Structural basis of the fibrinogen–fibrin transformation: contributions from X-ray crystallography☆

Russell F. Doolittle

Center for Molecular Genetics, University of California, San Diego, La Jolla, CA, USA

Abstract During the past several years, a number of crystal structures have been determined of fragments from fibrinogen and fibrin and, most recently, a structure of a native fibrinogen. One feature of the fibrinogen molecule that has emerged from these studies has to do with its "loose ends," segments of the molecule that are extremely mobile and not discernable by X-ray crystallography. Some, if not all, of this flexibility is functionally important. Small synthetic peptides based on mobile parts of fibrinogen exposed by the action of thrombin have contributed significantly to these studies and may yet prove useful therapeutically. In the end, although crystal structures have added greatly to our understanding of fibrin formation, much still needs to be unraveled about how clots form.

KEY WORDS: fibrinogen; fibrin; crystal structures; clots

INTRODUCTION

Blood clots and bleeding – thrombosis and hemorrhage – events familiar to every physician, are often the difference between life and death. Heparin, aspirin and vitamin K antagonists are dispensed to millions of persons throughout the world on a daily basis in an effort to keep the clotting process suppressed. Contrarily, a smaller number of persons have genetically impaired clotting – the hemophilias are the best known – but also including persons with variant fibrinogen molecules that do not clot properly. The question arises, what is the state of our knowledge of the molecular basis of the fibrinogen to fibrin conversion, a process studied by tens of thousands of researchers during the past century? Surely by now we should understand every detail. Unhappily, there is much that remains enigmatic. Were it not so, it might be possible to regulate the process more effectively than is now possible. In this article I review recent progress in determining the three-dimensional structures of fibrinogen and fibrin by X-ray crystallography, calling attention along the way to some flexible features that have not yielded to this approach.

DETERMINING CRYSTAL STRUCTURES

The starting point for determining a crystal structure by X-ray crystallography is obtaining crystals. Unfortunately, many proteins are extremely difficult to crystallize, and in some cases it may prove impossible. If a protein is small and compact, it may crystallize with ease under many conditions. But large, multi-domained proteins like fibrinogen can be gangly and disordered, defying the concerted efforts of an army of would-be crystallizers and never adopting the regular (periodic) arrangements that are the heart of the crystal lattice. One strategy that has been taken in such cases is to snip the protein into core domains that may be more compact and amenable to crystallization, although even then, there is no guarantee of success. In the case of fibrinogen, it is only within the last several years, after a long period of failure, that this approach met with success.

Once crystals are in hand, it is a matter of how well they diffract X-rays. This quality is usually described in terms of "resolution," the degree to which separate points in the reconstructed image can be resolved. For example, electron microscopy of proteins may yield images that have resolutions of the order of 20 Å. Reasonable structures determined by X-ray crystallography should have resolutions better than 3.0 Å, at which stage the individual side-chains of the amino acids can be distinguished. The process by which the diffraction data – tens of thousands of "spots" – are transformed into three-dimensional electron density maps corresponding to the molecules in the crystal, is arduous but wonderfully rewarding when completed.

Even when crystals are in hand that diffract X-rays strongly, some features of a molecule may not be visualized directly. In a crystal, every molecule is in contact with its neighbors in a regular three-dimensional lattice. Nonetheless, some dangling features may protrude from each unit and wobble incoherently. The situation is analogous to a person not sitting still for an old fashioned time-exposure group photograph, in which case his image will be blurred or, in the extreme, he may not show up in the picture at all. As it happens, some of the most important portions of fibrinogen molecules do not sit still for the photographer.

EARLY STUDIES ON FIBRINOGEN AND FIBRIN

Usually, a great deal is known about a protein in advance of a crystal structure determination, and a brief review of what was known about fibrinogen and fibrin is needed to put the crystallography contributions in perspective. Fibrinogen is a large distended molecule – much longer than it is wide – with a molecular weight of about 340,000 (hereafter 340 kDa) and an overall length of about 450 Å. Shadow cast electron micrographs long ago showed three globules, the connections between which could not be resolved.[1]

Fibrinogen is actually a covalent dimer composed of two each of three non-identical (but evolutionarily related) poly-

☆ *Abbreviations:* GPR-, Gly-Pro-Arg-derivatives; GPRPam, Gly-Pro-Arg-Pro-amide; GHRPam, Gly-His-Arg-Pro-amide; t-PA, tissue plasminogen activator.

peptide chains; the subunit formula is $\alpha_2\beta_2\gamma_2$. As it happens, the middle globule of the structure contains all six amino-terminals, and it was realized that the connectors between globules were each made up of the three non-identical chains. There was good reason to believe that these were interwoven α helices of the sort called ''coiled coils.''[2,3]

As is well known, thrombin cleaves peptide material–the fibrinopeptides A and B–from the amino-terminal regions of the α- and β-chains.[4] The two peptides are cleaved at different rates, and it has long been conjectured that the release of the fibrinopeptide A mainly encourages the linear growth of the fibrin polymer, while the slower release of the fibrinopeptide B is what allows the lateral growth needed for thicker fibers. In this regard, the initial polymerization occurs in a staggered half-molecule fashion to form two-molecule thick protofibrils about 15 U long.[5] These then aggregate laterally, also by a kind of staggered overlap, eventually yielding the mature anastomosing fibers that make up the clot.

The removal of the fibrinopeptides exposes new end-groups which take the form of positively charged ''knobs'' that fit into negatively charged ''holes'' on neighboring molecules, allowing the spontaneous polymerization to a fibrin gel. The A knob, exposed by removal of the fibrinopeptide A from the fibrin α-chain, begins Gly-Pro-Arg (abbreviated GPR), and the B knob, exposed when the fibrinopeptide B is cleaved away on the fibrin β-chain, begins Gly-His-Arg (abbreviated GHR). Remarkably, small synthetic peptides patterned on A-knob sequences only three or four amino acids in length can bind to fibrinogen and inhibit its polymerization.[6] Under most circumstances, however, synthetic B-knob sequences do not appreciably interfere with fibrin formation.

Although clots are initially held together only by non-covalent bonds, under physiological conditions they are quickly stabilized by the action of the transglutaminase known as factor XIII, itself activated by thrombin. Factor XIII introduces ε-amino-γ-glutamyl cross-links between the individual fibrin units, the first to form being between the carboxyl-terminal segments of neighboring γ chains.[7] Additional cross-links are subsequently formed between various portions of α-chains, but at a considerably slower rate.[8]

Fibrin clots are not meant to be permanent structures. Under physiological conditions, plasmin, itself derived from the precursor protein plasminogen, cleaves fibrin in an efficient manner leading to dissolution of the clot, chiefly by severance of the inter-domainal coiled-coil connectors. The conversion of plasminogen to plasmin can be catalyzed by several agents, chief among which is t-PA (tissue plasminogen activator). Fibrin, but not fibrinogen, enhances the activity of t-PA. In this regard, a skein of amino acids encompassing residues α151–158 of the human fibrin(ogen) α-chain has been found to be responsible.[9] There is immunological evidence that this region is inaccessible in fibrinogen but exposed in fibrin.[10]

In vitro, plasmin cleaves fibrinogen into a few major core fragments and a host of smaller peptides. The process begins with large portions of the α-chains being cleaved away, the totality amounting to the carboxyl-terminal two-thirds of the chain. At about the same time, the amino-terminal segments of β-chains are removed. The first major product is usually referred to as ''fragment X.'' Further digestion cuts across the now well exposed coiled coils, leading to the removal of one of the two large terminal globules, entities denoted fragments D, first from one end of a given molecule, then from the other. The residual central core domain is called fragment E. If cross-linked fibrin is digested under comparable circumstances, the products are much the same, except that the terminal fragments D of abutting units in the fibrin remain joined together (D-dimer, also called double-D).

Only a few of many important references were cited above, and readers should consult two popular symposium volumes[11,12] or other sources for a proper historical record. In one way or another, all of this background knowledge was critical in the efforts to determine the structures of fibrinogen and fibrin by X-ray crystallography.

CRYSTALLOGRAPHY STUDIES ON FIBRIN(OGEN) FRAGMENTS

Crystals of partially proteolyzed bovine fibrinogen equivalent to fragment X were reported as early as 1972.[13] Slow but steady progress was made on this project over the next twenty years, by which time a low resolution 18 Å structure of the 285-kDa entity was achieved.[14] Then, in 1995, crystals of the 85-kDa fragment D from human fibrinogen were reported that diffracted to 3 Å,[15] and it was anticipated that an atomic resolution structure would soon be available.[16]

The first fragment of a fibrinogen molecule to have its crystal structure determined at high resolution, however, was a recombinant form of the γC domain,[17] a 30-kDa portion of the γ-chain that extends from residues γ143–γ411. The structure was novel, no other reported proteins having a similar folding pattern. Its centerpiece was a five-stranded β sheet flanked by a few small α helices. A sub-domain composed of four prominent loops appeared to form a pocket which could bind exposed knobs from other molecules, a conjecture which was promptly validated by soaking the crystals in a solution of the knob-like peptide GPRP and determining the structure of the complex.[18]

Even though the recombinant γC domain was solved at high resolution (2.1 Å), the carboxyl-terminal region was quite disordered and could not be modeled with any certainty. This was disappointing because this region of the molecule had long been known to play a key role in fibrin stabilization,[7] as well as to be involved in the binding of fibrinogen to platelets and other cells.[19] This was the first hint that X-ray structures were not going to provide every detail about functionally important features of the protein.

Shortly thereafter, the anticipated structure of fragment D from human fibrinogen appeared.[20] The structure revealed the arrangement of the homologous βC and γC domains relative to the coiled coils that connect them to the central portion of the molecule, as well as the relative locations of the holes that each of them contained (Fig. 1). In this regard, the γ-chain ''hole'' was found to be conveniently disposed near the very end of the molecule, but the β-chain equivalent was directed back towards the central region, leaving unanswered the question of the source of its partnering ''B knob.''

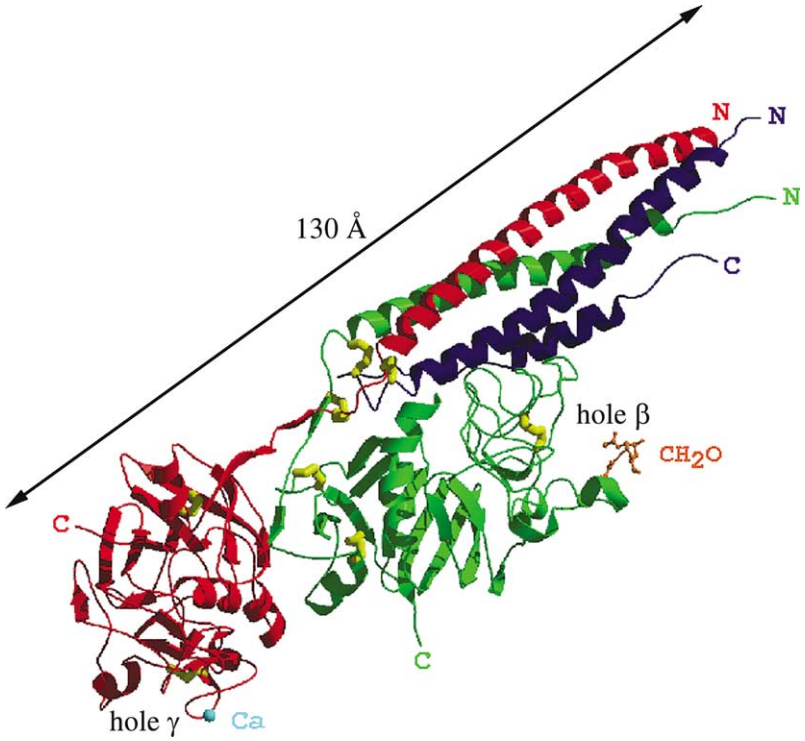

Fig. 1 **Ribbon model of fragment D from human fibrinogen showing the residual coiled coils and globular carboxyl-domains. Ca, calcium; CH_2O, carbohydrate cluster. The holes into which the GPR and GHR knobs fit are labeled on the γ- and β-chains, respectively (reprinted from Ref. 20 with permission).**

Numerous other features fell into place, however, including the three-dimensional whereabouts of the β-chain carbohydrate cluster. Of great interest, also, the α-chain makes an abrupt reversal of direction in the middle of the second disulfide ring, folding back in a way that helps shield the region on the α chain that had been implicated in the activation of t-PA.

The same study[20] reported the structure of D-dimer (also known as double-D) isolated from cross-linked fibrin, providing the first glimpse of the abutment known as the D:D interface (Fig. 2). Again disappointingly, the cross-links between the γ-chain carboxyl-terminal segments were not visible, the result of an inherent mobility, just as are the unlinked segments.

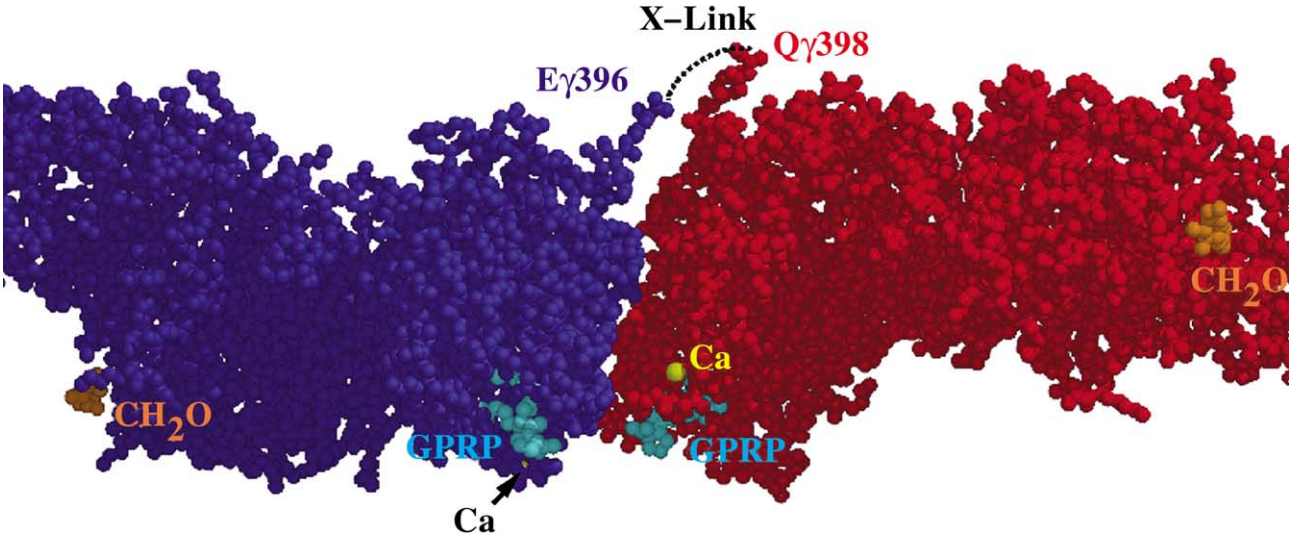

Fig. 2 **Space-filling model of D-dimer showing abutting ends at the D:D interface. The isopeptide cross-link between γ-chains was not visible in electron density maps and is denoted by the broken line (reprinted from Ref. 20 with permission).**

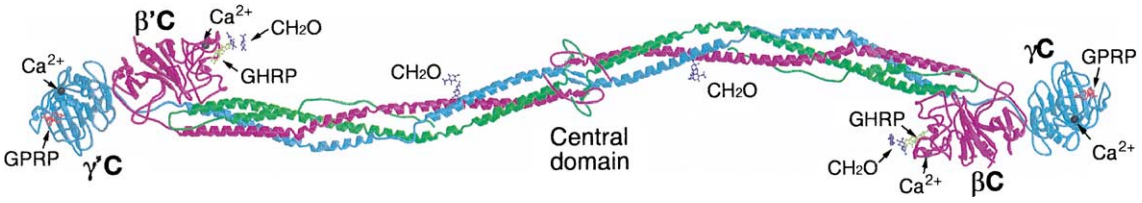

Fig. 3 **Ribbon model of native chicken fibrinogen solved at 2.7 Å resolution. α-chains are green, β-chains are red and γ-chains are blue (from Ref. 24 with permission).**

FULL-LENGTH STRUCTURES

Because the 85-kDa fragment D occurs twice in a given molecule of fibrinogen, it amounts to about half the mass of the native protein, or two-thirds of the mass of the proteolytically modified bovine fibrinogen mentioned above. As such, it was possible to improve the quality of the bovine structure by the method of molecular replacement, using the model of fragment D to phase the data to a resolution of about 4 Å.[21] The improved structure was the first to encompass the full sweep of the molecule, the resulting model lacking only the highly flexible αC domains and amino-terminal portions of the six chains. Its sigmoid silhouette was remarkably similar to what had been observed long ago in negatively stained electron micrographs.[22]

Meanwhile, frustrated by unsuccessful attempts to crystallize native human fibrinogen, others had turned to fibrinogen from chickens. This was not just a desperate attempt to try another species. Rather, the choice was based on the belief that the main barrier to crystallization of the native protein was the presence of the highly mobile αC domains, which in human fibrinogen are tethered to the main body of the molecule by a long series of 13-residue repeats. It is principally this region that is removed by limited proteolysis of the bovine protein. As it happens, amino acid sequence studies had revealed that chicken fibrinogen lacks the repeats linking the αC domain to the parent body, and it was thought their absence might be helpful. In fact, crystals were obtained, and a preliminary 5.5 Å structure of the native chicken fibrinogen was shown to be superimposable upon that of the modified bovine fibrinogen. The chicken structure was the first to show the arrangement of amino acids in the two disulfide rings which hold the sets of three chains together in the central part of the molecule.[23]

Eventually it was possible to obtain crystals of native chicken fibrinogen which diffracted to 2.7 Å, a resolution at which it was possible to show the full nature of the attachments that bind the two halves of the protein together, as well as numerous details of the overall structure (Fig. 3).[24] Nonetheless, the amino-terminal segments of the α- and β-chains, including the knobs and the fibrinopeptides that guard them, were unhappily missing. Not long afterwards, the structure of the 35 kDa fragment E from bovine fibrinogen was reported at 1.4 Å resolution.[25] Once again, the terminal regions of the α- and β-chains were in absentia, although in this case the flexible region of the β-chain and a substantial portion of the α-chain had already been removed by proteolysis during the generation of the fragment.

All of the structures mentioned above are stored in the Protein Data Bank and are publicly available, and readers are encouraged to use the internet to examine the many detailed renderings available (Table 1).

MOVING PARTS

Of the 2728 amino acid residues in native chicken fibrinogen, only 1959 were visualized in the 2.7 Å resolution crystal structure. As such, the positions of 769 amino acids are unaccounted for, or, because the molecule is a dimer, almost

Table 1 **Some reported crystal structures of fibrinogen and fibrin fragments**

Protein	Mol. wt. (kDa)	Resol. (Å)	PDB #[a]	Year	Refs.
rγC (human)	30	2.1	1FIB	1997	17
rγC (human) (GPRP)	30	2.1	2FIB	1997	18
Fragment D (human)	85	2.9	1FZA	1997	20
D-dimer (human) (GPRP)	170	2.9	1FZB	1997	20
D-dimer (human) (GPRP,GHRP)	170	2.3	1FZC	1998	48
rα_EC (human)	25	2.1	1FZD	1998	42
D-dimer (human) (GHRP)	170	2.9	1FZF	1999	38
D-dimer (human) (no ligands)	170	3.0	1FZE	1999	38
Fragment X (bovine)	285	~4	1DEQ	2000	21
Native fibrinogen (chicken)	320	2.7	1JFE	2001	24
Fragment E (bovine)	35	1.4	1JY2	2001	25
Fragment D (lamprey) (GHRP)	89	2.8	1LWU	2002	57

[a] Structures may be viewed or downloaded at www.rcsb.org/pdb/ by searching these index numbers.

400 on one half or the other. The missing portions occur in four general locations: (1) the αC domain, (2) the β-chain amino-terminal segment composed of residues β1–60, (3) the α-chain amino-terminal segment (res. α1–27), and (4) the γ-chain carboxyl-segment (res. γ394–409). Additionally, there are a few other regions of the protein – mainly loops – where the structure is ''loose,'' as typified by what the crystallographer calls ''high B factors.'' The B factor is a measure of thermal motion within the crystal.

The αC domain. As noted above, the carboxyl-terminal two-thirds of human fibrinogen is readily removed by many different proteases. Plasmin brings about this release by targeting a cluster of vulnerable sites in the region α206–239 (human numbering).[26] The X-ray structure of chicken fibrinogen extends to just about this same location, B-factors rising along the way until the electron density blurs to the point where the polypeptide chain can no longer be traced. The loose density that can just barely be discerned beyond that point is nestled within the confines of the sinuous coiled coils.[24]

Several recombinant versions of the αC domain from bovine fibrinogen have been thoroughly characterized biochemically, and although there may be some organized structure, there is little evidence for features like α helices or β sheets. This apparent lack of secondary structure is consistent with an unusually high sequence variability of this region from species to species.[27] Numerous attempts to crystallize such preparations have been undertaken in at least two laboratories; none has succeeded. On the other hand, there is some spectroscopic evidence that suggests the repeat region (the part absent in chicken fibrinogen) connecting the αC domain to the parent molecule is a loose spiral similar to an extended polyprolyl helix.[28]

As for the function of the αC domain, there is evidence that it plays some kind of secondary role in the bundling of protofibrils or fibers,[29] perhaps in a non-specific manner.[30] The domain also binds a number of other proteins, including t-PA and plasminogen.[31]

The β-chain amino-terminal segment. Residues β1–60 of the β-chain of chicken fibrinogen do not appear in the electron density maps and must be very flexible. In mammalian fibrinogens and fibrins, this region is an early target for plasmin and must be very exposed. The proteolytically modified bovine fibrinogen (equivalent to fragment X) lacks this segment, as does fragment E. The missing region encompasses the fibrinopeptide B (res. 1–15) and the B-knob (res. 16–19), as well as part or all of a thrombin-binding site,[32] a cadherin binding site,[33] a heparin-binding site,[34] and a segment involved in angiogenesis.[35]

The α-chain amino-terminal segment. The first 27 residues of α-chains were missing from the native chicken structure, even though one of the disulfide bonds that connects the two halves of the molecule occurs at αCys28. After the removal of the fibrinopeptide A, the two A knobs are flexibly tethered and must carve out a volume of space in which at the extremes they might be as much as 40 Å apart. The two holes at the D:D interface are approximately 25 Å apart (Fig. 2). How the two knobs find holes on two other molecules ''on the fly'' is not immediately obvious, but the flexibility may make it easier than if they were rigidly fixed in space.

The γ-chain carboxyl-terminal segment. As noted above, the γ-chain carboxyl-terminal region is loose and disordered in all the reported fragment D crystals. A set of innovative carrier-driven crystallization studies were carried out, however, in which fusion proteins were made of the terminal 14 residues of the human fibrinogen γ-chain with two different proteins that are readily crystallized, lysozyme and glutathione *S*-transferase. Crystals were obtained in both cases and the structures determined.[36,37] That both constructs yielded similar structures for the 14-residue adduct was reassuring. Nonetheless, the bulk of the evidence indicates that in native fibrinogen the segment is intrinsically flexible.

Furthermore, the factor XIII-induced cross-links could not be determined in the D-dimer structures either.[20] Apparently the linkages between units in fibrin are like handcuffs that allow one to move ones arms, rather than an overall restraint that locks the system in one place.

Other mobile regions. There are some other regions of the fibrinogen molecule that are flexible. Of particular interest are the loops that form the binding pockets for the knobs. The β-chain binding pocket, for example, is not fully formed until the GHRPam ligand is actually present,[38] and in the γ-chain the B factors for one of the loops comprising the hole (residues γ357–360) are very high, indicating significant mobility. Flexibility may be important for the ready accommodation of the holes to the moving knobs. Intriguingly, this particular region has been implicated in fibrinogen binding to the integrin $\alpha_V\beta 3$ found in endothelial cells.[39]

Portions of the inter-domainal coiled coils are also quite flexible.[21,23,24] Perhaps not surprisingly, the mobility as indicated by high B factors correlates with the principal plasmin attack points.[24] Flexibility in these regions may also contribute to the general elasticity of fibrin gels.

RELATED STRUCTURES

The βC and γC domains of mammalian fibrinogens are composed of 220–230 amino acids and are 50% identical in sequence. Some of the differences occur in the hole regions and must contribute to the discrimination between the A and B knobs, although the crystal structures have not yet revealed the underlying structural basis. Numerous homologs of these domains occur in other extra-cellular proteins found in animals,[40] including the α_EC domain of the minor fibrinogen known as fibrinogen-420, which in most vertebrate animals is the result of alternative splicing.[41]

The crystal structure of the α_EC domain from human fibrinogen-420 was determined at 2.1 Å.[42] Although the domain has an obvious binding pocket, the loops around the pocket are truncated compared with those around the holes in γC and βC domains. What kind of ligand it may bind remains unknown, as does the function of fibrinogen-420.

Sequence searching studies have shown that fibrinogen-related domains occur exclusively at the carboxyl terminals of the extra-cellular proteins, which themselves are usually multi-domainal.[43] They are often involved in binding to cells, and many are involved in angiogenesis. Among the invertebrate animals, several are known to be lectins involved in defense reactions.[44–46] One of these, a lectin from horseshoe

crab hemolymph, has had its crystal structure determined.[47] Although overall it is obviously similar to the βC and γC domains of fibrinogen, its sialic acid binding site is distinctly different. Indeed, the key to understanding the specificity of these proteins resides in the detailed architecture of the ''holes.''

CORRELATING CRYSTAL PACKING AND FIBER FORMATION

During the course of crystal studies on fragments D and D-dimer, it was found that the nature of the crystals differed greatly depending on whether or not synthetic knobs were present.[38,48] Different lattice arrangements were observed depending on whether the A knob (in the form of GPRPam), the B knob (in the form of GHRPam), or both knobs were present, suggesting that subtle conformational changes were occurring when these surrogate knobs are bound, and that these changes influenced how the molecules packed together. It seemed reasonable that the same subtle forces could be at play when the units packed themselves together in the fibrin clot.

Because the very nature of crystal formation depends on the same principle, albeit in three dimensions equitably instead of the preferred one-dimensional nature of fiber growth, we examined the nature of the packing between molecules in the various X-ray structures to see what kinds of interfaces might be involved. It was already apparent that a preferred end-to-end interface occurred between γ-chains in all structures containing a fragment D,[20,38,48] whether or not they had been cross-linked to D-dimer (Fig. 2). The same interface also occurred in the end-to-end packing of the modified bovine[21] and the native chicken fibrinogen structures.[23,24]

The D:D interface is asymmetric in that the two abutting molecules contribute different residues to the interaction (Fig. 2). The two abutting units are juxtaposed in a way that their two holes are exposed in the same general direction so that a pair of A knobs from the another molecule can fit into them and hold the two units together.

There is still debate about the role of the B knobs, and particularly as to whether they are involved in linkages within a given protofibril or between different protofibrils. Experiments with monoclonal antibodies directed against the amino-terminus of the fibrin β chain – i.e., the B knob – have been equivocal on the point in the sense that B knobs may not immediately find holes upon their exposure.[49,50] Because of the long history of fibrinopeptide B release being associated with the lateral growth of clots, the X-ray data were initially interpreted along the lines of B-knob involvement between protofibrils.[51]

In this regard, fibrin fibers assemble themselves in a very orderly fashion, to the extent that electron microscopy long ago revealed a periodicity of 230 Å, consistent with the half-molecule overlap that occurs during the initial polymerization. Some natural attribute of the starting molecules allows them to associate side-by-side in a repetitive manner throughout the fiber. The crystal packing suggested that the alignment could readily be attained by using a different face of the γC domain from the one involved in the initial D:D interface (Fig. 4).

The same model studies showed that the B-knobs may well be restricted to a single protofibril and that they may interact with the same two molecules as are pinned together by a pair of A knobs.[30] The model also indicated that a β–β interface homologous to the end-to-end γ–γ interface could be involved in the lateral association of protofibrils once the β-chain holes had been filled. Furthermore, the model showed how the binding of the B knob to the β-chain hole could facilitate the movement of the βC domain away from the reported t-PA activation site involving α-chain residues α 151–158.

Other workers have also invoked movement of the βC domains as being involved in the exposure of the t-PA activation site,[52] and support for the for the involvement of βC domains in lateral polymerization has also been recently reported.[53]

THERAPEUTIC POSSIBILITIES

Currently, most anticoagulant therapy is directed at thrombin. If agents were available that could arrest fibrin formation directly at the polymerization stage, other actions of thrombin like platelet activation might not be compromised and the risk of hemorrhage minimized.

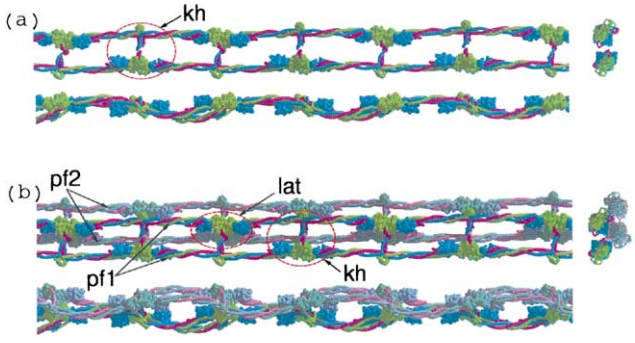

Fig. 4 **(a) Three views top, side and end-on of a single protofibril formed by knob-hole (k–h) interactions. Light green, γ-chains; blue, β-chains, red, α-chains. End-on view is at right. (b) Lateral association (lat) of two protofibrils (PF1 and PF2); (top, side, and end-on) views as in (a) (reprinted from Ref. 30 with permission).**

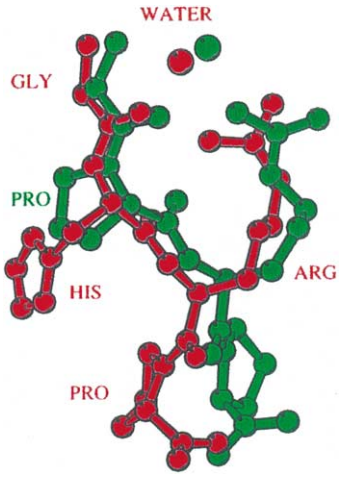

Fig. 5 **Superimposed structures of synthetic A and B knobs (GPRP is green and GHRP is red) as they sit in their respective holes (from Ref. 48 with permission).**

Some of the peptides that simulate the A-knob, particularly GPRPam (Fig. 5), have been very effective in stopping clotting in vitro but have not been exploited clinically. This is mostly because their binding to fibrinogen is relative weak,[54] and as a result the peptides are quickly lost from circulation. Attempts to prepare derivatives that maintain their anti-polymerant effect while binding more tightly to plasma albumin[55] were only modestly successful and have not been pursued further. Similarly, derivatives of the knob peptides that were covalently bound to albumin by linkers that are cleavable by thrombin have not been taken further.[56] Nonetheless, synthetic knobs ought to be useful in some limited settings like heart-lung machines, where clots continue to be a problem. In such situations the loss of small peptides is less a problem because of the limited time and re-circulation in the closed system.

What are needed in general, however, are analogs that bind much more tightly to the natural holes in fibrinogen than currently available peptides. More careful scrutiny of the knob-hole interactions in the crystal structures ought to lead to the design and construction of better synthetic knobs. The geometry of the β- and γ-chain holes has been well preserved during vertebrate evolution, hardly any change being evident upon comparison of the hole regions in the crystal structures of lamprey and human fragments D.[57]

One goal should be to find a small molecule that will imitate the B knob and bind tightly to the β-chain hole but not the γ-chain hole. This might allow clots to form when needed but be more readily lysed subsequently, either because lateral growth is limited or because t-PA activation is enhanced.

CONCLUSION

X-ray crystallography has provided a wealth of detail about the structures of fibrinogen and fibrin, including many aspects that were anticipated and others that were totally unexpected. The various crystal structures have detailed the basis of numerous human fibrinogens that exhibit defective clotting, including many variants that were already known[58,59] and others that were discovered after the structures became available.[60,61] The crystal structures have also given rise to model systems that detail the many interactions that occur during fibrin formation, providing a rational basis for fashioning agents that may be useful for regulating the process.

It may have been unreasonable to expect that all parts of the fibrinogen molecule would be rigidly fixed in space. Indeed, in recent years it has been found that there are many proteins that are ''native unfolded.''[62,63] In some of these cases, the inherent flexibility is what allows the protein to adopt a conformation complementary to a receptor.[64] In the case of fibrinogen, flexibility is what allows the knobs to locate holes without the entire parent molecule having to be perfectly oriented. This may be especially important in the case of the B knobs, in which cases the masking fibrinopeptides B are not removed until the stage where the units have polymerized to protofibrils. Similarly, the inherent flexibility of the γ-chain carboxyl-terminal segments doubtless aids in the factor XIII catalysis of cross-links, as well as in the binding of this region to integrins. The flexible nature of other parts of the molecule may contribute to the general elasticity of the clot, an important property of which is its deformability.

Acknowledgments

The work from the author's laboratory on the structures of fibrinogen and fibrin was supported by the US National Institutes of Health, Grant HL 26873.

Correspondence to: Russell F. Doolittle, Center for Molecular Genetics, University of California, San Diego, La Jolla, CA 92093-0634, USA.
Tel.: +858-534-4417; Fax: +858-534-4985;
E-mail: rdoolittle@ucsd.edu.

References

1. Hall CE, Slayter HS. The fibrinogen molecule: its size, shape and mode of polymerization. J Biophys Biochem Cytol 1959; 5: 11–17.
2. Bailey K, Astbury WT, Rudall KM. Fibrinogen and fibrin as members of the keratin-myosin group. Nature 1943; 151: 716–717.
3. Cohen C. Invited discussion at 1960 symposium on protein structure. J Polym Sci 1961; 49: 144–145.
4. Bailey K, Bettelheim FR, Lorand L, Middlebrook WR. Action of thrombin in the clottting of fibrinogen. Nature 1951; 167: 233–234.
5. Ferry JD. The mechanism of polymerization of fibrin. Proc Natl Acad Sci USA 1952; 39: 566–569.
6. Laudano AL, Doolittle RF. Synthetic peptide derivatives that bind to fibrinogen and prevent the polymerization of fibrin monomers. Proc Natl Acad Sci USA 1978; 75: 3085–3089.

7. Chen R, Doolittle RF. γ–γ cross-linking sites in human and bovine fibrin. Biochemistry 1971; 10: 4486–4491.
8. McKee PA, Mattock P, Hill RL. Subunit structure of human fibrinogen, soluble fibrin and cross-linked insoluble fibrin. Proc Natl Acad Sci USA 1970; 66: 738–744.
9. Schielen WJG, Voskuilen M, Tesser GI, Nieuwenhuizen W. The sequence Aα-(148–160) in fibrin, but not in fibrinogen, is accessible to monoclonal antibodies. Proc Natl Acad Sci USA 1989; 86: 8951–8954.
10. Schielen WJG, Adams HPHM, Voskuilen M, Tesser GI, Nieuwenhuizen W. The sequence Aα-(154–159) of fibrinogen is capable of accelerating the t-PA-catalyzed activation of plasminogen. Blood Coagul Fibrinolysis 1991; 2: 465–470.
11. Mosesson MW, Doolittle RF (eds.). Molecular biology of fibrinogen and fibrin. Ann NY Acad Sci 1983; 408.
12. Nieuwenhuizen W, Mosesson MW, De Maat MPM (eds). Fibrinogen. XVIth International Fibrinogen Workshop. Ann NY Acad Sci 2002; vol. 936.
13. Tooney NM, Cohen C. Microcrystals of a modified fibrinogen. Nature 1972; 234: 23–25.
14. Rao SPS, Poojary D, Elliott Jr BW, Melanson LA, Oriel B, Cohen C. Fibrinogen structure in projection at 18 Å resolution. Electron density by co-ordinated cryo-electron microscopy and X-ray crystallography. J Mol Biol 1991; 222: 89–98.
15. Everse SJE, Pelletier H, Doolittle RF. Crystallization of fragment D from human fibrinogen. Prot Sci 1995; 4: 1013–1016.
16. Doolittle RF, Everse SJ, Spraggon G. Human fibrinogen: anticipating a three-dimensional structure. FASEB J 1996; 10: 1464–1470.
17. Yee VC, Pratt KP, Cote HC, LeTrong I, Chung DW, Davie EW, Stenkamp RE, Teller DC. Crystal structure of a 30 kDa C-terminal fragment from the γ chain of human fibrinogen. Structure 1997; 5: 125–138.
18. Pratt KP, Cote HCF, Chung DW, Stenkamp RE, Davie EW. The fibrin polymerization pocket: three-dimensional structure of a 30 kDa C-terminal γ chain fragment complexed with Gly-Pro-Arg-Pro. Proc Natl Acad Sci USA 1997; 94: 7176–7181.
19. Hawiger J. Adhesive ends of fibrinogen and its antiadhesive peptides. The end of a saga? Semin Hematol 1995; 32: 99–101.
20. Spraggon G, Everse SJ, Doolittle RF. Crystal structures of fragment D from human fibrinogen and its cross-linked counterpart from fibrin. Nature 1997; 389: 455–462.
21. Brown JH, Volkmann N, Jun G, Henschen AH, Cohen C. The crystal structure of modified bovine fibrinogen. Proc Natl Acad Sci USA 2000; 97: 85–90.
22. Williams RC. Morphology of bovine fibrinogen monomers and fibrin polymers. J Mol Biol 1981; 150: 399–408.
23. Yang Z, Mochalkin I, Veerapandian L, Riley M, Doolittle RF. Crystal structure of a native chicken fibrinogen at 5.5 Å resolution. Proc Natl Acad Sci USA 2000; 97: 3907–3912.
24. Yang Z, Kollman JM, Pandi L, Doolittle RF. Crystal structure of a native chicken fibrinogen at 2.7 Å resolution. Biochemistry 2001; 40: 12515–12523.
25. Madrazo J, Brown JH, Litvonowich S, Dominguez R, Yakovlev S, Medved L, Cohen C. Crystal structure of the central region of bovine fibrinogen (E5 fragment) at 1.4-Å resolution. Proc Natl Acad Sci USA 2001; 98: 11967–11972.
26. Takagi T, Doolittle RF. Amino acid sequence studies on the α chain of human fibrinogen. Location of four plasmin attack points and a covalent cross-linking site. Biochemistry 1975; 14: 5149–5156.
27. Murakawa M, Okamura T, Kamura T, Shibuya T, Harada M, Niho Y. Diversity of primary structures of the carboxy-terminal regions of mammalian fibrinogen Aα-chains. Thromb Haemost 1993; 69: 351–360.
28. Tsurupa G, Latchezar L, Medved L. Structural organization of the fibrin(ogen) αC-domain. Biochemistry 2002; 41: 6449–6459.
29. Gorkun OV, Henschen-Edman AH, Ping LF, Lord ST. Analysis of Aα251 fibrinogen: the αC domain has a role in polymerization, albeit more subtle than anticipated from the analogous proteolytic fragment X. Biochemistry 1998; 37: 15434–15441.
30. Yang Z, Mochalkin I, Doolittle RF. A model of fibrin formation based on crystal structures of fibrinogen and fibrin fragments complexed with synthetic peptides. Proc Natl Acad Sci USA 2000; 97: 14156–14161.
31. Tsurupa G, Medved L. Identification and characterization of novel tPA- and plasminogen-binding sites within fibrin(ogen) αC-domains. Biochemistry 2001; 40: 801–808.
32. Koopman J, Haverkate F, Lord ST, Grimbergen J, Mannucci PM. Molecular basis of fibrinogen Naples associated with defective thrombin binding and thrombophilia. J Clin Invest 1992; 90: 238–244.
33. Gorlatov S, Medved L. Interaction of fibrin(ogen) with the endothelial cell receptor VE-cadherin: mapping of the receptor-binding site in the NH_2-terminal portions of the fibrin β chains. Biochemistry 2002; 41: 4107–4116.
34. Odrijin TM, Shainoff JR, Lawrence SO, Simpson-Haldaris PJ. Thrombin cleavage enhances exposure of a heparin-binding domain in the N-terminus of the fibrin β chain. Blood 1996; 86: 2050–2061.
35. Thompson WD, Smith EB, Stirk CM, Marshall FJ, Stout AJ, Kocchar A. Angiogenic activity of fibrin degradation products is located in fibrin fragment E. J Pathol 1992; 168: 47–53.
36. Donahue JP, Patel H, Anderson WF, Hawiger J. Three-dimensional structure of the platelet integrin recognition domain of the fibrinogen γ-chain obtained by carrier protein driven crystallization. Proc Natl Acad Sci USA 1994; 91: 12178–12182.
37. Ware S, Donahue JP, Hawiger J, Anderson WF. Structure of the fibrinogen γ-chain integrin binding and factor XIIIa cross-linking sites obtained through carrier protein driven crystallization. Prot Sci 1999; 8: 2663–2671.
38. Everse SJ, Spraggon G, Veerapandian L, Doolittle RF. Conformational changes in fragments D double-D from human fibrin(ogen) upon binding the peptide ligand Gly-His-Arg-Pro-amide. Biochemistry 1999; 38: 2941–2946.
39. Yokoyama K, Erickson HP, Ikeda Y, Takada Y. Identification of amino acid sequences in fibrinogen γ-chain and tenascin C C-terminal domains critical for binding to integrin $\alpha_v\beta$3. J Biol Chem 2000; 275: 16891–16898.
40. Doolittle RF. A detailed consideration of a principal domain of vertebrate fibrinogen and its relatives. Prot Sci 1992; 1: 1563–1577.
41. Fu Y, Cao Y, Hertzberg KM, Grieninger G. Fibrinogen α genes: conservation of bipartite transcripts and carboxy-terminal-extended α subunits in vertebrates. Genomics 1995; 30: 71–76.
42. Spraggon G, Applegate D, Everse SJ, Zhang J-Z, Veerapandian L, Redman C, Doolittle RF, Grieninger G. Crystal structure of a recombinant α_EC domain from fibrinogen-420. Proc Natl Acad Sci USA 1998; 95: 9099–9104.
43. Doolittle RF, Spraggon G, Everse SJ. Evolution of vertebrate fibrin formation and the process of its dissolution. In: Plasminogen-Related Growth Factors. CIBA Foundation Symposium, Vol. 212. New York: Wiley; 1997: 4–23.

44. Knibbs RN, Osborne SE, Glick GD, Goldstein IJ. Binding determinants of the sialic acid-specific lectin from the slux *Limax flavus*. J Biol Chem 1993; 268: 18524–18531.
45. Adema CM, Hertel LA, Miller RD, Loker ES. A family of fibrinogen-related proteins that precipitates parasite-derived molecules is produced by an invertebrate after infection. Proc Natl Acad Sci USA 1997; 94: 8691–8696.
46. Gokudan S, Muta T, Tsuda R, Koori K, Kawahara T, Seki N, Mizunoe Y, Wai SN, Iwanaga S, Kawabata S-I. Horseshoe crab acetyl group-recognizing lectins involved in innate immunity are structurally related to fibrinogen. Proc Natl Acad Sci USA 1999; 96: 10086–10091.
47. Beisel H-G, Kawabata S-I, Iwanaga S, Huber R, Bode W. Tachylectin-2: crystal structure of a specific GlcNac/GalNac binding lectin. EMBO J 1999; 18: 2222–2313.
48. Everse SJ, Spraggon G, Veerapandian L, Riley M, Doolittle RF. Crystal structure of fragment double-D from human fibrin with two different bound ligands. Biochemistry 1998; 37: 8637–8642.
49. Morris TA, Marsh JJ, Fagnani R, Hagan M, Moser KM. Degree of polymer orgainzation decreases the binding of a monoclonal antibody raised against the β-chain amino terminus of fibrin. Thromb Haemost 1997; 77: 704–709.
50. Lugovoskoi EV, Komisarenko SV. The use of monoclonal antibodies for studying the fibrin polymerization. Russian J Bioorg Chem 2000; 26: 883–891.
51. Doolittle RF. Fibrin packing arrangements inferred from crystal structures of fragments D and double-D complexed with synthetic peptide knobs. Jpn J Thromb Hemost 2000; 11: 123–130.
52. Yakolev S, Makogonenko E, Kurochkina N, Nieuwenhuizen W, Ingham K, Medved L. Conversion of fibrinogen to fibrin: mechanism of exposure of tPA- and plasminogen binding sites. Biochemistry 2000; 39: 15730–15741.
53. Lounes KC, Ping L, Gorkun OV, Lord ST. Analysis of engineered fibrinogen variants suggests that an additional site mediates platelet aggregation and "B–β" interactions have a role in protofibril formation. Biochemistry 2002; 41: 5291–5299.
54. Laudano AP, Doolittle RF. Studies on synthetic peptides that bind to fibrinogen and prevent fibrin polymerization. Structural requirements, number of binding sites, and species differences. Biochemistry 1980; 19: 1013–1090.
55. Kuyas C, Doolittle RF. Gly-Pro-Arg-Pro derivatives that bind to human plasma albumin and prevent fibrin formation. Thromb Res 1986; 43: 485–490.
56. Yin YC, Doolittle RF. A latent inhibitor of fibrin polymerization with ancillary anticoagulant activity. Thromb Res 2000; 97: 375–378.
57. Yang Z, Spraggon G, Pandi L, Everse SJ, Riley M, Doolittle RF. Crystal structure of fragment D from lamprey fibrinogen complexed with the peptide Gly-His-Arg-Pro-amide. Biochemistry 2002; 41: 10213–10224.
58. Everse SJ, Spraggon G, Doolittle RF. A three-dimensional consideration of variant human fibrinogens. Thromb Haemost 1998; 80: 1–9.
59. Cote HC, Lord ST, Pratt KP. γ-Chain dysfibrinogenemias: molecular structure-function relationships of naturally occurring mutations in γ chain of human fibrinogen. Blood 1998; 92: 2012–2195.
60. Fellowes AP, Brennan SO, Ridgway H, Heaton DC, George PM. Electrospray ionization mass spectrometry identification of fibrinogen Banks Peninsula (γ280Tyr>Cys): a new variant with defective polymerization. Brit J Haematol 1998; 101: 24–31.
61. Mullin JL, Brennan SO, Ganly PS, George PM. Fibrinogen Hillsborough: a novel γGly309Asp dysfibrinogen. Haemostas Thromb Vasc Biol 2002; 99: 3597–3601.
62. Schweers O, Schonbrunn HE, Marx A, Mandelkow E. Structural studies of tau protein and Alzheimer paired helical filaments show no evidence of *beta*-structure. J Biol Chem 1994; 269: 24290–24297.
63. Uversky VN, Gillespie JR, Fink AL. Why are "natively unfolded" proteins unstructured under physiological conditions? Proteins: Struct Funct Gen 2000; 41: 415–427.
64. Dyson HJ, Wright PE. Coupling of folding and binding for unstructured proteins. Curr Opin Struct Biol 2002; 12: 54–60.

