Searching for Differences between Fibrinogen and Fibrin that Affect the Initiation of Fibrinolysis

Russell F. Doolittle*

Depts. of Chemistry & Biochemistry and Molecular Biology, University of California, San Diego, La Jolla, CA 92093-0314, USA

Abstract: Although in a gross sense fibrin is merely a collection of fibrinogen molecules packed together in bundles, numerous small structural differences can arise as a result of the conversion of the soluble precursor into the gelled product. Some of the consequences are obvious, others more subtle. In one way or another, all these changes are the result of a sequence of events that includes the release of the fibrinopeptides A and B, the formation of protofibrils, the cross-linking of γ chains, the assembly into mature fibers and the cross-linking of α chains. Numerous immunologic differences between fibrinogen and fibrin have been cataloged, and putative sites for fibrin enhancing the activity of plasminogen activators have been identified. Although some conformational changes have been found by X-ray crystallography, the structural changes leading to the exposure of sites thought to bind t-PA and/or plasminogen remain to be demonstrated.

Key Words: Fibrinogen, fibrin, tissue plasminogen activator, plasminogen, conformational changes, synthetic peptide knobs.

INTRODUCTION

Fibrin clots are well known to contain the seeds of their own destruction. Something about the polymerization of fibrinogen changes its countenance such that specific regions of the resulting fibrin enhance the activation of tissue plasminogen activator [t-PA], even though the same regions are inactive in fibrinogen [reviewed in ref. 1, 2]. This mini-review focuses entirely upon what it is about fibrin--as opposed to fibrinogen--that encourages its own dissolution. In particular, the aim is to target conformational changes that are responsible for t-PA and plasminogen being bound to fibrin. There have been numerous articles published about these phenomena in the past, and the brief history described here is mainly for context; it should be regarded more as a representative sampling than a complete survey. Moreover, in the ultra-reductionist approach being adopted, we will ignore many agents that can influence the process under physiological conditions, including antithrombins and plasminogen activator inhibitors. Instead we will concentrate on simple systems, mostly involving thrombin and fibrinogen, as clot formers, and either a t-PA-plasminogen combination or plasmin as clot destroyers (Fig. **1**). During the development, we will also comment on the many ways by which fibrinolysis can be enhanced or delayed by various circumstances, *in vitro*.

EARLY HISTORY

The seminal observations about fibrin stimulating fibrinolysis were made in the early 1980's. In one case, Binder & Spragg [3] in a study of the conversion of iodine-labeled plasminogen to plasmin by a vascular plasminogen activator, found that, when added separately, neither thrombin nor fibrinogen had any effect on the process, but when added together the rate of plasmin generation increased by 10-20-fold. The observation was quickly confirmed by others [4-6]. During this period, also, it was reported that the plasminogen content of serum is lower than that of plasma, a significant portion of the loss being attributed to adherence to fibrin [7]. The sum result of these studies made it clear that fibrin itself contributed to the generation of plasmin under conditions where fibrinogen could not.

A further advance was the finding that the activation of plasminogen occurs in two stages, the second and more vigorous occurring after the fibrin had already experienced a degree of plasmic degradation [8]. A molecular explanation was provided by Christensen [9], who found that it was the carboxy-terminal lysines exposed by plasmic digestion that were serving as binding sites for more molecules of plasmin and plasminogen, thereby amplifying the lytic effect. The finding also explained the remarkable effectiveness of the affinity chromatographic purification of plasminogen by lysine-Sepharose and its elution by the lysine analog ε-amino caproic acid (EACA, a.k.a. aminohexanoic acid, ACA, is deaminated lysine), the presentation of the α-amino-attached lysine mimicking a carboxy-terminus [10].

Biochemically speaking, lysine had already figured prominently in considerations of plasminogen and plasmin. As early as 1959, EACA had been found to prevent the activation of plasminogen, as well as inhibiting the action of plasmin [11]. The events surrounding the early clinical use of EACA [12] are described in a charming memoir by Professor Okamoto [13]. The same Japanese group subsequently found that another lysine analog, tranexamic acid [aminomethylcyclohexanoic acid, AMACHA], is an even more powerful inhibitor of lysis [14]. As is the case for EACA, tranexamic acid does not act principally on the catalytic site of plasmin [15], even though the specificity of plasmin favors cleavage at lysine sidechains [16]. Rather, as implied in the earlier reports of EACA blocking plasminogen activation [11], it occupies a separate lysine binding site [LBS], later found to reside among subsidiary domains known as

*Address correspondence to this author at Depts. of Chemistry & Biochemistry and Molecular Biology, University of California., San Diego, La Jolla, CA 92093-0314, USA; Tel: 858 534-4417; Fax: 858 534-4985;
E-mail: rdoolittle@ucsd.edu

1871-5257/08 $55.00+.00

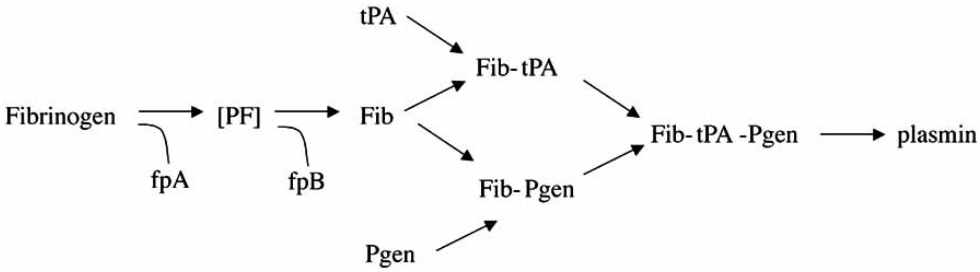

Fig. (1). Schema of events leading to fibrin-stimulation of tissue plasminogen activator and generation of plasmin. PF, protofibril; Fib, fibrin; tPA, tissue plasminogen activator; Pgen, plasminogen; fpA, fibrinopeptide A; fpB, fibrinopeptide B. Note that a ternary complex [Fib-tPA-Pgen] is needed for the initial conversion of plasminogen to plasmin.

kringles. Moreover, binding of lysine analogs like EACA or tranexamic acid was found to induce conformational changes in the kringle regions of plasminogen and t-PA [17]. It should be noted that, although plasminogen and t-PA are descended from a common ancestral protease, plasminogen has five kringles and t-PA only two; in addition, t-PA has a fibronectin finger domain and an EGF domain. Given these differences, it might be expected that t-PA and plasminogen would bind to different sites.

On another note involving lysine, Nesheim and colleagues [18] discovered a thrombin-activated carboxypeptidase B [designated TAFI, for thrombin activated fibrinolysis inhibitor] that removes carboxy-terminal lysines, bringing attention to yet another level of regulation for the fibrinolytic response, and confirming the importance of carboxy-terminal lysines.

METHODOLOGY

Many different approaches have been brought to bear on the matter of t-PA activation of fibrinolysis, but most fall into one of three general categories. The simplest involves mixing fibrin(ogen)-derived materials with t-PA and plasminogen in the presence of a chromogenic substrate that becomes colored in the presence of plasmin [19]. A second kind of experiment involves mixing thrombin and fibrinogen in the presence of t-PA and plasminogen and following clot formation and lysis turbidometrically. Many other experiments have involved solid phase binding of one or more of the principal components—-usually t-PA or plasminogen--followed by systematic exposure to other materials and the affinity for them measured by various means, including conventional enzyme-linked immuno-sorbent assays (ELISAs) or surface plasmon resonance (SPR). In many of these studies, the availability of lysine analog inhibitors like EACA, coupled with the use of the enzyme TAFI, can be put to good use in distinguishing lysine binding sites from lysine-independent sites, on the one hand, and distinguishing carboxy-terminal lysine-binding sites from internal lysines, on the other [20]. Thus, if the binding of a particular agent was inhibited by EACA, it has been presumed the binding involved lysine. If treatment with TAFI reduced the binding, it was presumed it was a carboxy-terminal lysine that was involved.

As it happens, native human fibrinogen, as it is secreted into the circulation, contains 208 lysine residues (being a dimer, it actually has 104 in each half), and none of them occurs at the carboxy-terminus of a polypeptide chain. Moreover, so far as is known, no lysine bonds are cleaved during the conversion of fibrinogen to fibrin. It could be presumed, then, that initiation of fibrinolysis does not require a carboxy-terminal lysine residue. In order to explore the problem meaningfully, it is necessary to review briefly some additional old but important observations.

PLASMIN DIGESTION OF FIBRINOGEN AND FIBRIN

The bonds targeted by plasmin during the digestion of fibrinogen and fibrin have been studied exhaustively, beginning with the report of Nussenzweig *et al.* [21] in the late 1950's that led to the enduring designation of the core fragments D and E, and, a decade later, the identification of intermediate fragments X and Y by Marder *et al.* [22] and their correlation of those fragments with the three-dimensional structure of fibrinogen provided by electron microscopy [23].

For the most part, the degradation of fibrin follows a similar course [24], αC fragments being removed early in the process, followed by severance of the coiled-coil connectors and the exposure of new carboxy-terminal lysine residues. A major difference between fibrinogen and fibrin degradation products, however, is the result of fibrin being cross-linked by factor XIII, γ-chains becoming reciprocally cross-linked near their carboxy-termini [25]. As a consequence, D-dimers are generated instead of simple fragments D. Actually, a product unique to the plasmic digestion of cross-linked fibrin is the entity D_2E, first discovered in 1974 [26], and later shown to comprise the bulk of digested fibrin [27]. Much of the discussion that follows will center on the details of how fragment E interacts with the covalently cross-linked fragments D known as D-dimer. Schematic diagrams of the D-dimer and fragment E are shown in (Fig. **2**).

THE HUNT FOR T-PA/PLASMINOGEN BINDING SITES ON FIBRIN

The search for what it is about fibrin that distinguishes it from fibrinogen with regard to stimulating fibrinolysis divided into two schools of thought. Some workers felt that it was the mere association of monomeric units that in itself provided an immobile scaffolding for plasminogen and/or t-PA; others were certain that the principal stimulus was because binding sites are buried in fibrinogen and only became exposed during the transformation to fibrin. Early support for the latter view included the observation that simply denaturing fibrinogen gave rise to material that markedly acceler-

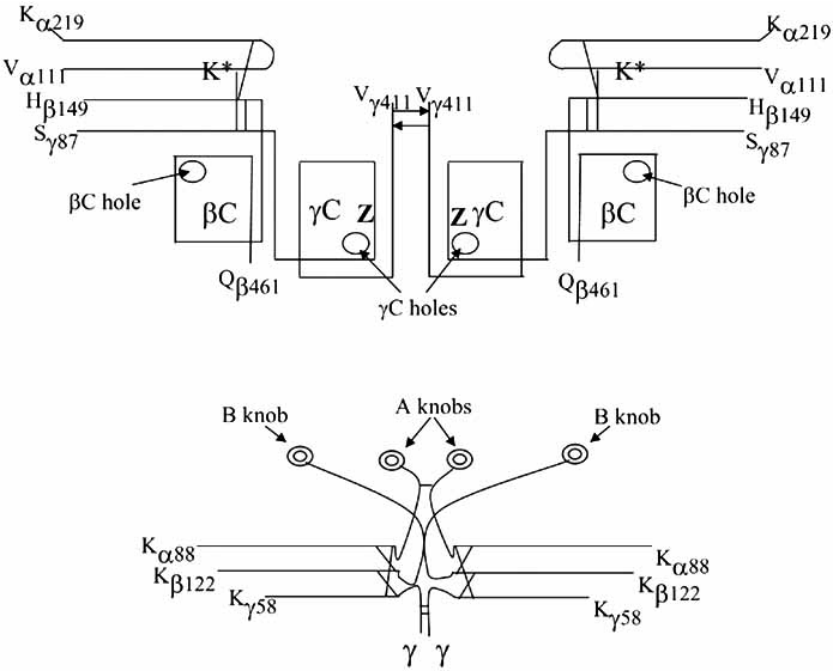

Fig. (2). Simplified depiction of fragments E (lower) and D-dimer (upper) showing key residues discussed in text. All the numbering is based on human fibrinogen. More highly degraded forms of fragments E and D exist than those shown. Lysine (K) carboxy-terminals are of particular interest. The site labeled **K*** corresponds (roughly) to the α-chain region 150-159, and those labeled **Z** to the γ-chain region 312-324.

ated plasmin generation [6]; disquietingly, denaturation of some other proteins, including immunoglobulins and ovalbumin, resulted in the same degree of stimulation [6]. Indeed, the decade that followed the discovery of fibrin activating its own lysis is littered with conflicting results, and, as will become evident, caution needs to be exercised in accepting any single observation on its own.

Although numerous laboratories turned their attention to finding the structural basis of the effect, arguably none contributed more to the effort than the Dutch teams at Leiden and Nijmegen. Like some others, their first experiments centered around plasmin-generated core fragments which mimicked the stimulatory action of fibrin, including fragments D, E and D-dimer [28]. They next turned their efforts to chemical fragmentation of fibrinogen with cynanogen bromide, following a regimen that had been developed by workers investigating the amino acid sequence of fibrinogen [29]. A segment of the α chain encompassing residues 148-197 (all numbering corresponds to human fibrinogen) was found to be particularly effective [30]. Further experiments involving synthetic peptides localized the active region to residues 148-160, a section that included a single lysine residue at position 157. Peptides in which the lysine was replaced by arginine or valine were totally ineffective [31]. Disconcertingly, subsequent experiments showed that glutamic acid could substitute for the lysine without any loss of activity [32].

On the other hand, a strong case for this region being involved in the t-PA activation site was made by the finding that the segment was accessible to monoclonal antibodies in fibrin but not fibrinogen [33]. Moreover, a synthetic hexapeptide corresponding to residues 154-159, the sequence of which is ..IDIKIR..,enhanced the rate of t-PA stimulation by six-fold [34].

Meanwhile, another cyanogen bromide fragment from fibrinogen was found to be capable of stimulating plasmin generation, in this case composed of two consecutive segments of the γ-chain, residues 311-336 and 337-379, which are connected by a disulfide bond [35]. Reduction of the disulfide bond abolished all activity. Binding studies with the full 69-residue cyanogen bromide fragment established that it bound t-PA in a lysine-independent manner and was not affected by EACA [20]. A portion of this region had previously been implicated as a plasminogen-binding site by others [36].

The authors noted that one section of this fragment had a charge distribution that was similar to what is found in the active α-chain peptide described above: ..WDNDNDKF.., for γ-chain residues 315-322 and ..LEVDIDIKI.. for α-chain residues 150-158. Given this tenuous resemblance, they raised monoclonal antibodies to a synthetic peptide stretching from γ-chain residues 312-324 and found that indeed they reacted with fibrin but not fibrinogen [35].

THE ROLE OF POLYMERIZATION

The possibility that the polymerization of fibrin units *per se* plays a role in the activation of fibrinolysis should not be discounted [4]. Part of the argument for polymerization playing a role is based on the premise that t-PA and plasminogen interact only weakly in solution and that localization by fibrin favors their association [37]. In line with this view, an elegant electron microscopy study that involved cross-linking plasminogen molecules to fibrinogen or fibrin [38] showed that much more plasminogen is bound to fibrin than to fibrinogen, and considerably more is bound to fragment X polymer than to fibrin. Fragment X polymer, unlike either fibrinogen or fibrin, ought to have a significant number of

carboxy-terminal lysines. As expected, fibrin purposely made with substantial amounts of fragment X is much more vulnerable to lysis [39].

Polymerization and localization cannot alone be the whole story behind fibrin activation of fibrinolysis, or the small peptides discussed above would not stimulate the process. Rather, the activity of those fibrin-derived materials in free solution implies an interaction with t-PA and/or plasminogen that affects their reactivity. We will have more to say on the matter below.

αC DOMAINS

Other portions of fibrin have been found to bind t-PA and fibrinogen besides those residing in the regions circumscribed by α-chain 150-159 and γ-chain 312-320. In particular, recombinant forms of the αC domain embodying residues 392-610 (the carboxy-terminal one-third of α chains) have been found to contain unique binding sites for t-PA and plasminogen [40]; they are independent in the sense that t-PA and plasminogen do not compete with each other. Moreover, the sites, which are not detected in fibrinogen, have been found to be lysine-dependent. Because the recombinant material ought not to have any carboxy-terminal lysines, the two dependencies must be associated with different lysine side chains among the 16 internal lysines in the constructs. Determining the exact locations of these sites remains a high priority, as would be the demonstration of how these disheveled structures can possibly differ in fibrin from fibrinogen. A clue may be provided by the observation that factor XIII cross-linked αC domains are twice as active as uncross-linked ones [40], keeping in mind that cross-linking ought to reduce the number of available lysine side chains [41].

Recent NMR studies [42, 43] have shown that αC domains have a small central core around its single intrachain disulfide bond, but beyond that, they are largely unfolded, in line with longstanding notions that this part of the molecule mostly lacks significant amounts of secondary structure [44]. On a related note, past studies have consistently shown that fibrin made from fibrinogens with truncated α chains--i.e., lacking the αC domain--are much more prone to lysis than normal fibrin [45]. In these defective fibrins it is likely that the greater vulnerability is attributable to defective polymerization and should not be considered as an argument against the importance of plasminogen and t-PA binding sites in the absent αC domains.

As it happens, a small but measurable fraction of fibrinogen molecules prepared from freshly drawn blood invariably shows evidence of *in vivo* proteolysis, in particular a 27-residue peptide having been cleaved from the carboxy-terminus of the α chain. The exposed carboxy-terminal lysine that results could well serve as an initiation point for fibrinolysis when these molecules are subsequently incorporated into fibrin [46].

MORE ABOUT D_2E

Summing up to this point, a host of biochemical experiments over the years has resulted in two particular regions of the fibrin(ogen) molecule becoming the centers of attention for various models explaining fibrin-activated fibrinolysis: α-chain residues 148-158 and γ-chain residues 314-325. As noted, in the latter case the site likely involves a neighboring region that may extend as far as γ-chain residue 380. Since both of these regions exist in fragments D and D-dimer, neither of which is active in this context, the focus has increasingly turned to D_2E. The questions before us are, how does D_2E differ from D-dimer alone, and what is it that stimulates the t-PA activation of fibrinolysis?

In a set of carefully wrought experiments, Yakovlev *et al.* [47] confirmed that D_2E binds to immobilized t-PA, but D-dimer does not (although in some experiments, the D-dimer "exhibited a weak stimulating effect" with regard to plasmin generation). A convincing case was also made for both t-PA and plasminogen binding to an ultra-core fragment D shorn of its γC domain, denoted Dγ, as well as to an even smaller fragment lacking both βC and γC domains, which is to say, the coiled-coil stub that encompasses the targeted α-chain residues 148-160. The results with the Dγ fragment imply that the removal of the γC domain has allowed the βC domain to swing away from the activation site in the coiled coil. These are very persuasive results favoring a principal role for α-chain residues 148-160.

Still, even though it can provoke t-PA activation, D_2E is not an exact stand-in for the situation in fibrin. In particular, D_2E contains carboxy-terminal lysines that are not present in fibrin. That these carboxy-terminal lysines play a role in the D_2E activation was long ago shown by Varadi and Patthy [48] who removed carboxy-terminal lysine from the β chain coiled coil remnant with carboxypeptidase B and showed that the ability to stimulate t-PA fibrinolysis was lost. Other workers have also demonstrated that treatment of D_2E with TAFI greatly reduces its ability to provoke a response [49].

Beyond that, experimental evidence has always argued against an intimate association between the fragment E portions of fibrin and the fragment D regions of other units to which they are bound. Scanning calorimetry long ago showed that the D and E domains of both fibrinogen and fibrin "melt" independently [50]. The only known interactions between the E domain and the D-dimer involve knob-hole interactions, and, of the two kinds, recent optical tweezer studies have shown that the one of these involving the A knob and γC hole interaction accounts for the vast majority of the binding energy [51]. Moreover, as will be made clear shortly, the knobs themselves are situated at the ends of highly flexible tethers.

INFLUENCE OF CLOT STRUCTURE

Clots can differ greatly in their character, mostly the result of the relative rates of linear and lateral fiber growth as protofibrils associate, as well with regard to the degree of branching [52, 53]. The relative rates of linear and lateral propagation are governed by a number of factors, including the differential release of the fibrinopeptides A and B. As is well known, removal of the fibrinopeptides A results in the exposure of a pair of A-knobs, which can bind to holes on the γC domains of neighboring molecules and hold them together in an end-to-end fashion. The growth of protofibrils continues in this linear, half-molecule overlapping fashion

until some limiting length is achieved. During this period, the cleavage of fibrinopeptides B greatly accelerates, leading to lateral associations of the protofibrils. The more lateral association that occurs, the more turbid, or coarser, the clot [54].

Generally speaking, anything that reduces the overall electronegative charge of protofibrils will encourage more lateral bundling and lead to more turbid clots. Among the ways, besides the simple release of the negatively charged fibrinopeptide B, are the addition of calcium ions, removal of negatively charged sialic acid residues, and, importantly, the binding of positively charged peptides.

For the most part, coarse clots tend to lyse faster than fine clots [55]. One explanation that has been offered in the past is that coarse clots have more protofibrils bundled together in a restricted volume, and once fibrinolysis is initiated, plasmin(ogen) molecules have the opportunity to "crawl" directly from protofibril to protofibril, gnawing away as they go [56].

INFLUENCE OF PEPTIDES ON CLOT STRUCTURE

It has long been known that synthetic peptides corresponding to the A and B knobs have very different effects when added to a polymerizing system. Not surprisingly, synthetic A knobs (peptides beginning with GPR-) inhibit polymerization [57]. It was surprising, however, that synthetic B knobs (beginning with GHR-) actually enhance the turbidity of clots [57]. Even more surprising was the belated discovery [58] that these particular coarse clots lyse very slowly (Fig. **3**).

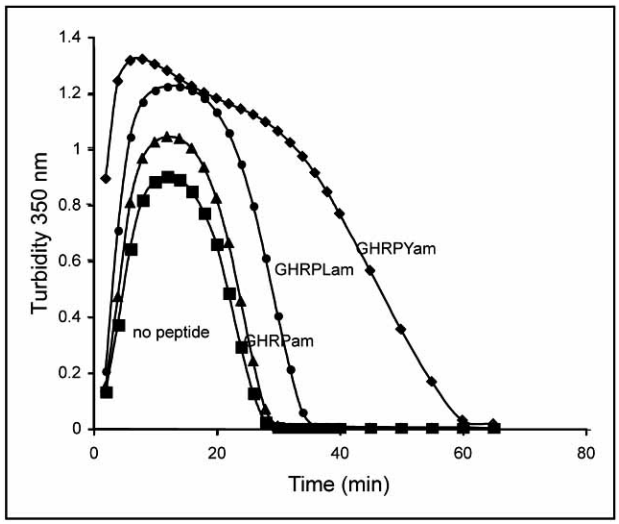

Fig. (3). Enhancement of fibrin polymerization by synthetic peptides patterned on the B-knob of fibrin followed by delay in t-PA activated fibrinolysis. Turbidity measured at λ = 350 nm. [from ref. 58].

Remarkably, also, was the recent finding that another positively charged peptide that occurs in fibrin(ogen), corresponding to γ-chain residues 369-380, also enhances the turbidity of clots and is even more powerful in its delaying of t-PA activated fibrinolysis [59]. Although these two quite different peptides clearly bind to different sites on fibrin(ogen), they appear to be influencing clot structure similarly, and in a way that makes the fibrin more refractory to lysis. How these coarse clots differ from the coarse clots generated in other circumstances, in which lysis occurs so much faster, remains unknown. It should be remarked that under some other circumstances coarse clots have been found to lyse more slowly than fine clots, also [60], reminding us of the many subtleties that influence this system.

There is a possibility that it is not the clot structure but rather some other influence of the peptides that is the cause of the delay. As it happens, the peptide corresponding to γ-chain residues 369-380 is a part of the cyanogen bromide fragment from the γ chain that was found to activate t-PA in fibrin-free chromogenic assays [35]. It would be interesting to find if the short peptides that activate t-PA in the free solution assays are inhibitory in assays in which fibrin formation and fibrinolysis are monitored turbidometrically, in which case they would be competing with the corresponding regions in fibrin.

X-RAY STRUCTURES

The advent of three-dimensional structures of fragments of fibrinogen and fibrin in the mid-1990's offered hope that validation of these many biochemical findings would be in hand. Although one of the most sought after structures, that of D_2E [61], has yet to materialize, the structures of a fibrinogen γC domain [62], fragments D and D-dimer [63], fragment E [64], a modified bovine fibrinogen [65] and native chicken fibrinogen [66] have all provided much useful information and stimulated a number of imaginative models for how fibrin stimulation of t-PA activation might occur.

As an example, when the structures of fragment D from fibrinogen and D-dimer from cross-linked fibrin became available [63], those parts of the protein that had previously been reported to stimulate fibrinolysis were immediately scrutinized. The first observation of note was that the segment involving α-chain residues 152-159, as had been predicted, was not exposed in fragment D. Neither was it in D-dimer derived from fibrin, however, nor were there any detectable differences between the two structures found for the implicated γ–chain regions.

In explanation, it was suggested that the protein might have relaxed into its initial posture as a result of the digestion that pared away various regions during the generation of the D-dimer, including the removal of the carboxy-terminal segments of α chains [63]. The X-ray structures also made it clear that there is no structural similarity between the proposed α-chain site and the γ-chain site, a similar arrangement of charged residues notwithstanding. In fact, the specified γ-chain segment overlaps substantially with the well known high affinity calcium binding site.

As implied above, early reports [28] about t-PA activation by D-dimer have been superceded by more recent studies that have shown that, although D_2E is apparently an activator of t-PA, D-dimer is not [67, 47]; as such, the similarity in the X-ray structures of fragments D and D-dimer might not be surprising. The answer to the question of what it is about complexing with fragment E that makes D-dimer ca-

pable of binding and/or stimulating the t-PA activation of plasminogen has been eclipsed by the corollary question, what is it about the binding of fragment E to D-dimer that makes it so difficult to crystallize? Thus, although numerous structures of D-dimer have been determined in the past dozen years, most in the presence of synthetic A and/or B knobs [62-66, 68-71], all attempts to crystallize D_2E have failed.

As it happens, the first crystal structure of D-dimer had been determined with a synthetic peptide "A knob" bound in its γ-chain "holes" [63]. Subsequent structures were determined with both synthetic A and B knobs [68, 69] in the γ- and β-chain holes, respectively. A hint of what might be happening with regard to t-PA activation was gotten when it was found that a distinct conformational change occurs upon binding the "B knob." [69]. Thus, in the absence of B knobs, the βC domain is pinned back against the coiled coil by a calcium bridge. One of the residues that ligates the calcium is βAsp398, the side chain of which swings away along with its neighbor βGlu397 to form a part of the binding pocket when the B knob is present. The polypeptide backbone of the structure stays irresolutely in place, however, and--tantalizing as the situation may be--the nearby αLys157 and its neighboring residues are not significantly changed vis-à-vis the situation when the B knob is absent.

The synthetic knobs in these crystal preparations are free and untethered, however, and the suggestion has been made that binding of the natural B knob in the βC holes may tip the balance in a way that leads to βC-βC interactions between associated protofibrils and favor enough movement to expose the alleged binding site on the α chain [72, 58].

MODELING THE ACTIVATION PROCESS

The availability of crystal structures for fibrinogen and various fragments gave rise to a variety of models aimed at explaining how t-PA activation occurs. In the study by Yakovlev *et al.* [47], for example, a model was proposed in which the βC domains in D_2E move away from the coiled coil and expose the α-chain binding site. Independently, a similar model was described on the basis of a modeling study of fibrin formation [72]. The main difference between these two models is that, in the one case, the wherewithal for exposure of the α-chain site is entirely encompassed in D_2E alone and does not involve B knobs, whereas in the fibrin model the movement of βC domains is the result of a β–β interaction that occurs during the lateral association of protofibrils and invokes the action of B knobs being bound to βC holes.

Similar models have been published about how t-PA might bind to one of these sites and plasminogen to the other [2], giving rise to the anticipated ternary complex that leads to active plasmin (Fig. **1**). A picture of D_2E was published recently that has the β-chain tethers nicely wrapped around D-dimer [73]; it is attractive but as yet totally without experimental support.

Among the questions that models need to address are, which of the two implicated regions binds the t-PA and which the plasminogen? It has been argued that although both of these proteins bind equally well to the α-chain segment that includes Lys-157, the fact that there will always be more plasminogen available than t-PA implies that plasminogen will win out and, by default, t-PA would be bound on the γC domain [2]. Such an arrangement is somewhat at odds with electron microscopy results that have shown plasminogen to be situated at very ends of fibrin units where γC domains are located [38].

It needs to be recalled that the peripheral domains involved in binding differ for t-PA and plasminogen, and in other situations the two proteins bind to unique sites [40]. Another matter to consider is, if the designated regions are binding sites for bringing t-PA and plasminogen into juxtaposition for the interaction between them, how is it that small peptides like α-chain 152-159, described above, are able to stimulate the process on their own? We are reminded that binding to fibrin, like peptides acting on their own, must induce conformational changes in the t-PA.

KRINGLES AND FINGERS

Up until this point we have focused mostly on the structural aspects of the fibrin side of the interaction. There have been at least as many studies directed at the peripheral domains of plasminogen and t-PA, and especially their kringles, each of which represents a potential lysine binding site. In plasminogen, kringle-4 has been found to be most important with regard to fibrin binding. The predilection of kringle-4 for carboxy-terminal lysines is reflected by its strong affinity for lysine-Sepharose.

At this point, crystal structures of four of the five plasminogen kringles have been determined individually at high resolution, including several complexed with EACA [74], and other crystal structures have shown that it is possible for kringles to bind internal lysine, as well [75]. There are also structures available for clusters of several contiguous kringles, including a set of three that reflect the overall folding in plasminogen [76].

On the other hand, the binding of t-PA to fibrin has been shown to depend on both the terminal finger domain and the second kringle domain, these two being separated in the sequence by an EGF domain and the first kringle [77]. The structure of kringle-2 from t-PA has been determined by X-ray crystallography [75], and structures of finger domains have been determined by NMR. Several different constructs involving the various t-PA peripheral domains have been described and their ability to bind fibrin determined [78]. Interestingly, it was found that a key lysine binding site may not bind directly to fibrin, but rather is involved *intra*molecularly in a way that stabilizes the active conformation. The basis of EACA inhibition, and presumably that of tranexamic acid, apparently resides in binding to this site [78].

Lipoprotein(a)

Apolipoprotein(a) contains a long cluster of kringles that are very similar to those found in plasminogen, and particularly to kringle-4 [79]. The protein, which is a variable component of the low density lipoprotein fraction (LDL), is thought to inhibit or regulate plasminogen activity. Many of the kringles found in apolipoprotein(a) have had their crystal

structures determined, including some complexed with EACA [80].

In one of the many studies directed toward understanding this inhibition, a 17-kringle construct was found to inhibit clot lysis in a dose-dependent manner, apparently blocking the binding of either t-PA or plasminogen or both [81]. The prolongation of lysis time, with no apparent direct influence on plasmin itself, is very similar to what is observed with synthetic B knobs [58].

In other studies, a yeast two-hybrid approach used various kringles as bait to find the portions of fibrin that were binding to apo(a) [82]. Interestingly, neither of the regions of fibrin(ogen) corresponding to the previously reported cyanogen bromide fragments that activate fibrinolysis [32, 35] were implicated by this method. Instead, a section of the γ chain encompassing residues 207-235 was identified, and, with less certainty, the homologous section of the β chain [82].

URGENTLY NEEDED STRUCTURES

In spite of the determined efforts of scores of laboratories over the past quarter century to find the basis of fibrin-stimulated fibrinolysis, key points remain mysterious. The three-dimensional structures of various components of the system that have been determined so far--revealing as they may have been on other fronts and useful as they have been for devising hypothetical models--haven't shown how fibrin stimulates t-PA activated fibrinolysis. What are needed are crystal structures of the key interactants complexed with each other.

The persistent appearance of contradictory reports alleging what it is about fibrin that stimulates t-PA should give us pause. The mere fact that denatured proteins other than fibrinogen are stimulatory [6] raises a red flag. In particular, we should be careful about regarding the α-chain segment encompassing residues 148-160 as being a proven site in *native* fibrin, no matter how active it may be on its own.

In this regard, Kranenburg *et al.* [83] while confirming that this α-chain peptide does indeed enhance t-PA activation of plasminogen, also showed that it can form fibrillar structures *in vitro* similar to the cross-beta structures found in amyloid deposits. Those authors [83] reminded us, also, that in the past a variety of amyloid proteins, characteristically rich in β-structure, has been found to activate t-PA [84-86], and that fibrin is reportedly richer in β structure than fibrinogen [87]. Is it possible that efforts to devise models for exposing the α-chain region around Lys-157 have been chasing a phantom? Will it turn out that it is mainly newly formed associations of αC domains in fibrin that are the main source of the t-PA enhancing activity [40, 43]? The only way these questions will be answered satisfactorily will be by determining the three-dimensional structures of complexes involving fragments of fibrin and fragments of t-PA or plasminogen.

There are several conceivable complexes—-if they could be determined either by X-ray crystallography or possibly by NMR—-that would shed considerable light on the problem at hand (Table **1**). Foremost among these would be a structure of the complex entity D_2E. As noted, persistent attempts in our laboratory to crystallize D_2E over the course of many years have been uniformly unsuccessful. Other groups with fresh imaginations need to take up the challenge. It is possible, for example, that D_2E can be stabilized by the presence of stoichiometric amounts of t-PA and/or plasminogen in inactivatable forms. Alternatively, the use of appropriate kringles to cap off carboxy-terminal lysines might be the answer. There are many possible avenues to explore.

Table 1. Needed Three-Dimensional Structures

Structures Already On Hand	Some Complexes to Aim For
fragment E (bovine)	D_2E
γC domain	γC domain-t-PA finger
fragment D	D_2E-kringles
D-dimer	fragment E-kringle
D-dimer (with A knobs)	kringle-αC domain
D-dimer (with B knobs)	kringle-α chain peptide
D-dimer (with A and B knobs)	kringle-fibrinogen
proteolyzed fibrinogen (bovine)	kringle-proteolyzed fibrinogen
native fibrinogen (chicken)	fragment Dγ-kringle
kringle IV kringle kringle IV-EACA	γ domain-apo(a) kringle
finger domain (NMR)	
αC domain (NMR)	

Another set of structures that ought to be attempted involves kringles complexed with the lysine-containing peptides reported to stimulate t-PA enhancement, and particularly the α-chain peptide 148-160. Other fibrin fragments with carboxy-terminal lysines ought to be prime targets, also. Indeed, it should be possible to crystallize fragment E with kringles bound to the outermost of the carboxy-terminal lysines at the ends of its coiled-coil stumps.

Virtually all the components necessary for assembling such complexes already exist. For example, good crystal structures for a variety of kringle domains complexed with EACA have long been available. Why haven't structures been determined for complexes of these same kringles with the lysine-containing peptides that provoke the t-PA activation of plasminogen? Is this because no one has tried, or is it because those peptides don't interact with the LBS?

Some of the most sought after complexes may be fleetingly transient, defying efforts to trap them in a crystallizable form. Furthermore, many of the proteins involved have loose and floppy sections that impede crystal formation. As an example, in order to obtain crystals of the bovine fragment E for which a high resolution structure has been determined, it was necessary to whittle it down to a bare-bones core, to the point where it had neither A nor B knobs or their tethers, and its truncated coiled coils contained a lone carboxy-terminal

lysine on each of its α chains [64]. As such, it is not an entity that can be expected to react with D-dimer to form a D_2E complex. It might, however, be able to complex with a suitable kringle by way of its residual carboxy-terminal lysines. The need is urgent, and the effort must be made.

CONCLUDING REMARK

Although there have been a plethora of experiments implicating certain regions of fibrin with the t-PA activation of plasminogen, direct comparisons of the regions that reflect the structural differences in fibrinogen and fibrin have not yet emerged. Nor have any complexes of fragments of fibrin and portions of plasminogen and/or t-PA had their structures determined. The lack of direct structural evidence remains a "loose end" in this extremely important area of hemostasis.

ACKNOWLEDGEMENTS

This review was written while the author was receiving support from the National Institutes of Health.

REFERENCES

[1] Nieuwenhuizen, W. *Ann. N.Y. Acad. Sci.*, **2001**, *936*, 237.
[2] Medved, L.; Nieuwenhuizen, W. *Thromb. Haemostasis,* **2003**, *89*, 409.
[3] Binder, B.R.; Spragg, J. In *Protides Biological Fluids;* Peeters, H. ed.; Pergamon Press: Oxford, **1980**; Vol. *28*, pp. 391-398.
[4] Hoylerts, M.; Rijken, D.C.; Lijnen, H.R.; Collen, D. *J. Biol. Chem.,* **1982**, *257*, 2912.
[5] Ranby, M. *Biochim. Biophys. Acta,* **1982**, *704*, 461.
[6] Radcliffe, R. *Biochim. Biophys. Acta,* **1983**, *743*, 422.
[7] Cederholm-Williams, S.A. *J. Clin. Pathol*., **1981**, *34*, 979.
[8] Norman, B.; Wallen, P.; Ranby, M. *Eur. J. Biochem*., **1985**, *149*, 193.
[9] Christensen, U. *FEBS Letts*., **1985**, *182*, 43.
[10] Deutsch, D.; Mertz, E.T. *Science,* **1970**, *170*, 1095.
[11] Alkjaersig, N.; Fletcher, A.P.; Sherry, S. *J. Biol. Chem*., **1959**, *234*, 832.
[12] Kinjo, K.; Hirata, T.; Okamoto, S. *Kobe J. Med. Sci*., **1963**, *18*, 51.
[13] Okamoto, S. *Strategies for Creating New Medicines;* Kobe Research Project on Thrombosis and Hemostasis: Kobe, **2003**.
[14] Iwamoto, M.; Abiko, Y.; Shimizu, M. *J. Biochem*., **1968**, *64*, 759.
[15] Iwamoto, M. *Thromb. Diath. Haemorrh*., **1975**, *33*, 573.
[16] Weinstein M.J.; Doolittle, R.F. *Biochim. Biophys. Acta*, **1972**, *258*, 577.
[17] Markus, G.; Priore, R.L.; Wissler, F.C. *J. Biol. Chem*., **1979**, *254*, 1211.
[18] Bajzar, L.; Manuel, R.; Nesheim, M.E. *J. Biol. Chem*., **1995**, *270*, 14477.
[19] Mattler, L.E.; Bang, N.U. *Thromb. Haemostasis,* **1977**, *38*, 776.
[20] Grailhe, P.; Nieuwenhuizen, W.; Angles-Cano, E. *Eur. J. Biochem*., **1994**, *219*, 961.
[21] Nussenzweig, V.; Seligmann, M.; Pelimont, J.; Grabar, P. *Ann. Inst. Pasteur.,* **1961**, *100*, 377.
[22] Marder, V.J.; Shulman, N.R.; Carroll, W.R. *J. Biol. Chem.* **1969**, *244*, 2111.
[23] Hall, C.E.; Slayter, H.S. *J. Biophys. Biochem. Cytol*., **1959**, *5,* 11.
[24] Dudek, G.A.; Kloczewiak, M.; Budzynski, A.Z.; LaTallo, Z.S.; Kopec, M. *Biochim. Biophys. Acta,* **1970**, *214*, 44.
[25] Chen, R.; Doolittle, R.F. *Biochemistry,* **1971**, *10*, 4486.
[26] Hudry-Clergeon, G.; Paturel, L.; Suscillon, M. *Pathol. Biol*., **1974**, *22*(suppl.), 47.
[27] Gaffney, P.J.; Joe, F. *Thromb. Res*., **1979**, *15*, 673.
[28] Verheijen, J.H.; Nieuwenhuizen, W.; Wijngaards, G. *Thromb. Res.,* **1982**, *27*, 377.
[29] Blomback, B.; Blomback, M.; Henschen, A.; Hessel, B.; Iwanaga, S.; Woods, K.R. *Nature,* **1968**, *218*, 130.
[30] Nieuwenhuizen, W.; Vermond, A.; Voskuilen, M.; Traas, D.W.; Verheijen, J.H. *Biochim. Biophys. Acta,* **1983**, *748*, 86.
[31] Voskuilen, M.; Vermond, A.; Veeneman, G.H.; van Boom, J.H.; Klasen, E.A.; Zeggers, N.D.; Nieuwenhuizen, W. *J. Biol. Chem.,* **1987**, *262*, 5944.
[32] Schielen, W.J.; Voskuilen, M.; Tesser, G.I.; Nieuwenhuizen, W. *Proc. Natl. Acad. Sci. USA,* **1989**, *86*, 8951.
[33] Schielen, W.J.; Adams, H.P.; Voskuilen, M.; Tesser, G.J.; Nieuwenhuizen, W. *Biochem. J.,* **1991**, *276*, 655.
[34] Schielen, W.J.; Adams, H.P.; Van Leuven, K.; Voskuilen, M.; Tesser, G.I.; Nieuwenhuizen, W. *Blood,* **1991**, *77*, 2169.
[35] Schielen, W.J.; Adams, H.P.; Voskuilen, M.; Tesser, G.I.; Nieuwenhuizen, W. *Blood Coag. Fibrinolysis,* **1991**, *2*, 465.
[36] Varadi, A.; Patthy, L. *Biochemistry,* **1983**, *22*, 2440.
[37] Nieuwenhuizen, W. *Thromb. Res*., **1994**, *75*, 343.
[38] Weisel, J.W.; Nagaswami, C.; Korsholm, B.; Petersen, L.C.; Suenson, E. *J. Mol. Biol*., **1994**, *235*, 1117.
[39] Schaefer, A.V.L.; Leslie, B.A.; Rischke, J. A.; Stafford, A.R.; Fredenburgh, J.C.; Weitz, J.I. *Biochemistry,* **2006**, *45*, 4257.
[40] Tsurupa, G.; Medved, L. *Biochemistry,* **2001**, *40*, 801.
[41] Sobel, J.H.; Gawinowicz, M.A. *J. Biol. Chem*., **1996**, *271*, 19288.
[42] Burton, R.A.; Tsurupa, G.; Medved, L.; Tjandra, N. *Biochemistry,* **2006**, *45*, 2257.
[43] Burton, R.A.; Tsurupa, G.; Hantgan, R.R.; Tjandra, N.; Medved, L. *Biochemistry,* **2007**, *46*, 8550.
[44] Doolittle, R.F.; Kollman, J.M. *Proteins,* **2006**, *63*, 391.
[45] Collet, J.-P.; Moen, J.L.; Veklich, Y.I.; Gorkun, O.V.; Lord, S.T.; Montalescot, G.; Weisel, J.W. *Blood,* **2005**, *106*, 3824.
[46] Cottrell, B.A.; Doolittle, R.F. *Biochem. Biophys. Res. Comm.* **1976**, *71*, 754.
[47] Yakovlev, S.; Makogonenko, E.; Kurochkina, N.; Nieuwenhuizen, W.; Ingham, K.; Medved, L. *Biochemistry,* **2000**, *39*, 15730.
[48] Varadi, A.; Patthy, L. *Biochemistry,* **1984**, *23*, 2108.
[49] Stewart R.J.; Fredenburgh, J.C.; Rischke, J.A.; Bajzar, L.; Weitz, J.I. *J. Biol. Chem.,* **2000**, *275*, 36612.
[50] Donovan, J.W.; Mihalyi, E. *Proc. Natl. Acad. Sci. USA,* **1974**, *71*, 4125.
[51] Litvinov, R.; Gorkun, O.V.; Owen, S.; Shuman, H.; Weisel, J.W. *Blood,* **2005**, *106*, 2944.
[52] Weisel, J.W.; Nagaswami, C. *Biophys. J.,* **1992**, *63*, 111.
[53] Weisel, J.W. *Biophys. J*., **2004**, *112*, 267.
[54] Ferry, J.D.; Morrison, P.R. *J. Am. Chem. Soc*., **1947**, *69*, 388.
[55] Carr, M.; Alving, B.M. *Blood Coag. Fibrinolysis,* **1995**, *6*, 567.
[56] Weisel, J.W.; Veklick, Y.; Collet, J.-P.; Francis, C.W. *Thromb. Hamostasis,* **1999**, *82*, 277.
[57] Laudano, A.P.; Doolittle, R.F. *Proc. Natl. Acad. Sci. USA,* **1978**, *75*, 3085.
[58] Doolittle, R.F.; Pandi, L. *Biochemistry,* **2006**, *45*, 2657.
[59] Dempfle, C.-E.; Bindeballe, N.; Munchback, M.; Blume, C.; Borgreffe, M.; Mosesson, M.W. *J. Thromb. Haemostasis,* **2007**, *5*, 1707.
[60] Kolev, K.; Tenekedjiev, K.; Komorowicz, E.; Machovich, R. *J. Biol. Chem.,* **1997**, *272*, 13666.
[61] Doolittle, R.F.; Everse, S.J.; Spraggon, G. *FASEB J.,* **1996**, *10*, 1464.
[62] Yee, V.C.; Pratt, K.P.; Cote, H.C.; LeTrong, I.; Chung, D.W.; Davie, E.W.; Stenkamp, R.E.; Teller, D.C. *Structure,* **1997**, *5*, 125.
[63] Spraggon, G.; Everse, S.J.; Doolittle, R.F. *Nature,* **1997**, *389*, 455.
[64] Modrazo, J.; Brown, J.; Litvinovich, S.; Dominguez, R.; Yakovlev, S.; Medved, L.; Cohen, C. *Proc. Natl. Acad. Sci. USA,* **2001**, *98*, 11967.
[65] Brown, J.H.; Volkmann, N.; Jun, G.; Henschen, A.H.; Cohen, C. *Proc. Natl. Acad. Sci. USA,* **2000**, *97*, 85.
[66] Yang, Z.; Kollman, J.M.; Pandi, L.; Doolittle, R.F. *Biochemistry,* **2001**, *40*, 12515.
[67] Weitz, J.I.; Leslie, B.; Ginsberg, J. *J. Clin. Invest*., **1991**, *87*, 1081.
[68] Everse, S.J.; Spraggon, G.; Veerapandian, L.; Riley, M.; Doolittle, R.F. *Biochemistry,* **1998**, *37*, 8637.
[69] Everse, S.J.; Spraggon, G.; Veerapandian, L.; Doolittle, R.F. *Biochemistry,* **1999**, *38*, 2941.
[70] Kostelansky, M.; Betts, L.; Gorkun, O.V.; Lord, S.T. *Biochemistry,* **2002**, *41*, 12124.
[71] Doolittle, R.F.; Chen, A.; Pandi, L. *Biochemistry,* **2006**, *45*, 13962.
[72] Yang, Z.; Mochalkin, I.; Doolittle, R. F. *Proc. Natl. Acad. Sci. USA,* **2000**, *97*, 14156.

[73] Lugovskoy, E.V.; Gritsenko, P.G.; Kapustianenko, L.G.; Kolesnikova, I.N.; Chernishov, V.I.; Komisarenko, S.V. *FEBS J.,* **2007**, *274*, 4540
[74] Mulichak, A.M.; Tulinsky, A. *Blood Coag. Fibrinolysis,* **1990**, *1*, 673.
[75] de Vos, A.M.; Ultsch, M.H.; Kelly, R.F.; Padmanabhan, K.; Tulinsky, A. *Biochemistry,* **1992**, *31*, 270.
[76] Abad, M.C.; Arni, R.K.; Grella, D.K.; Castellino, F.J.; Tulinsky, A.; Geiger, J.H. *J. Mol. Biol*., **2002**, *318*, 1009.
[77] Horrevoets, A.J.G.; Smilde, A.; de Vries, C.; Pannekoek, H. *J. Biol. Chem.,* **1994**, *269*, 12639.
[78] Bakker, A.H.F.; Weening-Verhoeff, E.J.D.; Verhejen, J.H. *J. Biol. Chem*., **1995**, *270*, 12355.
[79] Angles-Cano, E.; de la Pena Diaz, A.; Loyau, S. *Ann. N.Y. Acad. Sci*., **2001**, *936*, 261.
[80] Mochalkin, I.; Cheng, B.; Klezovitch, O.; Scanu, A.; Tulinsky, A. *Biochemistry,* **1999**, *38*, 1990.
[81] Sangrar, W.; Bajzar, L.; Nesheim, M.E.; Koschinsky, M.L. *Biochemistry,* **1995**, *34*, 5151.
[82] Klose, R.; Fresser, F.; Koch, S.; Parson, W.; Kapetanopoulos, A.; Fruchart-Najib, J.; Baier, G.; Utermann, G. *J. Biol. Chem*., **2000**, *275*, 38206.
[83] Kranenburg, O.; Bouma, B.; Kroon-Batenburg, L.M.J.; Raijerkerk, A.; Wu, Y.-P.; Voest, E.E.; Gebbink, F.B.G. *Curr. Biol*., **2002**, *12*, 1833.
[84] Kingston, I.B.; Castro, M.J.; Anderson, S. *Nat. Med*., **1995**, *1*, 138.
[85] Wnendt, S.; Wetzels, I.; Gunzler, W.A. *Thromb. Res*., **1997**, *85*, 217.
[86] Van Nostrand, W.E.; Porter, M. *Biochemistry,* **1999**, *38*, 11570.
[87] Marx, J.; Hudry-Clergeon, G.; Capet-Antonini, F.; Beernard, L. *Biochim. Biophys. Acta,* **1979**, *578*, 101.

Received: 28 December, 2007 Revised: 05 February, 2008 Accepted: 26 February, 2008