. U.S.A. 75*, 3085−3089.
Laudano, A. P., Cottrell, B. A., & Doolittle, R. F. (1983) *Ann. N.Y. Acad. Sci. 408*, 315−329.
Lee, M. H., Kaczmarek, E., Chin, D. T., Oda, A., McIntosh, S., Bauer, K. A., Clyne, L. P., & McDonagh, J. (1991) *Blood 78*, 1744−1752.
Lewis, S. D., Shields, P. P., & Shafer, J. A. (1985) *J. Biol. Chem. 260*, 10192−10199.
Liu, C. Y., Nossel, H. L., & Kaplan, K. L. (1979) *J. Biol. Chem. 254*, 10421−10425.
Lord, S. T., Byrd, P. A., Hede, K. L., Wei, C., & Colby, T. J. (1990) *J. Biol. Chem. 265*, 838−843.
Maekawa, H., Yamazumi, K., Murmatsu, S. I., Kaneko, M., Hirata, H., Takahashi, N., Arocha-Pinango, C. L., Rodriguez, S., Nagy, H., Perez-Requejo, J. L., & Matsuda, M. (1992) *J. Clin. Invest. 90*, 67−76.
Mann, K. G., Krishnaswamy, S., & Lawson, J. H. (1992) *Semin. Hematol. 29*, 213−216.
Marsh, H. C., Jr., Meinwald, Y. C., Lee, S., Martinelli, R. A., & Scheraga, H. A. (1985) *Biochemistry 24*, 2806−2812.
Marshall, G. R., & Merrifield, R. B. (1965) *Biochemistry 4*, 2394−2401.
Martin, P. D., Robertson, W., Turk, D., Huber, R., Bode, W., & Edwards, B. F. P. (1992) *J. Biol. Chem. 267*, 7911−7920.
Matsueda, G. R., & Margolies, M. N. (1986) *Biochemistry 25*, 1451−1455.
Mosesson, M. W., DiOrio, J. P., Muller, M. F., Shainoff, J. R., Siebenlist, K. R., Amrani, D. L., Homandberg, G. A., Soria, J., Soria, C., & Samama, M. (1987) *Blood 69*, 1073−1081.
Mosesson, M. W., Siebenlist, K. R., Amrani, D. L., & DiOrio, J. P. (1989) *Proc. Natl. Acad. Sci. U.S.A. 86*, 1113−1117.
Ni, F., Scheraga, H. A., & Lord, S. T. (1988) *Biochemistry 27*, 4481−4491.
Niewiarowski, S., Regoeczi, E., Stewart, G. J., Senyi, A. F., & Mustard, J. F. (1972) *J. Clin. Invest. 51*, 685−700.
Procyk, R., Kudryk, B., Callender, S., & Blomback, B. (1991) *Blood 77*, 1469−1475.
Rasmussen, U. B., Vouret-Craviari, V., Jallat, S., Schlesinger, Y., Pages, G., Pavirani, A., Lecocq, J. P., Pouyssegur, J., & Van Obberghen-Schilling, E. (1991) *FEBS Lett. 288*, 123−128.
Root-Bernstein, R. S., & Westall, F. C. (1984) *Proc. Natl. Acad. Sci. U.S.A. 81*, 4339−4342.
Ruggeri, Z. M. (1995) *Thromb. Haemostasis 74*, 460−463.
Runge, M. S., Bode, C., Matsueda, G. R., & Haber, E. (1987) *Proc. Natl. Acad. Sci. U.S.A. 84*, 7659−7662.
Shafer, J. A., & Higgins, D. L. (1988) *CRC Crit. Rev. Clin. Lab. Sci. 26*, 1−41.
Siebenlist, K. R., Mosesson, M. W., DiOrio, J. P., Tavori, S., Tatarsky, I., & Rimon, A. (1989) *Thromb. Haemostasis 62*, 875−879.
Siebenlist, K. R., DiOrio, J. P., Budzynski, A. Z., & Mosesson, M. W. (1990) *J. Biol. Chem. 265*, 18650−18655.
Vali, Z., & Scheraga, H. A. (1988) *Biochemistry 27*, 1956−1963.
Veklich, Y. I., Gorkun, O. V., Medved, L. V., Nieuwenhuizen, W., & Weisel, J. W. (1993) *J. Biol. Chem. 268*, 13577−13586.
Voter, W. A., Lucaveche, C., Blaurock, A. E., & Erickson, H. P. (1986) *Biopolymers 25*, 2359−2373.
Weisel, J. W. (1986) *Biophys. J. 50*, 1079−1093.
Weitz, J. I., Hudoba, M., Massel, D., Maraganore, J., & Hirsh, J. (1990) *J. Clin. Invest. 86*, 385−391.
Wilner, G. D., Danitz, M. P., Mudd, M. S., Hsieh, K. H., & Fenton, J. W., II (1981) *J. Lab. Clin. Med. 97*, 403−411.
Yamazumi, K., & Doolittle, R. F. (1992) *Proc. Natl. Acad. Sci. U.S.A. 89*, 2893−2896.

BI962741B