Binding of Synthetic B Knobs to Fibrinogen Changes the Character of Fibrin and Inhibits Its Ability To Activate Tissue Plasminogen Activator and Its Destruction by Plasmin†

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ABSTRACT: Synthetic peptides corresponding to the amino-terminal sequence of the β chain of fibrin increase the turbidity of fibrin clots, whether they are generated by the direct interaction of thrombin and fibrinogen or by the reassociation of fibrin monomers. The turbidity of batroxobin-induced clots, which are characteristically "fine," is increased even more dramatically. Pentapeptides are more effective than tetrapeptides. Surprisingly, the same peptides also delay fibrinolysis, whether activated by exogenously added plasmin or from the fibrin-enhanced stimulation of tissue plasminogen activator (tPA) activation of plasminogen. The peptides have only a very slight effect on the plasmic hydrolysis of a chromogenic peptide, either by the direct addition of plasmin or by plasmin generated from plasminogen by tPA. The synthetic peptides mimicking the B knobs appear to exert their action in two ways. First, they render fibrin less vulnerable to attack by plasmin. Second, they delay the fibrin activation of tPA. The latter is attributed to their ability to prevent the binding of the authentic B knob, which itself is located at the end of a flexible 50-residue tether and which needs time to find its elusive "hole". We propose that, when after a while the tethered knob does become inserted, it locks the βC domain in a conformation that allows access to tPA-plasminogen-binding sites, whereas the untethered synthetic knobs restrict the fibrin to a conformation in which those sites remain inaccessible. Thus, although the interaction involving the A knob and γC hole is the basis for the polymerization of fibrin, the comparable but delayed interaction involving the B knob and the βC hole is ultimately directed at preparing the clot for its eventual destruction.

Our laboratory has a longstanding interest in small synthetic peptides that can bind to fibrinogen and either prevent fibrin formation or change the character of the fibrin that is formed (*1*, *2*). Thus, peptides beginning with the sequence Gly-Pro-Arg-, which typifies the amino-terminal sequence of most fibrin α chains (the A knob), inhibit fibrin formation, but peptides beginning with the sequence Gly-His-Arg-, which occurs at the amino terminus of fibrin β chains in mammals (the B knob), actually enhance the turbidity of fibrin clots (*1*). It is also known that calcium greatly affects the binding of the synthetic B knob (*3*, *4*) and that fibrin formed by batroxobin, in which case the fibrinopeptide B is not removed, exhibits enhanced turbidity in the presence of a synthetic B knob (*4*).

During the course of examining the influence of various synthetic B knobs on fibrinolysis, we were surprised to find that clots formed in the presence of these peptides were significantly more resistant to fibrinolysis than ordinary clots. Moreover, the more tightly a peptide bound to fibrin(ogen), as manifested by the turbidity of the clot, the greater the impact on subsequent fibrinolysis, whether that fibrinolysis was produced by exogenous plasmin or the fibrin-stmulated tissue plasminogen activator (tPA)[1] activation of plasminogen. This was surprising because it is widely believed that turbid clots are "coarse clots" (*5*) and that "coarse clots" are more rapidly lysed than "fine clots" (*6*−*8*, *inter alia*).

Among the peptides that we have tested are two pentapeptides patterned on the amino-terminal sequences of the β chains from human fibrin (GHRPL-) and bovine fibrin (GHRPY-). Independent of the species from which the fibrinogen was obtained, the latter turned out to be considerably more active as an inhibitor of fibrinolysis, whether the lysis was initiated by adding plasmin or tPA. Because tPA is unusual in that it also manifests a fibrin-independent activity (*9*, *10*), it was necessary to show that the peptides, none of which contains lysine, were not directly inhibiting either tPA or the plasmin generated by it. At the concentrations employed, the peptides do not significantly inhibit the activity of either plasmin by itself or generated by a solution containing tPA and plasminogen, in hydrolyzing small substrates such as the chromogenic peptide D-Val-Leu-Lys-*p*-nitroanilide (dVLKpna). The results, combined with some other papers from the past, have led us to a new interpretation of a major role for the interaction between the B knob and

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[1] Abbreviations: tPA, tissue plasminogen activator; GHRPam, Gly-His-Arg-Pro-amide; GHRPLam, Gly-His-Arg-Pro-Leu-amide; GHRPYam, Gly-His-Arg-Pro-Tyr-amide; GHPRYam, Gly-His-Pro-Arg-Tyr-amide; dVLKpna, D-Val-Leu-Lys-*p*-nitroanilide; PEG, poly(ethylene glycol); SDS, sodium dodecyl sulfate; cc-αc, coiled-coil α chain, residues 148−160 of the fibrin(ogen) α chain.

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its hole on the βC domain and provide another reason for why fibrin but not fibrinogen is able to interact with and activate tPA.

MATERIALS AND METHODS

The peptides Gly-His-Arg-Pro-amide (GHRPam), Gly-His-Arg-Pro-Leu-amide (GHRPLam), and Gly-His-Arg-Pro-Tyr-amide (GHRPYam) were prepared by BOC chemistry (*11*) with a Beckman model 990 automatic peptide synthesizer; purity was assessed by electrospray mass spectrometry and HPLC. The initial concentrations of stock peptide solutions synthesized were determined by quantitative amino acid analysis on a Beckman model 6300 amino acid analyzer; subsequent measurements were determined by HPLC analysis in conjunction with stock reference solutions. The control peptide Gly-His-Pro-Arg-Tyr-amide (GHPRYam) (in which residues 3 and 4 are reversed) was purchased from Sigma−Genosys. The concentrations of peptides purchased from Genosys were determined from the dry weights supplied by the vendor. The concentrations of GHRPYam and GHPRYam solutions were verified by spectrophotometry, with the molar extinction coefficient of tyrosine at $\lambda = 274.5$ being taken as 1340 M^{-1} cm^{-1} (*12*).

Fibrinogen was prepared from outdated plasma, purchased from the San Diego Blood Bank, by a cold ethanol method (*13*). Although the fibrinogen was greater than 95% clottable, it contained biologically active amounts of plasminogen and factor XIII, and we refer to this as "untreated" fibrinogen. In experiments involving calcium, 1.5 mM iodoacetamide was used to inactivate any traces of factor XIIIa that might be generated; the effectiveness of this treatment was demonstrated by running sodium dodecyl sulfate (SDS) polyacrylamide gels (*14*) of fibrin formed in the presence or absence of calcium and the presence and absence of iodoacetamide (Figure S1 in the Supporting Information). In some experiments, plasminogen was removed from plasma or fibrinogen by passage over lysine−Sepharose (*15*). This proved to be especially important in experiments in which exogenous plasmin was added.

The chromogenic peptide dVLKpna was purchased from Sigma−Aldrich. Thrombin was obtained from Enzyme Research; tPA was obtained from Sigma−Aldrich; and batroxobin was obtained from CenterChem, Inc. Plasmin was purchased from Chromogenix; 2 mg of powder was dissolved in 2.5 mL of 50% glycerol (0.8 mg/mL = 9 μM) and stored at −20 °C. Plasminogen was a gift from Dr. Alan J. Johnson, Department Medicine, New York University School of Medicine; the vial containing 4.5 mg of protein powder was dissolved in 2.5 mL of 30 mM Tris at pH 7.5.

Working Stocks. Working stock solutions of thrombin, tPA, plasminogen, and fibrinogen were stored as aliquots at −78 °C. In the case of thrombin, these were prepared by diluting the purchased material (2.8 mg/mL) 100-fold with 0.1% poly(ethylene glycol) (PEG)3350−0.2 M NaCl and storing 50 μL aliquots at −78 °C. The batroxobin stocks contained 20 units/mL. Working stock solutions of tPA were prepared by dissolving the initial vial contents of 10 μg in 0.5 mL of 0.05 M imidazole−0.15 M NaCl buffer at pH 7.0 and storing 50 μL aliquots of the 20 μg/mL solution at −78 °C. Fibrinogen working stocks were prepared by dissolving fibrinogen to concentrations of about 8 mg/mL in 0.05 M Tris at pH 7.0 and 0.15 M NaCl, with 1.0 mL aliquots being stored at −78 °C; the protein was diluted appropriately with the same buffer at the time of use.

Assays. Thrombin−fibrinogen clotting and lysis assays were conducted in 1-mL disposable cuvettes (1-cm path length) in a Helios spectrophotometer at room temperature ($t = 22$ °C), with turbidity being read at 350 nm. Up to six different clotting/lysis mixtures could be monitored simultaneously, although typically, experiments were limited to four or five. Not unexpectedly, there was some variation from experiment to experiment because of small differences in the relative rates of reactions attributable to different batches of fibrinogen or other stock solutions. Unless stated otherwise, all figures depict results obtained in a single experiment. Fibrin monomer reassociation experiments (*16*) were conducted with fibrin that had been dispersed in 1 M NaBr and 0.05 M Na acetate buffer at pH 5.3. Aliquots were diluted 20-fold at time 0 with 0.08 M phosphate buffer at pH 6.8, and the ensuing turbidity followed in a Zeiss spectrophotometer at 350 nm, also at room temperature.

Thrombin−plasmin−fibrinogen assays were conducted under solution conditions similar to those described by others (*17*). Typically, the final solutions contained 0.2 NIH units/mL thrombin, 0.29 μg/mL tPA (5 nM), and 1.1 mg/mL fibrinogen (3.3 μM). Thrombin−fibrinogen−plasmin assays employed plasminogen-free fibrinogen; the concentrations of the three components were thrombin, 0.8 NIH unit/mL; plasmin, 3 nM; and fibrinogen, 3.3 μM. Suitable controls and various concentration dependencies were determined. No lysis occurred in any of these tests unless plasmin or tPA was added (Figure S2 in the Supporting Information), nor did it occur with tPA when plasminogen-free fibrinogen was used (Figure S3 in the Supporting Information). The amounts of thrombin used in these control experiments were the same as described for all of the other experiments.

A large number of experiments were also conducted with an assay employing recalcified citrated plasma, thrombin, and tPA as described by Gombas et al. (*17*); the approach was set aside in favor of the assay involving fibrinogen−thrombin and tPA. A sample of the citrated plasma results, all of which were comparable to those obtained with fibrinogen−thrombin−tPA, is provided as Figure S4 in the Supporting Information).

Chromogenic assays for plasmin with the peptide dVLKpna (final concentration = 0.1 mg/mL) were conducted at pH 7.5, with the yellow color being read at $\lambda = 405$ nm; these included a two-stage assay for plasmin generation, which used a microtiter plate in the second stage. Plates were read in a Molecular Devices, Corp. autoreader set at $\lambda = 405$ nm.

Apparent Dissociation Constants. The apparent dissociation constants of peptides for fibrinogen and fibrin were estimated in two ways. In one, increases in turbidity in the early stages (2 min time points) of the polymerization of fibrin were determined at various peptide concentrations and used as the binding function. In the other, the binding function was calculated from half-fibrinolysis times at various peptide concentrations. Half-fibrinolysis times have been used similarly by others (*18*).

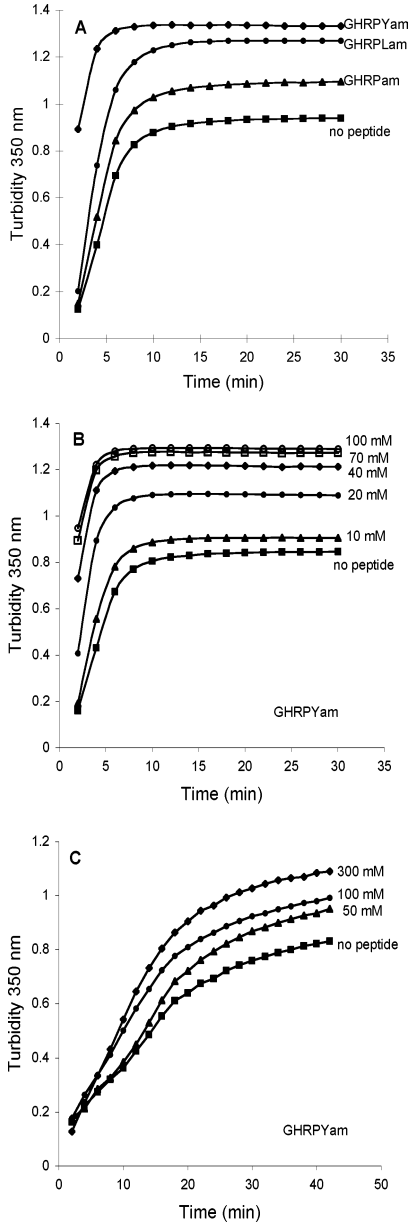

FIGURE 1: Enhancement of thrombin-catalyzed fibrin polymerization by three different synthetic peptides patterned on the B knob of fibrin. (A) GHRPam, GHRPLam, and GHRPYam all at 100 μM final concentration. The initial concentration of fibrinogen was 3.3 μM, with no added calcium. (B) Various concentrations of GHRPYam from 10 to 100 μM, with no added calcium. The data were collected in two consecutive experiments. (C) Effect of various amounts of GHRPYam on the reassociation of fibrin monomers by the method of LaTallo et al. (*16*). The concentration of the fibrin monomer after dilution was 0.65 mg/mL (2.0 μM). Turbidity was measured at $\lambda = 350$ nm.

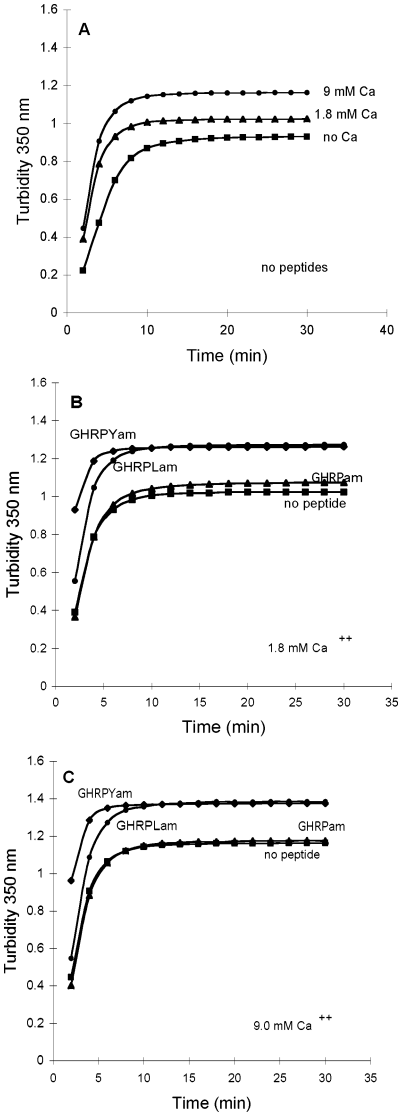

FIGURE 2: Influence of calcium ions on thrombin-catalyzed fibrin polymerization in the presence and absence of synthetic peptide knobs (all at 100 μM). (A) Influence of the calcium concentration on the clot turbidity in the absence of synthetic peptides (data are the same as "no peptide" plots in B and C). (B) Influence of three synthetic peptides (100 μM) on the clot turbidity in the presence of 1.8 mM calcium. (C) Same three synthetic peptides (100 μM) at 9.0 mM calcium. Turbidity was measured at $\lambda = 350$ nm.

RESULTS

Effect of GHRP Peptides on Fibrin Formation. Three different GHRP peptides were compared with regard to their ability to enhance the turbidity observed during the thrombin-catalyzed polymerization of fibrin. The effectiveness of these peptides followed the sequence GHRPYam $\gg$ GHRPLam $>$ GHRPam (Figure 1A). The most effective peptide, GHRPYam, generated a significant increase in turbidity at concentrations as low as 10 μM when the concentration of fibrinogen was 3.3 μM (Figure 1B). Its effectiveness during the reassociation of fibrin monomers was only slightly less, even though the solution environments (pH and ions) differ in the two kinds of experiment (Figure 1C).

The enhancement of clot turbidity by these peptides was influenced by calcium ions, being substantially greater in calcium-free circumstances. As is well-known, calcium on its own tends to increase clot turbidity and, in the absence of a peptide, increased the turbidity of fibrin to about the same extent as did the peptides alone (Figure 2A). The effects were not additive, however, and at increasing concentrations of calcium, the enhancement resulting from the presence of the peptides was decreased (parts B and C of Figure 2).

Turbidity Effects with Batroxobin-Initiated Fibrin. The same three synthetic peptides exhibited the same relative order of potency in enhancing the turbidity of clots formed by the action of batroxobin, but because batroxobin-induced clots tend to be transparent (*19*, *20*), the effects were even more striking (Figure 3A). The turbidity of batroxobin-generated fibrin is also enhanced by calcium ions (Figure

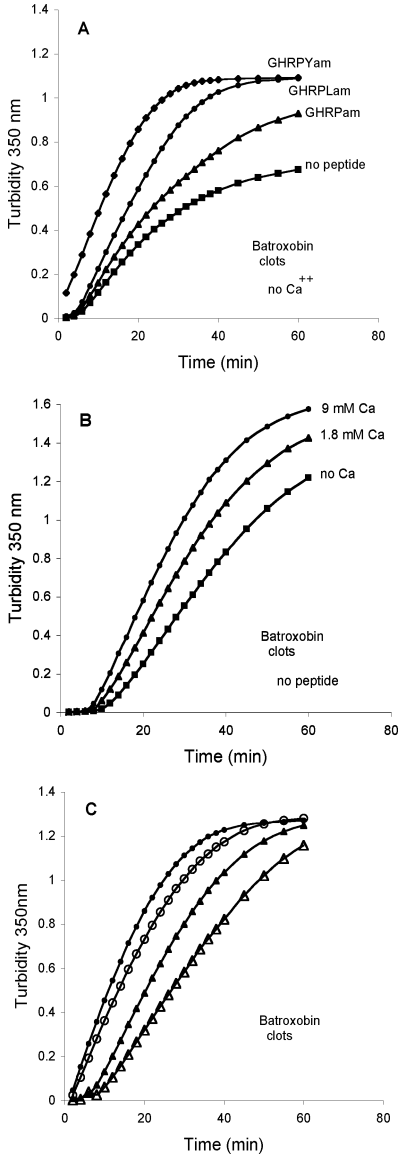

FIGURE 3: Influence of synthetic B knobs on batroxobin-catalyzed polymerization of fibrin. (A) GHRPam, GHRPLam, and GHRPYam all at 100 μM final concentration. The initial concentration of fibrinogen was 3.3 μM, with no added calcium. (B) Influence of calcium on batxrobin-catalyzed fibrin formation in the absence of synthetic peptides. (C) Combined effects of calcium and the peptide GHRPYam on batroxobin-catalyzed fibrin formation. Open symbols, no calcium present; closed symbols, 1.8 mM calcium. Circles denote the presence of 100 mM GHRPYam, and triangles denote no peptide. Turbidity was measured at $\lambda = 350$ nm.

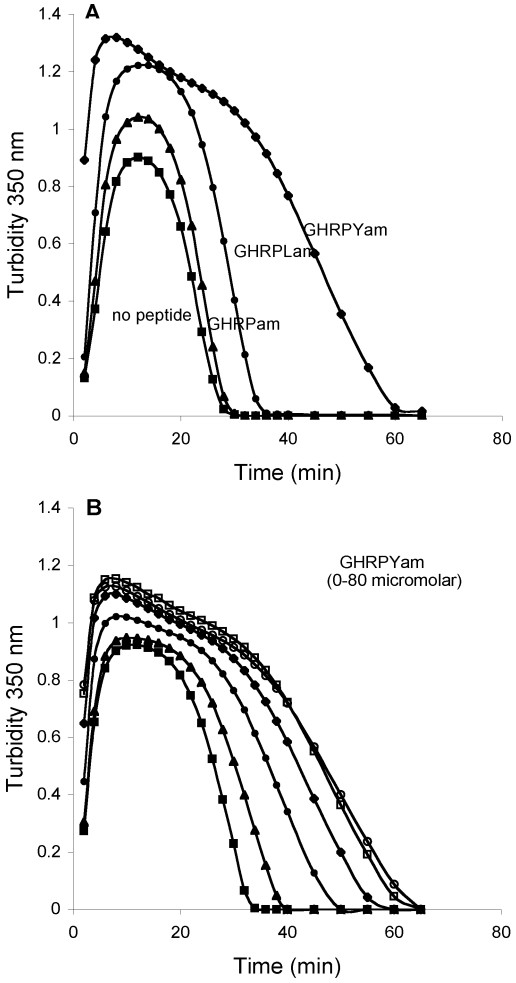

FIGURE 4: Inhibition of fibrin-stimulated tPA activation of plasminogen by synthetic B knobs. The concentration of fibrinogen at time 0 was 3.3 μM. A mix of thrombin and tPA was added such that their final concentrations were 0.08 and 0.29 μg/mL, respectively. (A) GHRPam (▲), GHRPLam (●), and GHRPYam (◆) all at 100 μM final concentration. (■) No peptide, with no added calcium. (B) Same conditions as in A, except that that the concentrations of GHRPYam ranged from 0 to 80 μM [from left to right, 0 (■), 10 (▲), 20 (●), 40 (◆), 60 (○), and 80 (□) μM]. Observations were made in two consecutive experiments.

3B), and as in the case of thrombin-generated clots, the enhancement of turbidity by the synthetic peptides is diminished as the calcium concentration is raised (Figure 3C).

Effect of GHRP Peptides on Fibrin Stimulation of tPA. In experiments with thrombin, tPA, and (untreated) fibrinogen, the presence of the synthetic peptides led to distinct delays before the commencement of fibrinolysis, with the magnitude of the effect corresponding to the order of turbidity enhancement (parts A and B of Figure 4). In the case of GHRPYam, significant delays were observed at concentrations as low as 10 μM (Figure 4B), about the same concentration at which the enhancement of turbidity is first observed (Figure 1B). The control peptide GHPRYam, in which the sequence of residues 3 and 4 is inverted, had no effect on fibrin turbidity or the tPA-activation of fibrinolysis when tested in the same concentration range (Figure S5 in the Supporting Information).

It is notable that even though calcium and these synthetic peptides both have the same effect with regard to the enhancement of turbidity of clots, calcium does not cause any delay in the fibrin-stimulated activation of plasminogen by tPA (Figure 5). Indeed, a small acceleration in lysis is consistently observed (Figure 5).

Two-Stage Assay Monitoring Plasmin Generation. When fibrin is formed *in vitro* in the presence of tPA, plasmin is generated from endogenous plasminogen contained in the untreated fibrinogen preparation. A two-stage assay was employed to demonstrate two important aspects of the system (a) that even in the absence of synthetic peptides there is a lag in plasmin generation accompanying fibrin formation and (b) that the peptide GHRPYam further delays the appearance of this plasmin activity.

To this end, sets of tubes (10 in each set) containing 150 μL of a solution containing thrombin, tPA, and untreated

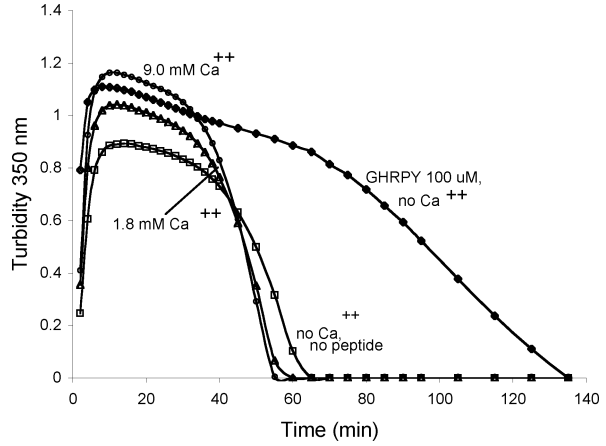

FIGURE 5: Comparison of fibrin-stimulated tPA activation of fibrinolysis in the presence and absence of two different concentrations of calcium ions (■ = 0, △ = 1.8 mM, and ○ = 9.0 mM). Note that even though calcium results in enhanced clot turbidity, the tPA-provoked fibrinolysis is not delayed. The same reaction with 100 μM GHRPYam (◆) (no calcium) was conducted simultaneously.

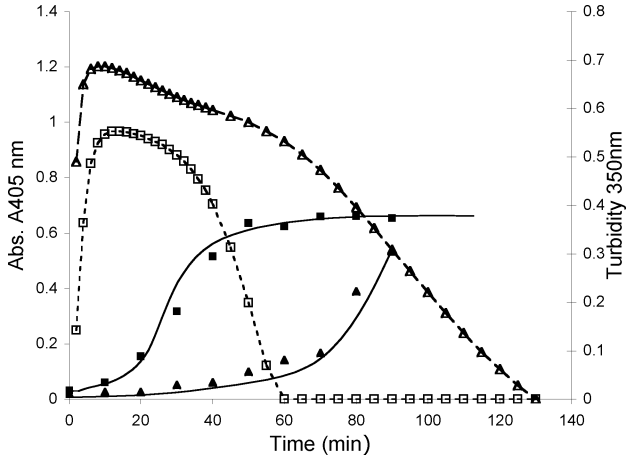

FIGURE 6: Two-stage assay of plasmin generation and its inhibition by 150 μM GHRPYam. Readings taken from microtiter plate at λ = 405 nm over the course of a 90-min experiment with sets of 10 tubes, each of which contained thrombin, tPA, and (untreated) fibrinogen; one set (▲) contained 150 μM GHRPYam, and the other (■) was without a peptide. Turbidities of the clots in the presence (△) and absence (□) of the peptide GHRPYam were monitored at λ = 350 nm in a simultaneous assay, where each cuvette had the same components as the tubes from which the aliquots for the microtiter plate assay were taken (see the text for a further explanation).

fibrinogen (i.e., containing residual plasminogen), with or without GHRPYam (final concentration = 150 μM), were prepared so that the respective times at which clotting was initiated were staggered at intervals of 10 min (covering a span of 90 min). At the designated completion time, 100 μL of the chromogenic peptide was added to each and the tubes incubated for 10 min at 37 °C, immediately after which tubes were cooled, with any remaining clots removed on wooden sticks, and 200 μL aliquots were transferred to a microtiter plate for immediate reading (Figure 6). Parallel assays for following turbidity in solutions of the same composition were conducted simultaneously in cuvettes at λ = 350.

In the set without added peptide, maximum plasmin activity was reached at about 50 min; fibrinolysis in the parallel assay in a cuvette was complete in just under 60 min (Figure 6). In the presence of 150 μM GHRPYam,

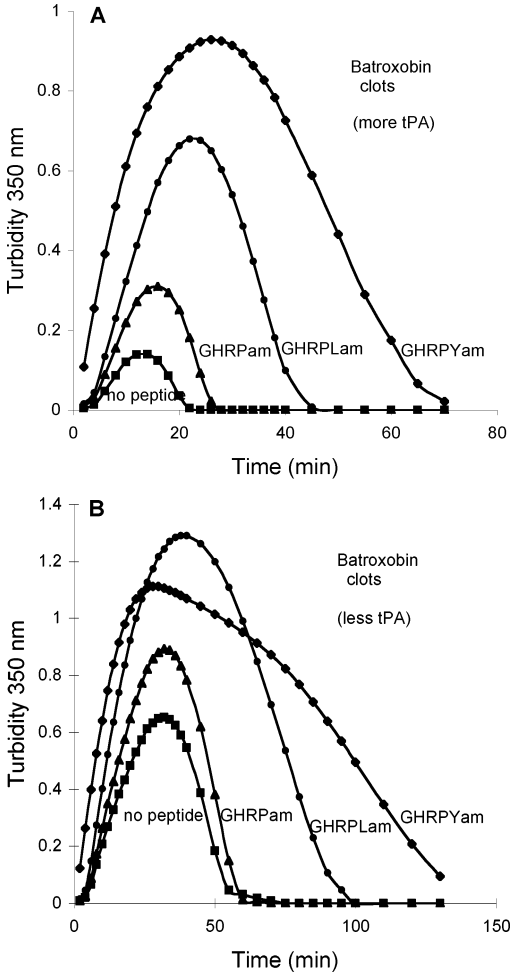

FIGURE 7: Inhibition of tPA-induced fibrinolysis by synthetic B knobs when batroxobin (0.18 unit/mL) is used to bring about polymerization of fibrin. The tPA concentration was 0.29 μg/mL, and the concentration of fibrinogen at time 0 was 3.3 μM. (A) GHRPam, GHRPLam, and GHRPYam all at 100 μM final concentration with no added calcium. (B) Repeat experiment, except that the amount of tPA was reduced to 0.09 μg/mL.

however, plasmin generation was delayed by an additional 45−55 min; once begun, the activity rose sharply, reaching its maximum at about 100 min. Complete lysis in the parallel assay in a cuvette with GHRPYam present was not attained until 130 min.

Lysis of Batroxobin-Initiated Fibrin. Synthetic B knobs had a marked effect on the lysis of fibrin initiated by batroxobin (Figure 7A). Because lysis occurs more rapidly in fibrin produced by batroxobin than when fibrin is produced by thrombin, experiments were also conducted with lesser amounts of added tPA, the concentration of which was clearly the rate-determining factor (Figure 7B). The relative effectiveness of the three different synthetic peptides in delaying fibrinolysis followed the same pattern (GHRPYam ≫ GHRPLam > GHRPam).

Effect of GHRP Peptides on Fibrinolysis by Exogenous Plasmin. The peptides GHRPam, GHRPLam, and GHRPYam were compared with regard to their ability to influence fibrinolysis in systems composed of thrombin, plasmin, and plasminogen-free fibrinogen (Figure 8). Again, the three peptides delayed fibrinolysis in roughly the same proportion as they enhanced the turbidity of fibrin clots formed with thrombin (or batroxobin) or delayed tPA-

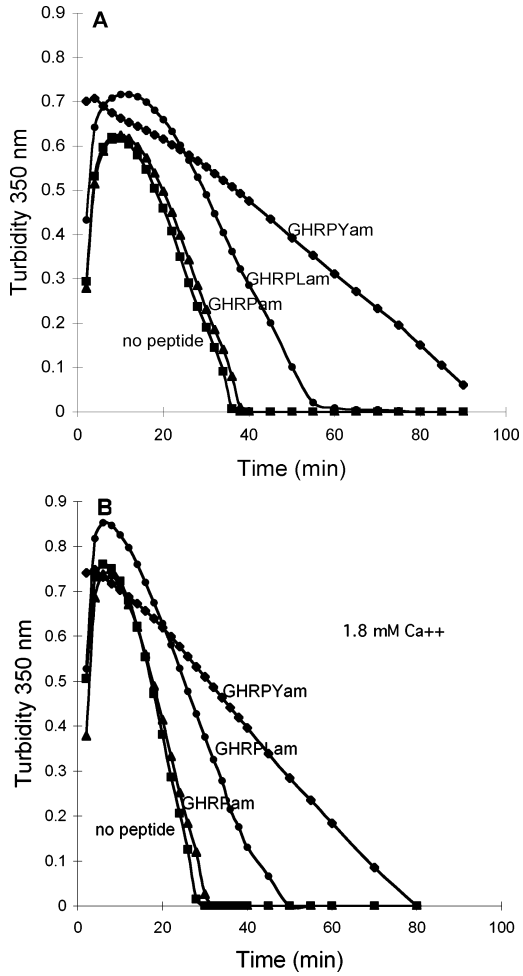

FIGURE 8: (A) Effect of GHRPam, GHRPLam, and GHRPYam (each at 100 μM) on fibrinolysis brought about by adding a thrombin−plasmin mixture to plasminogen-free, iodoacetamide-treated fibrinogen in the absence of calcium (final concentrations: thrombin, 0.8 NIH unit/mL; plasmin, 0.03 μM; fibrinogen, 3.3 μM). (B) Conditions the same as in A, except for presence of 1.8 mM calcium.

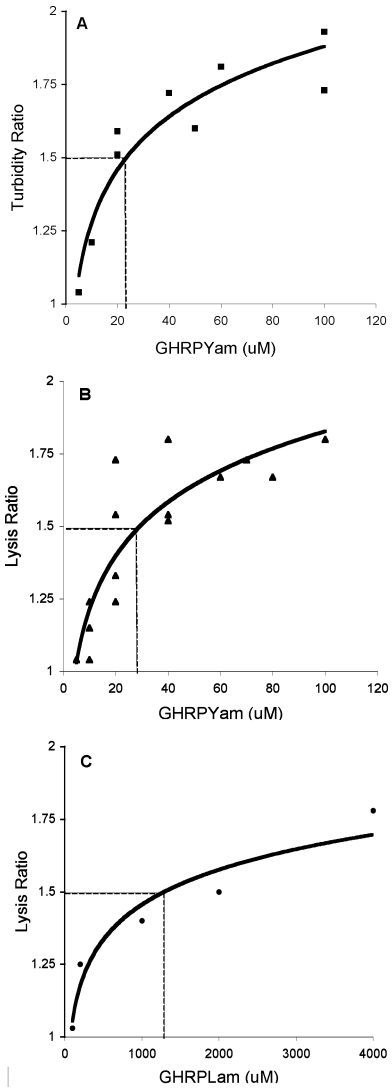

FIGURE 9: Estimation of apparent dissociation constants. (A) Plot of initial turbidity changes versus concentrations of GHRPYam. The values are the ratios of the initial time points (2 min time points) of peptide-containing reactions to the control without a peptide (data from Figure 1B). (B) Plot of changes in tPA-provoked fibrinolysis versus concentrations of GHRPYam. The values are the ratios of the half-lysis times observed in the peptide-containing reactions to that of the control without a peptide (data from Figure 4B). In both cases, an apparent dissociation constant of about 30 μM was estimated from the $^1/_2$ maximum responses. (C) Plot of changes in tPA-provoked fibrinolysis versus concentrations of GHRPLam. Ratios determined as in B. Note the difference in the abscissa scale (concentrations).

activated fibrinolysis. The kinetic profile of lysis differed from when tPA was the limiting factor, however. Because the plasmin is added directly, there is no significant lag time, with lysis beginning as soon (or sooner) as the maximum turbidity point is reached. Interestingly, there is a slight acceleration of the lysis in the presence of calcium ions (Figure 8B), just as was observed in the experiments involving the activation of tPA. It is intriguing, also, that, although the synthetic B knobs prolong the time needed for overall fibrinolysis, there is a brief initial period of rapid vulnerability in which the peak turbidity declines sharply (denoted by arrows in Figure S6 in the Supporting Information).

Relative Affinities of the Synthetic B Knobs for Fibrin(ogen). In all of the different assays, whether involving fibrin formation or lysis, the three peptides exhibited the same relative order of effectiveness, and it was possible to estimate apparent dissociation constants in various ways. In the case of GHRPYam, roughly the same values were found by measuring the turbidity enhancement during fibrin formation (Figure 9A) and by delays in tPA-provoked fibrinolysis (Figure 9B). In both cases, values in the 20−30 μM range were found for the concentrations of peptide at which the respective effects were half of their maximum values. As an index of how much more effective GHRPYam is than GHRPLam, it required an approximately 20-fold higher concentration of the latter to obtain the same delay in fibrinolysis (Figure 9C).

Chromogenic Tests for Plasmin Activity. None of the three synthetic peptides had a significant effect on the plasmin-catalyzed hydrolysis of the chromogenic peptide, dVLKpna. Only a relatively minor effect could be observed at concentrations 10 times higher than those used in polymerization and lysis assays, as shown for GHRPYam in Figure 10A. Similarly, at the same excess concentrations, only a slight inhibition of fibrin-independent activation of plasminogen by tPA resulted when the latter was determined by substitut-

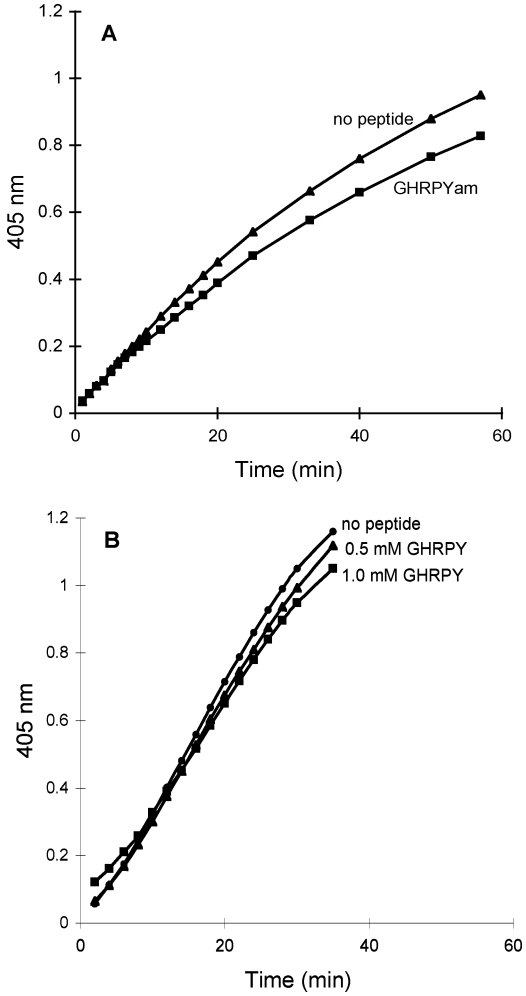

FIGURE 10: (A) Hydrolysis of chromogenic peptide (dVLKpna) by plasmin in the presence of high concentrations of synthetic B knobs. GHRPYam = 500 μM. (B) Hydrolysis of chromogenic peptide by fibrin-independent tPA-activated plasminogen as measured in the presence of GHRPYam (500 μM and 1.0 mM). The color was read at λ = 405 nm.

ing the chromogenic peptide substrate for fibrinogen (Figure 10B).

DISCUSSION

Synthetic peptides based on the sequence of the B knobs of fibrin enhance the turbidity of fibrin clots, whether generated from mixtures of thrombin and fibrinogen, mixtures of batroxobin and fibrinogen, or the reassociation of fibrin monomers. The effect is more dramatic with batroxobin, doubtless because clots induced by that enzyme, in the absence of these peptides, are so transparent. As is well-known, calcium ions also lead to more turbid clots (*4*, *21*, *inter alia*). At the concentrations employed (1.8 and 9.0 mM), the effects are not additive with those brought about by the peptides. It can be presumed that in both cases the enhanced turbidity is due to increased lateral association of growing protofibrils.

The same peptides also render fibrin more resistant to fibrinolysis, whether by the fibrin stimulation of the activation of plasminogen by tPA or by directly added plasmin. Their effectiveness in this regard is roughly proportional to their ability to enhance the turbidity of the resulting clots. This is surprising because it is widely accepted that turbid or "coarse" clots are more readily lysed than "fine" clots (*6*−*8*). The turbid clots that form in the presence of the synthetic B knobs differ from the turbid clots formed in the presence of calcium in this regard; although synthetic B knobs greatly delay the activation of plasminogen by tPA, calcium ions do not (Figure 5).

There are two fundamental questions to be addressed. (a) How do the synthetic B knobs promote lateral aggregation? (b) How do they prevent the onset of fibrinolysis?

Clot Turbidity. The lateral aggregation of protofibrils is the basis of clot turbidity; it is affected by many different factors, of course, including ionic strength (*5*), pH (*5*), calcium concentration (*4*), chloride concentration (*22*), fibrinogen or thrombin concentration (*6*), differential rates of release of the fibrinopeptides A and B (*23*), dextran presence (*24*), and deglycosylation (*25*), among other factors. Despite these many variables, the association of protofibrils needs to employ very specific interactions to keep units in exact register on the way to the uniformly banded fibrin fibers that always ensue.

The clots with enhanced turbidity described in this study appear to be the same as those observed in past experiments under various conditions except that the βC holes are occupied by positively charged synthetic peptides. In some way that occupancy also renders these clots refractory to fibrinolysis. Unlike the natural, intramolecularly tethered B knobs, these synthetic knobs are free and untethered. They can bind to fibrinogen in advance of the action of thrombin or other treatments. To appreciate the impact of these synthetic peptides on fibrinolysis, a brief review is needed of how fibrin is thought to stimulate the activation of plasminogen by tPA.

Activation of tPA by Fibrin. It is well-established that polymerized fibrin but not fibrinogen can enhance the activity of tPA (*26*−*31*). Specific regions of human fibrin have been found to contribute to the activation process, including sites encompassing residues 148−160 in the α chain and residues 320−324 of the γ chain (reviewed in ref *32*). Experiments involving X-ray crystallography have shown that the putative tPA (or plasminogen) binding site on the α chain, which is near the carboxy-terminal end of the coiled coil, is buried in fragment D. It is blocked on one side by a fourth α helix proceeding antiparallel to the coiled coil and on the other by the βC domain (*33*, *34*). These X-ray structures also revealed that the implicated region of the α chain in the covalently joined D dimer derived by digestion of *fibrin* is also inaccessible. As such, it was suggested that whatever structural feature that changed to expose the activation site during the conversion of fibrinogen to fibrin must have relaxed back into its original position during the digestion used to generate the cross-linked fragment (*33*). The challenge is to understand the mechanism by which the site on the α chain within the coiled-coil (from here on abbreviated as the cc-αc site) becomes accessible as a result of the conversion of fibrinogen to fibrin.

The Different Stages of Fibrinolysis. It has also been long appreciated that the stimulation by fibrin of the activation of plasminogen by tPA occurs in more than one step. As early as 1985, Norrman et al. (*35*) described two distinct phases of fibrinolysis. The second and more rapid occurs after the system has been exposed to a certain degree of plasmic degradation. Presciently, they noted that the existence

of the slower starting rate might be a mechanism to protect a clot from premature lysis. At about the same time, Christensen (*36*) provided a molecular explanation for the increase in the rate. She found that it was the carboxy-terminal lysine residues generated by plasmic digestion that were serving as binding sites for additional molecules of plasminogen (and plasmin), thereby providing an amplifying effect. Weisel et al. (*37*), using electron microscopy and photochemical cross-linking, followed the time course of polymerizing fibrin and binding of plasminogen to it. They focused on the initial phases of polymerization and found that plasminogen was bound to the end-to-end junctions within the growing protofibrils, a location well-positioned both for activation by a tPA molecule bound at the coiled-coil site noted above and for appropriate attack on fibrin after its activation. This model was subsequently extended to offer an explanation of the lytic advantage of coarse fibrin networks where plasmin could "walk" directly from one protofibril to another in the clot (*38*). A more recent model devised by others (*32*) reverses the relative locations of plasminogen and tPA, both of which utilize kringle domains for binding to fibrin (*39*, *40*).

What is lacking in these models is any mechanism for delaying the initiation of the stimulation of fibrinolysis produced by fibrin. Surely, it would not be advantageous to initiate the fibrinolytic response coincident with the start of polymerization. We are now proposing that an extended interval between the time when B knobs are first exposed by thrombin and when they eventually find a βC hole can provide the needed delay.

B-Knob Binding and Conformational Changes. It is known that a conformational change occurs in the βC domain upon binding the peptide GHRPam (*41*). A bridge formed by a calcium ion was identified in crystallographic models of fragments D and the cross-linked D dimer linking the βC domain and the coiled coil. The ligands are residues Aspβ398, Aspβ261, Glyβ263, and Gluγ132. In models of the same fragments complexed with the peptide GHRPam, the side chains of Aspβ398 and the adjacent Gluβ397 are rotated approximately 180° around the backbone to form part of the βC "hole". Importantly, even though the bridge is disrupted by the conformational change, the backbones of the polypeptide chains remain in virtually the same positions when the GHRPam is bound as when it is not. These observations have been repeated by others who used recombinant forms of human fragment D (*42*). In both studies, the binding of synthetic peptides mimicking the B knobs results in an important local conformational change but is without long-range consequence, at least in the crystalline state.

Recombinant Mutant Fibrinogens. A connection between clot turbidity and the interactions just discussed has been reported in studies of recombinant fibrinogens in which key residues in the βC hole were substituted by alanine (*43*, *44*). The mutated forms in which either Gluβ397 or Aspβ398 were changed gave rise to fibrin that was less turbid than the wild type. This result implies that these mutations decrease lateral associations within the clots (*43*). In contrast, when a recombinant fibrinogen in which Gluγ132, a key ligand for the calcium bridge between the βC domain and the coiled coil, was changed to alanine, the fibrin clots that formed were much *more* turbid than those of the wild type, particularly in the absence of calcium (*44*). As might be expected, this substitution, away from the βC hole, did not affect the binding of a synthetic B knob, and no global changes were found in the structure of a fragment D prepared from the mutant fibrinogen. Nonetheless, the authors attributed the increased turbidity to enhanced "B/b" interactions (i.e., between the B knob and its hole), even though in the fragment D there was no effect on the binding of the peptide GHRPam and no shift in the βC domain away from the coiled coil in the crystal structure.

A Different Interpretation. Recourse to a previously published model describing how protofibrils may associate during fibrin formation (*45*) would have led to a different interpretation of the excessive turbidity exhibited by fibrin formed from the Gluγ132Ala mutant fibrinogen. The anticipated result of the complete disruption of the calcium bridge, on the basis of that description, would have the βC domain becoming unhinged from the coiled coil, thereby allowing $\beta-\beta$ associations between different protofibrils. In our view, these secondary interactions, which should not be confused with knob−hole interactions, must contribute greatly to the increased turbidity observed with the mutant fibrin. A similar increase in turbidity should occur when the calcium bridge is disrupted by the binding of peptides to the βC hole, and such an increase is observed (Figure 1).

It needs to be emphasized that the thrombin-generated clots described in this study are turbid even in the absence of synthetic B knobs; the peptides merely increase the turbidity. The amount of turbidity of a clot is a reflection of the competition between linear and lateral growth of the fibrin fibers.

Our explanation for the increases in turbidity is based on a detailed consideration of the different intermolecular associations that occur in crystal packing in a variety of crystallographic models of fragments D and the cross-linked dimer, complexed or not with synthetic A or B knobs or both (*45*). In that proposal, lateral associations of protofibrils are initially accomplished by secondary interactions between γC domains of a sort not involving the knob−hole interactions that lead to the formation of the protofibril itself. A concomitant feature of these $\gamma-\gamma$ lateral associations is that the βC domains become positioned tantalizingly close to where they could also form associations between protofibrils. All that is required is that they be pulled away from the coiled coil. Initially, we supposed a corollary benefit would be exposure of the cc-αc tPA/plasminogen binding site, and it was proposed that the B knob must be involved in holding the βC domain away so that the cc-αc site remained accessible (*45*). Some revision is now in order in light of our new findings.

Different Roles for Different Holes. The fibrinopeptides A, two in each fibrinogen molecule, are located at the ends of short tethers that are joined by a disulfide bond that is located a dozen residues carboxy-terminal to the junction on the α chain that is cleaved by thrombin. Release of the fibrinopeptides A exposes the amino-terminal "A knobs" with the sequence Gly-Pro-Arg-, which are then able to find "holes" located at the ends of neighboring molecules of fibrin(ogen) molecules and bridge them together (*46*). Crystal structures have shown that synthetic peptides mimicking the A knobs bind to holes on the γ subunits of abutting molecules and are perfectly placed for bridging fibrin units together,

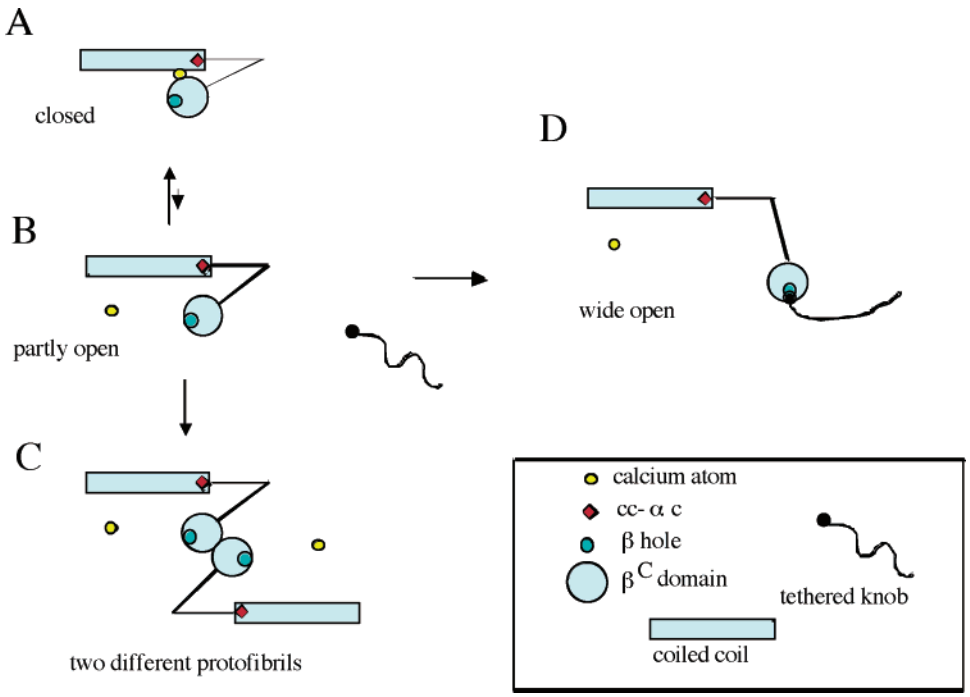

FIGURE 11: Cartoon depicting three different conformations of the βC domain relative to the coiled coil. Normally, there is always a small population of molecules in which the calcium bridge is disrupted and the partly open form exists. This form can engage in associations with βC domains on other protofibrils and enhance turbidity. The even smaller fraction of molecules in a wide-open state can be ensnared by tethered B knobs and give rise to the activation of plasminogen by tPA.

with the two-modeled tethers meeting in a nearby hypothetical E domain as required (*33*).

The two fibrinopeptides B are located at the ends of significantly longer tethers that are joined by a disulfide bond about 50 residues carboxy-terminal to the junction on the β chain that is cleaved by thrombin. In contrast to the situation for A knobs, the holes into which the B knobs fit are very remote from each other, with their nearest approach within the modeled protofibril being approximately 14 nm (*45*). Furthermore, the gates to these βC holes are guarded by large carbohydrate clusters (*33*, *34*). Also, the tethers leading to the B knobs are not visible in maps of electron density and must be extensively disordered in the crystals of native fibrinogen (*47*).

How long does it take the B knobs at the ends of the long, flexible tethers to find these guarded βC holes after the removal of the fibrinopeptides B? The fact that the association of the B knob and its hole is not instantaneous is demonstrated by the fact that antibodies to the amino terminus of the fibrin β chain (GHRP-) react with fibrin clots, even though antibodies to the fibrin α chain (GPR-) do not (*48*). This implies that the release of the fibrinopeptide B does not mean that the resulting B knob automatically and immediately finds a hole that coincidentally protects it from the antibody. As fibrin matures, however, the B knobs gradually become unavailable to antibodies (*49*).

Recent experiments designed to measure the strength of knob−hole interactions in fibrin have shown that the binding of the B knob to its hole does not contribute significantly to the strength of a clot (*50*). Virtually all of the bond energy holding the polymer together is contributed by the A-knob−γC hole interactions, with no apparent benefit being observed from B-knob interactions (*50*).

Although the release of the fibrinopeptide B certainly leads to the lateral association of protofibrils, if only by reducing much of the Coulombic repulsive charge between these entities, that is not what promotes access to the tPA activation site. Rather, it is the delayed capture of the mobile tethered B knob exposed by thrombin that is crucial. The tether is very long; the wandering knob is unconfined; the insertion of the knob in the hole needs to be coordinated with the local conformational change involving residues β397−398 as well as the more global change in which the βC domain is in a shifted position away from the coiled coil, a population of molecules that must constitute only a small fraction of the βC population; and the shift is hindered by the calcium bridge (Figure 11). For all of these reasons, it takes substantial time for a productive encounter to occur between a B knob and an appropriately situated βC domain, but when it does occur, the cc-αc site becomes permanently exposed.

The question then becomes, what is the molecular difference between the binding of a free peptide to the cc-αc site and occupation of the same site by the authentic knob? The binding of the latter promotes the activation of tPA and plasminogen, while the binding of the former inhibits this activation. Both provide electrostatic compensation to the inherent negative charge of the region, and both should disrupt the bridge formed by calcium. It must be because the authentic knob is on a tether that is anchored at its other end to a fixed location on a molecule of fibrin. When an authentic B knob snags a βC domain in its infrequently occupied position far away from the coiled coil, the capture prevents it from relaxing back into the position blocking access to the cc-αc site. The tether is like the flexible fishing line that becomes taut when the hook snags the mouth of a fish (Figure 11).

Reconciling All of These Matters. Clearly, there must be, at least, three different conformations of fibrin in this region. In one, the cc-αc site is fully accessible to tPA or plasminogen or both; in another, it is not. There is another

intermediate form where the cc-αc site is still inaccessible but in which the calcium bridge is disrupted. In "early" fibrin, including a period in which protofibrils are fully associated, the three conformations are in equilibrium, but the wholly inaccessible form is highly favored. After the removal of the fibrinopeptide B, however, the wandering B knob eventually finds the elusive βC hole and locks the system into the wide-open form and access to the cc-αc site is fully realized.

An untethered synthetic B knob, on the other hand, which can rapidly bind to a βC hole under any circumstance, although it is able to disrupt the calcium bridge, does not significantly increase the concentration of the wide-open conformation. Instead, the presence of the synthetic B knob leads to the βC domain becoming trapped in associations between protofibrils (Figure 11).

Regarding Direct Plasmic Degradation. The scenario described above may explain how synthetic B knobs delay the stimulation of the tPA activation of plasminogen, but it does not explain why fibrin in the presence of these same peptides is more resistant to *direct* plasmin degradation. One possibility is that the cc-αc site is also involved in the binding of plasmin. If this were so, then degradation would occur at a slower but steady rate, much as is observed (Figure 8).

Batroxobin-Generated Fibrin. In addition, the model does not explain our observations with batroxobin, an enzyme that does not release the fibrinopeptide B. It has been reported that fibrin generated by batroxobin enhances tPA activity (*51*, *52*), results that are more in accordance with the idea that all that is needed for the binding of plasminogen, tPA, or both is an end-to-end junction in an individual protofibril. It is possible that the batroxobin clots stimulate the activation of plasminogen by tPA by utilizing the tiny fraction of fibrin in which the βC domain is in the wide-open form that can exist even before the fibrinopeptide B is released, with the small amount of plasmin that results being sufficient to digest the flimsy clot.

GHRPYam Tight Binding. Finally, we should comment on why the pentapeptide GHRPYam, patterned on the sequence that occurs in bovine fibrin, is so much more effective than the other peptides tested, both with regard to enhancing the turbidity of clots and delaying fibrinolysis, even when human fibrinogen is used. Inspection of published crystallographic molecular models of human fragment D complexed with GHRPam (*34*, *41*) suggests that a tyrosine side chain carboxy-terminal to the proline at position 4 would interact with that of βArg406, a highly favored interaction (*53*).

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SUPPORTING INFORMATION AVAILABLE

Figure S1, SDS polyacrylamide gels; Figure S2, demonstration of the requirement for added tPA for fibrinolysis in the thrombin−fibrinogen assays used throughout this study; Figure S3, demonstration of the requirement for residual plasminogen for fibrinolysis in the thrombin−fibrinogen assays used in many experiments reported in this paper; Figure S4, effect of GHRPYam (■) on fibrinolysis with the assay using recalcified citrated plasma, thrombin, and tPA as described by Gombas et al. (*17*); Figure S5, comparison of GHRPYam (■) and GHPRYam (▲), both at 150 mM concentrations, with regard to fibrin stimulation of tPA activation of plasminogen; Figure S6, effect of GHRPam (▲), GHRPLam (●), and GHRPYam (◆) on fibrinolysis produced by exogenously added plasmin. This material is available free of charge via the Internet at http://pubs.acs.org.

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