Natively Unfolded Regions of the Vertebrate Fibrinogen Molecule

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ABSTRACT **Although it has long been realized that a large portion of the fibrinogen α chain has little if any defined structure, the physiological significance of this flexible appendage remains mysterious. Proteins 2006;63:391–397.**

Key words: fibrinogen; fibrin; intrinsically unfolded domains

INTRODUCTION

In their invitation to this special issue, the Editors noted that authors ought to consider George Rose's research interests in their choice of topic. In line with that request, some explanation may be needed for our chosen subject.

As it happens, in 2002 George Rose edited a volume of *Advances in Protein Chemistry* on the subject of unfolded proteins.[1] The volume, which contains articles from some of the same individuals contributing to this special issue of *Proteins*, was comprehensive. Nonetheless, we were surprised to find that a protein that has been the subject of study for a hundred years—and that for more than the last thirty has been thought to have a disproportionate amount of unfolded structure—was never mentioned. Nor was it listed elsewhere in various tallies of protein sequences designated as "intrinsically unstructured" or "natively unfolded."[2–4] The present manuscript is intended to remedy these apparent oversights by reviewing evidence for a lack of structure in certain sectors of the vertebrate fibrinogen molecule, particularly the carboxyl-terminal "domains" of the α chains. At issue are two points: (1) what constitutes a "natively unfolded" entity? And (2) do the fibrinogen α-chain carboxyl-terminal regions meet the requirements? In recent years, criteria for designating a protein as "disordered" or "unfolded"—in whole or part—have usually been based on sensitivity to proteases, on the one hand, or lack of secondary structure as determined by physical methods like circular dichroism, on the other.[5] Another commonly cited attribute is the absence of interpretable structure in electron density maps calculated from X-ray diffraction data. As we will show, the αC domains of fibrinogen qualify on all these counts and some others as well, including rapid evolutionary change and distinctive amino acid compositions. At the same time, there are some contrary data that imply a minimally folded core structure that need to be discussed also.

STRUCTURAL ASPECTS

Vertebrate Fibrinogen

Fibrinogen is the large glycoprotein that circulates in the blood plasma of all vertebrate animals and is the precursor of fibrin blood clots. It is a six-chained entity composed of two pairs each of three polypeptide chains joined by a complex set of disulfide bonds ($\alpha_2\beta_2\gamma_2$). Physical-chemico studies and electron microscopy long ago revealed an extended multi-domained structure approximately 45 nm in length. A plethora of biochemical experiments over the course of half a century gave flesh to that skeletal structure and provided a general if conceptual model of its structure (Fig. 1). One longstanding feature of the model was that the carboxyl two-thirds of the α chains were devoid of secondary structure, oft being referred to as "free-swimming appendages".[6]

The initial basis for this characterization was that the designated segments were easily removed and destroyed by a wide variety of proteases. In the ensuing three decades, an enormous amount of data supporting a mostly unfolded structure has been provided by other means. What follows is a summary of the evidence for that view and some speculation about how inherent flexibility may play a role in fibrin formation.

The Core Fragments D and E

In 1961, a French group digested fibrinogen with the enzyme plasmin and passed the products over an ion exchange column.[7] Five distinguishable peaks were observed, denoted A–E. Two of these, D and E, accounted for the bulk of the applied material and were destined to become the well studied core fragments D and E. Fragments D, of which there are two per molecule, and E (the central domain) together account for two-thirds of the mass of the starting fibrinogen. The missing one-third, which in most mammalian molecules amounts to about a thousand residues per native dimer, is mostly from the carboxyl-terminal segments of the two α chains. Fragments D and E also accounted for the main morphological features observed in shadow-cast electron micrographs of fibrinogen.[8,9]

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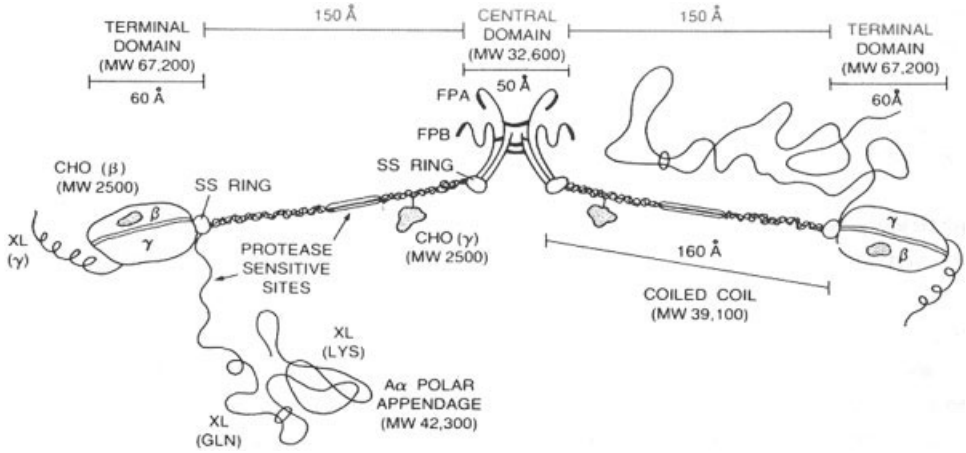

Fig. 1. An old model of fibrinogen concocted on the basis of early electron microscopy studies and sundry biochemical data. FPA, fibrinopeptide A; FPB, fibrinopeptide B; SS RING, disulfide rings; XL, cross-linking sites; CHO, carbohydrate. Aα, β, and γ denote the α, β, and γ chains of the dimeric protein, respectively. Note the minimally folded αC regions. (reprinted from Marder, Francis and Doolittle[71])

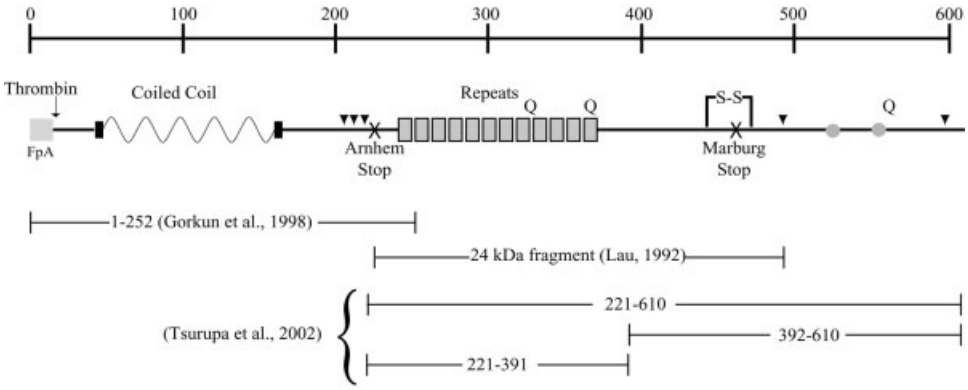

Fig. 2. Diagram of various regions of human fibrinogen α chain. Residue numbers denote some key points. A proteolytic fragment[43] and two recombinantly expressed regions[28,56] are labeled by authors and year. Positions corresponding to changes that lead to truncated α chains in some variant human fibrinogens are marked "X" (fibrinogens Arnhem[48] and Marburg[51]). Positions at which changes lead to amyloid formation are denoted by shaded circles.

Moreover, when fibrinogen was subjected to limited proteolysis, the percent of secondary structure remaining in the core structure, as determined by circular dichroism, increased greatly.[10] This observation was consistent with the notion that the three globules observed in electron micrographs were likely connected by α-helical "coiled coils."[11] Conversely, CD studies on the material released gave a signal characteristic of a "random coil".[12,13]

On another front, elegant scanning calorimetry experiments showed that there were two discrete moieties in fibrinogen that "melted" at very different temperatures, two widely separated heat-absorbing peaks being entirely accounted for by the fragments D and E with no other obvious features being evident.[14]

Amino Acid Sequence Studies

When the human fibrinogen α chain was sequenced,[15] it was found to have three quite characteristic zones: a coiled-coil domain that was homologous to the β and γ chains, a region consisting of ten 13-residue imperfect repeats, and an approximately 250-residue long terminal domain corresponding to what is now generally referred to as the αC domain (Fig. 2). The sequence was wholly consistent with the model shown in Figure 1 that had a terminal αC region connected to the main-frame of the molecule by a flexible tether composed of imperfect repeats, both sections being susceptible to release by proteases.

Sequence studies on other species revealed that the carboxyl-terminal regions of α chains have experienced a very rapid rate of evolutionary change, not only with regard to amino acid substitution, but also in the way of numerous deletions and insertions.[16] A region containing a single disulfide bond is modestly conserved (Fig. 3), but even it changes faster than the average plasma protein. For example, the 40-residue conserved segment containing the disulfide is only 67% identical in humans and chickens, whereas the entire 86-kDa fragments D of these two

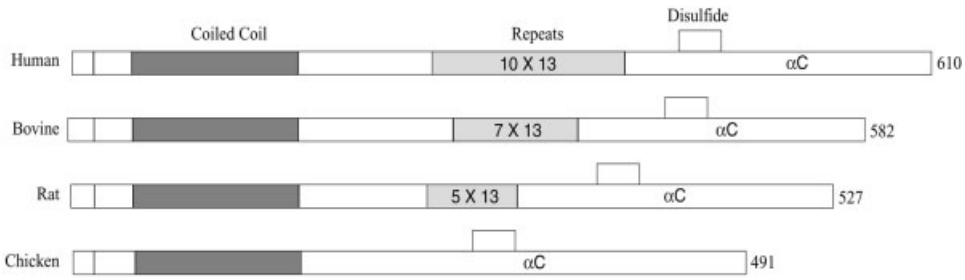

Fig. 3. Schematic depiction of human, bovine, rat and chicken fibrinogen α chains. The regions with the variable numbers of repeats and the αC domains are both readily removed by limited proteolysis. Note that the chicken α chain lacks the repeat region which in other species provides a connecting tether for the terminal αC domain.

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Human      ...GSFRPDSPGS G NARPNNPDWG      TFEE    VSGNVSPGT    RREYH    TEKLVTSKGDK
Rat        ...GSSRP SSGS G NLKPSNPDWG      EFSE    FGGSSSPAT    RKEYH    TGKLVTSKGDK
Cow        ...SSFRPDSSGH G NIRPSSPDWG      TFRE     EGSVSSGT    KQEFH    TGKLVTTKGDK
Chicken    ...PSPEMQAMSAFN NIKQMQV         VLERPETDHVAEARGD    SSPSH    TGKLITSSHRR
Xenopus    ...KDINQYSFVLEG                 TKEE    VKGS SIGT    SSVVFPSGSGKDVDFQGSKG
Zebrafish  ...LDKQMDNLNAMRVQSVETVSSLGIMKSRPLKD VLVPTIYKSGTGKAEQKLLFGDVGQMQLSLEAEG

                                          *     *         *              *   *  *
Human        ELRTGKEKV TS  GSTTTTRRS CSKTVTK TVIGPDG HKEVTKEVVTS EDGSDC         P
Rat          ELLIGNEKV TS  TGTSTTRRS CSKTITK TVLGNDG HREVVKEVVTS DDGSDC         G
Cow          ELLIDNEKV TS  GHTTTTRRS CSKVITK TVTNADG RTETTKEVVKS EDGSDC         G
Chicken      ESPSLVDKT SS    ASSVHR  CTRTVTKKVISGPDGPREEIVEKMVSS  DGSDC
Xenopus    TQSGTVKGGDQS TK  VITESRTVS CTKTKKKRVIITPEG PKE   EIIESYSGGAECEKLGKFSTG
Zebrafish  STAESAATVSKFPTSGTGSTSQTSSKQCTKSVRKVITHTKDGPVEKV  EVSSS   GPGC         D

Human      EAMDLG      TL  SGIGTLDGFRHRHPDEAAFF  DTASTGKTFPGFFSPMLGEFVSETESRGSE S
Rat        DGMDLGL     TH  SFSGRLDELSRMHPELGSFY  D     SRF GSLTSNFKEFGSKT    SD S
Cow        DA DFDW     HHTFPSRGNLDDFFHRDKD                DFFTRSSHEFDGRT    GL A
Chicken    SHLQGGREG  STYHFSGTGDFHKLDRLLPDLESFFTHDSVSTSSRHSIGSSTSSHVTGAGSSHLGTGGK
Xenopus    DTFEAGSDGTR VIKISGSSSLGDIS KIPSFEEFL   SSSGKKTQTSQTSSSSSSSSSTYSKGVT H
Zebrafish  DLGKLGIS   DSEFLTAAKDGKDLSR   DGLKI   TVTGGDEKSLTSLSHSTSDLGG    FGGD

             *
Human      GIFTNTKES   SSHHPGI  AEF   PSRGK  SSSYSKQ F TSSTSYNRGDSTFESKSYKMADEAGS
Rat        DIFTDIENP   SSH   V  PEF   SSSSK  TSTVRKQ V TKS              YKMADEAAS
Cow        PEFAALGES   GS             SSSK  TSTHSKQFV SSSTTVNRGGSAIESKHFKMEDEAES
Chicken    DKFTDLGEEE  EDDFGGLQPSGF    AAG   SASHSKTVLTSSSSSFNKGGSTFETKSLKTRETSEQ
Xenopus    DAFSDLGEDEFDDFSHTGLDSPSF   TPIQKEGSSTYTKTVVSSSSRGSTKDGTQFDIKSMKSGPVFDD
Zebrafish   FFKNLGTDNRKYASSSSSTSTKTVSDGSKSYSKTTLTSDPIFGDDLGAFMRGDVEEDMPDIHARSVKS

Human       EADHEG THSTKRGHAKSRPV
Rat         EAHQEGDTRTTKRGRARTMRGIHA
Cow        LEDLGFKGAHGTQKGHTKAPRA
Chicken    LGGVQHDQSAEDTPDFKARSFRPA
Xenopus    FGPIQHDNSEEDRPDFQARTVRKE
Zebrafish      QDDRKAGFVGGGTLE...
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Fig. 4. Sequence comparisons of αC domains from six assorted vertebrates. The extensive differences reflect the rapid evolutionary change that occurs in this region of the fibrinogen molecule. Only six residues in the approximately 240-residue segment are absolutely conserved (denoted by asterisks). Those positions in which five of the six residues are identical are emboldened. The most conserved region, delineated by the two cysteine residues that form the single disulfide bond, is shaded.

species are almost 80% identical. In general, the amino acid compositions of the αC domains in various species—their rapid change not withstanding—are consistent with what is now being deigned as characteristic of intrinsically or natively unfolded proteins.[17,18] Rapid evolution has been reported for other proteins with long disordered regions.[19] As it happens, also, some intrinsically disordered domains have been shown to be the result of evolutionary expansion of repeat regions.[20] In this regard, it is significant that the number of repeats in the central region of these chains varies widely from species to species (Fig. 4). Indeed, even the sizes of the repeats differ, being 13 residues in most species, but 15 residues in marsupials, and 18 residues in dogs[16] and lampreys.[21] Birds were found to lack the repeated region completely,[22] as has now been found to be the case for bony fish and amphibians.[23] It has been proposed that in those molecules that do have the repeats they serve as an accordion or spring-like tether for the αC domain.[21]

Evidence Favoring a Compact αC Domain.

In calorimetry studies conducted subsequent to the those cited above, bovine fibrinogen was found to exhibit additional small but reproducible heat effects that were

consistent with some minimal structure in the αC domains. These data were regarded as indicative of a compact domain connected to the main body by a flexible tether.[24,25] Subsequent studies on human fibrinogen did not find the same thermal effects, however.[26] Nonetheless, the view has persisted that these are *compact globular* domains.[27] The question arises as to whether the descriptors "compact" and "globular" imply a fixed and folded structure. Although "globular" is a broad, all-embracing term that can accommodate both folded and unfolded domains, "compact" almost always implies a degree of order.

In line with this view, more recent studies employing fluorescence methods, differential scanning calorimetry, and CD were conducted on recombinant constructs of both the bovine and human αC domain, with and without the connecting tethers[28] (Fig. 2). Although thermally-induced changes in ellipticity were detected, virtually no conventional secondary structure (i.e., α-helix or β-structure) was found. Instead, the evidence favored the existence of an extended helical poly(L-proline) type II structure, mostly associated with the connector (tether) region. It is notable that the polyproline II helix is now regarded as a hallmark of the unfolded state.[1,29] It might be noted, also, that not all α-chain repeats in different species are proline-rich; but then again, polyproline II structure is often observed in other proline-poor proteins, as well.[30] As pointed out to us by a reviewer, enthalpy changes during scanning calorimetry are given to various interpretations and must be considered with great caution.[31]

Electron Microscopy

There have been conflicting reports over the years about the whereabouts of the αC domain as determined by various electron microscope approaches, some of which contend that the αC domains are associated with the central domain,[32,33] and others, mostly utilizing antibodies (or FAB fragments) directed against peptides in the αC region, which find the immuno-reactive material in varied and more distal locations.[34,35] In the latter experiments, only the antibodies (or FAB fragments), and not the αC regions themselves, appeared to be shadowed. However, there have been more definitive studies on isolated αC domains, particularly focused on a 40-kD fragment from bovine fibrinogen.[36]

A recent report utilizing scanning-transmission electron microscopy (STEM) has focused on a minor form of fibrinogen that has alternatively spliced α chains with carboxyl-terminal globular domains (denoted αC′) homologous to the β and γ chains located distal to (in a sequence sense) the usual αC domains.[37] In those micrographs allegedly including both kinds of domain, the globular αC′ domains, which contain about the same number of residues as αC domains, dwarf the images of any neighboring material.

Crystal Structures

More than three decades ago, it was serendipitously found possible to crystallize bovine fibrinogen that had been trimmed down by limited proteolysis, even though it was not possible to crystallize the native protein.[38] Eventually, a structure of this modified protein—lacking αC domains—was determined at a nominal 4 Å resolution.[39]

The fact that chicken fibrinogen totally lacks the middle-region tether[22] (Fig. 4) prompted efforts to crystallize that protein. Indeed, the native protein was crystallized and a structure determined at 2.7 Å resolution.[40] Not surprisingly, the αC domains were not discernable. It is indisputable that the αC domains in these crystalline settings are moving, whether or not they are intrinsically unfolded, and this in a fibrinogen whose α chains lack the repetitive region thought to be the flexible tether. Efforts to crystallize recombinant constructs corresponding to the αC domains from both bovine and chicken fibrinogens have been wholly unsuccessful.[41]

All of these observations are in accord with the αC domains of fibrinogen having the properties of "natively unfolded proteins" as currently defined. The questions arise, why are these regions of fibrinogen unfolded, and what is their raison d'etre?

FUNCTIONAL ASPECTS

Fibrin Formation

There is general agreement that αC domains play a role in fibrin formation. Antibodies directed to that region of the molecule interfere with clotting,[42] as do purified fragments themselves.[36,43,44] Moreover, if the αC domains are absent, whether removed by proteases or—as we will discuss further below—the result of genetic aberration, clotting is greatly slowed.[45] What the role may be, however, is not at all clear.

Put very briefly, fibrin formation occurs in two stages. In the first, thrombin removes the fibrinopeptides A from the amino-terminal segments of α chains, thereby exposing a set of knobs in the central region of fibrinogen that can fit into holes at the extremeties of other molecules and bridge them together in an overlapping fashion to form a two-molecule thick protofibril. In the second stage, thrombin removes the fibrinopeptide B from the amino-terminal segment of the β chain, the result of which is the lateral association of the protofibrils and the formation of mature fibers. The involvement of the readily removed αC domain appears to be limited to this second stage.

In this regard, it has long been known that fibrinogen prepared by conventional means from normal plasma contains separable fractions that have different degrees of solubility.[46] Comparisons of "high-soluble" and "low-soluble" fractions have been traced to differences in the carboxyl-terminal regions of the α chains, the more soluble fractions having distinctly shorter chains, the result of in vivo proteolysis.[47] Similar results are observed when limited proteolysis is performed in vitro.

As it happens, however, proteolysis—especially when conducted in vitro—removes other parts of the fibrinogen molecule, most notably a flexible 40–50 residue segment from the amino-terminus of the β chain, and the possible contribution of these missing segments to the slowed clotting could not be discounted, even though in some reports great care was taken to show that the β chains

were intact.[47] The ambiguity was resolved, to a degree, when individuals were identified who had genetic defects in this region of fibrinogen, some of whom had the region in question wholly deleted (Fig. 2) and whose blood clotting was seriously disrupted.

Genetically Defective Fibrinogens

Genetically variant forms of human fibrinogen have been helpful in assessing the functions of the αC domain. A number of different variants result in truncated α chains, the most informative of which are arguably those that occur in the region just before the beginning of the ten imperfect repeats and which ought to be equivalent to those generated by limited proteolysis in vitro. One of these is fibrinogen Arnem,[48] in which a residue ordinarily cleaved by plasmin, αLys-219, has its codon mutated to a terminator. The patient experienced bleeding problems, and the fibrinogen was definitely slow to clot when treated with thrombin. Similar situations occur in a host of other variants in which the α-chain changes are more distal. On their own, then, variant human fibrinogens lacking all or part of the αC domain demonstrate the importance of that structure in fibrin formation.

Some of the genetically variant fibrinogens provide evidence for the flexible character of αC domains, as well as providing circumstantial evidence about their unfolded nature. In this regard, a variety of changes lead to the presence of unpaired cysteine residues in the αC region. These come about in two ways. In one these, noncysteine residues are simply mutated to cysteines. An example is Fibrinogen Dusart, which has a substitution of a cysteine for an arginine at position α554.[49,50] In the other situation, termination codons occur in the 29-residue segment between cysteines α442 and α472 (Fig. 2). An example is fibrinogen Marburg which has a stop codon in place of the normal lysine at position α461,[51] and as a result it not only lacks the carboxyl-terminal 150 amino acids that encompass most of the αC domain but has an unpaired cysteine at α442 as well.

Invariably these unpaired cysteines form disulfide bonds with plasma albumin, the most abundant of the plasma proteins. Burdened by that extra 67-kDa entity, these fibrinogens exhibit markedly abnormal polymerization. Significantly, none has ever been found to be disulfide-linked to its partner αC domain in the same or other molecules. It is of interest, also, that electron microscopy readily identifies the attached plasma albumin moieties but not the αC domains themselves.[52]

It should be mentioned in passing that several variant human fibrinogens in the region of α-chain residues 522–554 result in the formation of amyloid deposits.[53–55] Interestingly, unfolded domains are often associated with amyloid formation.[2] Finally, adding another note to the puzzle is the observation that a recombinant fibrinogen engineered to have its α chains terminate at position α251 was only modestly affected with regard to polymerization.[56]

Factor XIII Cross-Linking

In its early stages, fibrin formation is driven by the formation of noncovalent interactions. As the molecules polymerize and specific regions are brought into juxtaposition, the transglutaminase known as factor XIIIa incorporates isopeptide linkages between certain lysine and glutamine side chains. In particular, sets of reciprocal cross-links are rapidly formed between the carboxyl-terminal segments of abutting γ chains—in a time course measured in seconds or minutes—and while the first stage of fibrin formation is still in progress.[57,6] In contrast, a network of the same kind of cross-links (ε-amino-γ-glutamyl lysines) is formed very much more slowly—over the course of hours or days—between αC domains. The latter involve a variety of lysine and glutamine side-chains[58] and lead to a multimerically cross-linked system.[59] Studies with recombinant αC domains and exogenous factor XIII have yielded similar results.[60,61]

Unquestionably, the cross-linking of the αC domains contributes to the stability of clots, even though the wide variety of different cross-links formed reflects an almost random nature of encounter consistent with a lack of specific structure.[62]

The αC Domain as a Binding Site

It is well established that the αC domains in fibrinogen have binding sites for factors that are involved in the destruction of fibrin clots, namely, tissue plasminogen activator (t-PA) and plasminogen.[63] It is also known that inhibitors of such proteolytic enzymes can bind to these regions.[64] Moreover, these same regions of the α chains are also known to be involved in the binding of fibrinogen to blood platelets,[65] fibroblasts,[66] endothelial,[67] and other lymphoid cells.[68] The αC domain is also the site for cross-linking fibrinogen to fibronectin,[69] a process vital to wound healing.

A Guardian Function

A region of a protein can have multiple functions, of course. One interesting but almost forgotten suggestion was that these flexible appendages—like leashed hounds—guard the otherwise vulnerable coiled coils against proteolytic attack. A careful kinetic analysis of the proteolysis of both bovine and human fibrinogens concluded that such a role was indeed reasonable.[70]

SUMMARY

In spite of the evidence for an intrinsically disordered structure marshaled above, the popular view remains that αC domains are compact and globular.[27,28] That in most species they are tethered to the main frame of the parent molecule by a flexible connector is not in dispute. How these regions can be associated *intra*-molecularly in fibrinogen and be in motion at the same time remains mysterious. It may be that these are matters of degree: we need to ask, how disordered and how much motion?

Beyond that, the argument may be largely semantic, different images being provoked by the terms “compact globular” on the one hand, and “intrinsically unfolded” on

the other. In this regard, reference is made in discussions of other natively unfolded proteins to "an ensemble of interchanging conformations."[18] Surely this must be the case for the αC domain, defined loops of as yet unknown conformations being able to serve as specific binding sites for other proteins. Other loops must be able to form loose associations with themselves or other features on the neighboring αC domains. What these domains do not do is form stable structures with defined geometries that form *specific* associations with themselves; if they did we would expect readily defined cross-links to be introduced by factor XIII, if only because of the very limited number of glutamines that can act as acceptors. We would also expect to see the *intra*-molecularly associated structures in X-ray diffraction patterns. Finally, we would expect regions of association—whether *intra*- or *inter*-molecularly—to have amino acid sequences that are at least moderately conserved in an evolutionary sense.

In the end, there is good reason to believe that the αC domains of vertebrate fibrinogen are intrinsically unfolded. Even so, their mobility must be greatly constrained after the conversion of fibrinogen to fibrin, and especially after the introduction of covalent cross-links between them. The fact that individual cross-links involve different glutamine and lysine participants underscores the randomized encounters expected from these highly mobile entities. Apart from this ultimate stabilization, the general whereabouts and precise function of these segments remain controversial matters.

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