Domain Unfolding Plays a Role in Superfibronectin Formation*

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Superfibronectin (sFN) is a fibronectin (FN) aggregate that is formed by mixing FN with anastellin, a fragment of the first type III domain of FN. However, the mechanism of this aggregation has not been clear. In this study, we found that anastellin co-precipitated with FN in a ratio of ~4:1, anastellin:FN monomer. The primary binding site for anastellin was in the segment III1–3, which bound three molecules of anastellin and was able to form a precipitate without the rest of the FN molecule. Anastellin binding to III3 caused a conformational change in that domain that exposed a cryptic thermolysin-sensitive site. An additional anastellin binds to III11, where it enhances thermolysin digestion of III11. An engineered disulfide bond in III3 inhibited both aggregation and protease digestion, suggesting that the stability of III3 is a key factor in sFN formation. We propose a three-step model for sFN formation: 1) FN-III domains spontaneously unfold and refold; 2) anastellin binds to an unfolded domain, preventing its refolding and leaving it with exposed hydrophobic surfaces and β-sheet edges; and 3) these exposed elements bind to similar exposed elements on other molecules, leading to aggregation. The model is consistent with our observation that the kinetics of aggregation are first order, with a reaction time of 500–700 s. Similar mechanisms may contribute to the assembly of the native FN matrix.

Fibronectin (FN) is an extracellular matrix protein and is also present in a soluble form in blood and tissue fluid. FN molecules assemble into an insoluble supramolecular structure known as the FN matrix, which appears during embryonic development, wound healing, and also in cell culture. The biological importance of FN during development and wound healing has been demonstrated by generating conventional and conditional FN knock-out mice and alternative splicing domain (EDA) knock-out and knock-in mice. FN matrix fibrils may be one of the least understood macromolecular protein assemblies. Whereas a large number of protein structures can be self-assembled from purified subunits (e.g. microtubules, actin filaments, collagen fibrils, and viruses capsids), FN fibrils have only been produced by living cells in culture. The cells in tissue culture can assemble FN matrix fibrils either from FN that they synthesize or from soluble FN added exogenously. Fibril assembly takes place on the cell surface and requires integrins.

A fundamental deficit is our lack of knowledge of the structure of FN fibrils. FN molecules must be attached to each other to form the fibrils, but we do not know even the sites of contact between molecules nor the types of bonds that hold them together.

Several attempts at in vitro assembly of FN have resulted in aggregates that may be related to FN matrix fibrils. For instance, aggregates could be formed when FN was partially denatured in guanidine HCl and incubated over time. However, these aggregates seemed to be mediated by disulfide bonding of two free cysteines, which are not involved in native FN fibril formation. When FN is partially denatured and sheared in solution, the FN aggregates into mats with a distinctly fibrillar substructure. An intriguing alternative method of fibril formation involved pulling fibrils from the surface of a FN solution. This method did not involve solution denaturation, but surface denaturation may have played a role. These in vitro systems appear to have some relation to FN matrix assembly in vivo, but comparisons have not yet been followed up.

A decade ago, Morla et al. (16) reported that a small FN fragment, anastellin (originally called III1c), was able to induce aggregation and precipitation of FN in vitro. The aggregates had a partially fibrillar substructure that resembled FN fibrils at the light microscopic level. When FN was coated on plastic in the presence of anastellin, it had enhanced cell adhesion activity. The authors named the aggregate superfibronectin (sFN). Subsequent studies of anastellin have revealed potentially important biological activities when injected peritoneally into mice, including inhibition of angiogenesis and tumor growth. These activities required plasma FN (18), so it is likely that sFN plays a role. When tested in cell culture, Bourdoulous et al. (19) found that high concentrations of anastellin (20 μM) blocked FN matrix formation and caused an established matrix to disappear after 16 h of treatment. In contrast, Klein et al. (20) found that 20 μM anastellin caused no change in the FN matrix, except for loss of a particular epitope in EDA. However, their cultures were examined only after 1–2 h. Changes in cytoskeleton organization and signaling pathways have also been documented following treatment with anastellin.

In contrast to these studies on biological activities of anastellin, the biochemistry and structure of sFN itself has been little studied. The fibrillar structure of sFN aggregates suggested that the in vitro aggregation of anastellin and FN might be related to the assembly of FN fibrils in cell culture. Thus, understanding the structure of sFN may lead to insights on the structure and assembly of FN fibrils. A crucial step is to map the binding sites and determine the stoichiometry of anastellin binding to FN. Ingham et al. (22) have shown that an anastellin-like peptide binds several proteolytic FN fragments. However, anastellin-binding sites on FN for sFN aggregation have not been mapped.

In the present study we used a range of overlapping recombinant fragments to precisely map the binding sites for anastellin. We discovered that some segments of FN formed an aggregate with anastellin similar to sFN. This allowed us to determine the stoichiometry of binding. We also used a disulfide mutant to show that the unfolding of an FN-III domain is a key factor in aggregation. These results advance our understanding of the structure and function of fibrils.
understanding of the structure of sFN and provide new directions for determining the structure and assembly of native FN fibrils.

EXPERIMENTAL PROCEDURES

Bacterially Expressed Proteins—Diagrams of all of the recombinant FN fragments used in this study are shown in Fig. 1. The anastellin expression vector was kindly provided by Dr. Paula McKeown-Longo (Albany Medical College) (20). III7–10, III12–14, and III7–14 were recloned from pET11b expression vectors (23, 24) into pET15b expression vectors (Novagen) to generate a hexahistidine tag at the N terminus. The additional sequence at the N terminus is MGSSHHHHHSS-GLVPRGSHM. Fragments III1–12 (SGPV . . . TTLE; the FN sequence is underlined), III1–5 (SGPV . . . LQPGTS), III1–3 (SGPV . . . QQETC), III1–2 (SGPV . . . QTTAC), III1 with the C-terminal linker (SGPV . . . TTPFC), III2–12 (SPLV . . . TTLE), III2–5 (SPLV . . . LQPGTS), III2–3 (SPLV . . . QQETC), III2 (SPLV . . . QTTAC), III3–12 (APDA . . . TTLE), III3–5 (APDA . . . LQPGTS), III3 (APDA . . . QQETC), III4–12 (TVPS . . . TTLE), III4–7 (TVPS . . . TIIP), III4–5 (TVPS . . . LQPGTS), III10–12 (VSDV . . . TTLE), and III13–15 (NVSP . . . TDDS) were also cloned into pET15b expression vectors as described previously (23). Some of the constructs have additional residues at the C terminus; Cys was added for potential labeling, and Thr-Ser were created by the insertion of a restriction enzyme site for potential cloning. Following expression in Escherichia coli M15 for anastellin and BL21(DE3) for the FN fragments, most of the recombinant proteins were soluble and were purified with a cobalt-agarose column (TALON; Clontech) using standard procedures. However, anastellin was always in the insoluble pellet of the bacterial lysate and required renaturation. The pellet was solubilized in 4 M guanidine HCl in 20 mM Tris buffer (pH 8.0) and passed over a cobalt column. The protein was immobilized onto the column and renatured before elution. For renaturation, the column was sequentially washed with 4, 3, 2, and 0.5 M guanidine HCl in 20 mM Tris buffer (pH 8.0) and finally with 20 mM Tris buffer containing 150 mM NaCl (TBS; pH 8.0). Then the renatured soluble fractions were eluted with 1 M imidazole in TBS. Although a high concentration of imidazole was used for elution, anastellin was spread out over a large number of fractions and had to be concentrated with a Centricon-10 (Millipore) as described previously (19). Anastellin (with a His6 tag) from the pQE-70 vector (20) was also recloned into pET11b for expression in BL21(DE3) to improve solubility. However, the majority of anastellin was still insoluble and required renaturation. After renaturation, anastellin was quite stable in TBS and was able to induce sFN formation. Renatured anastellin also bound to a heparin-agarose column (Sigma) at the physiological salt concentration of 150 mM NaCl, as reported previously (25).

Mammalian Cell Expression Protein—The 1–9 (N-terminal 70-kDa domain) expression vector was constructed by modifying the pAIPFN-GFP vector (26). First a fragment containing 5–10 (i.e. most of the FN and green fluorescent proteins) was removed from the vector by Xbal
with Chinese hamster ovary K1 cells. The transfected cells were cultured into the expression vector was transfected with Lipofectamine (Invitrogen) into the XbaI site on the vector. We call this vector pAI1–9. The His6 tag was added onto a gelatin-agarose column (Sigma), and eluted with 6M urea in TBS.

After 16 h, the aggregates were analyzed by light microscopy and SDS-PAGE. Fluorescence (A) and differential interference contrast (B) images showed that aggregates contained FN-YFP and had a fibrillar substructure. A Coomassie-stained SDS-PAGE gel (C) showed that the aggregate was mainly composed of FN and anastellin. s, supernatant; P, pellet.

FIGURE 3. sFN formation in the conditioned medium from FN-YFP-transfected cells. 20 µm anastellin was added to the conditioned medium from FN-YFP-transfected cells. After 16 h, the aggregates were analyzed by light microscopy and SDS-PAGE. Fluorescence (A) and differential interference contrast (B) images showed that aggregates contained FN-YFP and had a fibrillar substructure. A Coomassie-stained SDS-PAGE gel (C) showed that the aggregate was mainly composed of FN and anastellin. s, supernatant; P, pellet.

Digestion to reduce vector size. Fragment 5–9 was PCR-amplified with a His6 tag, a stop codon and an XbaI site on the 3' end and inserted back into the XbaI site on the vector. We call this vector pAI1–9. The His6 tag was added at the C terminus for purification purposes (QAQQ ... YPSSGGHHHHHHC, the FN sequence is underlined). To add a neo-mycin selection cassette, the EYFP-C1 vector (Clontech) was inserted into the KpnI site in the pAI1–9 expression vector (this vector, pAI1–9/Neo, does not express YFP, because of a modification). The I1–9 expression vector was transfected with Lipofectamine (Invitrogen) into Chinese hamster ovary K1 cells. The transfected cells were cultured with 5×-minimal essential medium (BioWhittaker) containing 10% fetal calf serum (Hyclone) in the presence of genetin (G418, 0.75 mg/ml; Invitrogen). The highest expression clone was identified by Western blotting. The conditioned medium from this clone was collected, loaded onto a gelatin-agarose column (Sigma), and eluted with 6 M urea in TBS. The eluted fractions contained both I1–9 fragments and FN from the fetal calf serum and so were run over a cobalt-agarose column for further purification. The I1–9 fragments were eluted with 0.2 M imidazole in TBS. We typically obtained 0.1–0.2 mg of purified protein from 1 liter of culture medium.

Pelleting Assay—The concentrations of purified proteins were estimated from their absorbance at 280 nm using the extinction coefficient of each protein calculated by the Protein computer program (DNAstar Inc.). The concentration of plasma FN (pFN) is stated for the monomer. Prior to the pelleting assay, proteins were centrifuged at 20,000 × g for 10 min to remove minor aggregates. The proteins of interest were mixed in a final volume of 25 µl with TBS containing 5 mM EDTA and incubated at room temperature for 16 h. EDTA was added because some metal ions, such as cobalt and copper, are able to precipitate a small fraction of FN (27). After incubation, the samples were centrifuged at 20,000 × g for 10 min, and the supernatants were collected. The pellets were rinsed with 100 µl of TBS and resuspended with SDS-PAGE loading buffer. SDS-PAGE was performed using standard procedures.

Turbidity Assay—Turbidity measurements were performed with a spectrophotometer (Shimadzu, UV-2401PC). Prior to the assay, the proteins were centrifuged at 20,000 × g for 10 min to remove minor aggregates. The proteins of interest were mixed in a final volume of 500 µl with TBS containing 5 mM EDTA. The samples were illuminated with 550-nm light, and data were collected at 20-s intervals for 1,800 s.

Protease Assay—Proteins of interest were digested with thermolysin (10 µg/ml) in a final volume of 25 µl with TBS containing 10 mM CaCl2 at room temperature for 1 h. Prior to the digestion, the proteins were incubated with or without 40 µM anastellin at room temperature for 2 h. The samples were analyzed by SDS-PAGE.

Disulfide Mutations—To design an intrachain disulfide bond in III3, structural models of potential mutants were created by SWISS-MODEL (an automated protein homology modeling server) (28). For this modeling, tenasin FN-III domain 3 (29) and fibronectin FN-III domains (an automated protein homology modeling server) (28). For this modeling, tenasin FN-III domain 3 (29) and fibronectin FN-III domains 7–10 (30) were used as template structures. Two pairs of residues, residues Ser795 and Ser837 and residues Ala782 and Ser859, were selected for mutagenesis (Fig. 2), based on the proximity of modeled cysteine residues (i.e. the distance between sulfur atoms was estimated to be 2.0–2.5 Å). Site-directed mutagenesis was performed on the I1–5 expression vector using Pfu turbo DNA polymerase (Stratagene). Mutant proteins were purified as the wild type protein described above. Disulfide bond formation in these mutants was confirmed by nonreducing SDS-PAGE in which disulfide mutants migrated slightly faster than wild type. 5,5’-Dithio-bis(2-nitrobenzoic acid) assays (31, 32) also showed that there was no significant amount of free sulfhydryl under denaturing conditions, indicating that these disulfide bonds are completely formed.

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FIGURE 4. Concentration of anastellin needed for sFN formation. Increasing concentrations of anastellin were mixed with pFN (1 µM), incubated 16 h, and centrifuged. To efficiently form the sFN precipitate, 20 – 40 µM anastellin was required. s, supernatant; P, pellet; Std, BenchMark™ protein ladder (Invitrogen). The top band is 220 kDa, and the bottom two are 15 and 10 kDa.

Turbidity Assay—Turbidity measurements were performed with a spectrophotometer (Shimadzu, UV-2401PC). Prior to the assay, the proteins were centrifuged at 20,000 × g for 10 min to remove minor aggregates. The proteins of interest were mixed in a final volume of 500 µl with TBS containing 5 mM EDTA. The samples were illuminated with 550-nm light, and data were collected at 20-s intervals for 1,800 s.

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3 T. Ohashi and H. P. Erickson, unpublished observation.
Cell Culture and Microscopy—FN-YFP transfected 3T3 cells were maintained with Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% calf serum (Hyclone) (33). Cell suspensions (0.2 ml; 8 x 10⁵ cells/ml) were dropped onto 22-mm square cover glasses in 35-mm culture dishes and incubated at 37 °C for 30–60 min to allow cells to settle. The medium was then changed to Dulbecco’s modified Eagle’s medium containing 10% fetal calf serum depleted of FN, with or without recombinant FN fragments. After cells were cultured for 16 h, the samples were fixed with 3.7% formaldehyde in phosphate-buffered saline (pH 7.4), washed, and mounted on slides. The samples were observed with a light microscope (Zeiss Axiophot) using a YFP filter set (41028, Chroma), and the images were captured with a cooled CCD camera (CoolSNAPHQ; Roper Scientific).

RESULTS

Anastellin-binding Sites on FN and Stoichiometry of Anastellin Binding to FN and Recombinant Fragments—When anastellin was mixed with the conditioned media from cells secreting FN-YFP, the mixture became turbid and formed visible aggregates. Fluorescence microscopy showed that the aggregates contained FN-YFP and anastellin (Fig. 3A). Most aggregates were large globular pellets as seen in Fig. 3 (A and B, upper right corners). The fibrillar substructure was best seen in thinner extensions at the edges. Differential interference contrast images frequently showed fibrillar structures even in the large globular aggregates (Fig. 3B). SDS-PAGE showed that the aggregates consisted primarily of FN and anastellin (Fig. 3C). These results indicate that anastellin specifically interacts with FN to form sFN and does not precipitate proteins in general.

We next tested a range of concentrations of anastellin and found that 20–40 μM anastellin was needed to efficiently precipitate 1 μM purified pFN (Fig. 4). 40 μM anastellin was able to precipitate up to 4 μM purified pFN (data not shown). This suggests that the absolute concentration of anastellin rather than the ratio relative to FN is important. To determine the stoichiometry of anastellin to FN in the pellet, we scanned the Coo massie Blue-stained SDS gels from the pelleting assay along with ones containing FN and anastellin over a range of concentrations. We found an average of four anastellins/FN monomer (mean ± S.E. = 4.13 ± 0.12, n = 6) in the sFN pellet (Fig. 5). A recent study reported that anastellin co-precipitated with FN, in a ratio of 5–10:1 (34), similar to our results. In both cases the stoichiometry was estimated from scanning gels, which may not be the most accurate technique. However, we believe that our use of separate calibration curves for each protein should be accurate to within one subunit.

Several conditions were found to inhibit sFN formation: high salt (0.5 M NaCl), glycerol (40%), detergent (1% Triton X-100), and also high pH (CAPS buffer, pH 11.0). To resuspend sFN after it was formed, urea, guanidine HCl, or CAPS buffer (pH 11.0) was required, suggesting that the sFN aggregate is very stable. If left in TBS for several days, however, half of the aggregate would go back onto solution.
To map the anastellin-binding sites on FN, recombinant FN fragments were tested to see whether they inhibited sFN formation. Selected fragments are shown in Fig. 6, and the results for all the fragments are summarized in TABLE ONE. I1–9 did not interfere with sFN formation, although it co-precipitated with sFN (approximately one I1–9/sFN). Notably, I1–9 also co-precipitated with I1–5 plus anastellin (data not shown), consistent with a previous finding that a primary binding site for I1–9 is in I1–2 (35, 36) and indicating that this site is not destroyed or blocked by anastellin binding.

FN fragments that contained domain I1–3 (I1–12, I1–5, I1–3, I2–12, I2–2–3, I3–12, I3–3–5, and I3 itself) inhibited SFN formation. Interestingly, I1–12, I1–5, and I1–3 not only inhibited SFN formation but also replaced FN in the pellet with anastellin. I1–2 co-precipitated with SFN without interfering with SFN formation. FN fragments that contained domain I1–11 (I4–12, I4–7, and I10–12) also interfered with SFN aggregation. I7–10 and I12–14 had no effect on SFN formation, suggesting that anastellin interacts with I1–11. Domains I1–1, I2–4–5, I4–7, and I3–13–15 did not inhibit SFN formation, nor did they co-precipitate with SFN.

Surprisingly, FN fragments that contained I1–2 (I1–12, I1–5, I1–3, and also I1–2 itself) were able to aggregate with anastellin in the absence of FN (Fig. 6B), although fragments that also contained I1–3 (I1–12, I1–5, and I1–3) aggregated with anastellin more efficiently than I1–2 did. I1–2 did not inhibit I1–3 aggregation with anastellin, although a small fraction of I1–2 co-precipitated with it. Anastellin co-precipitated with I1–12 in a ratio of 4:1 (mean ± S.E. = 3.58 ± 0.16, n = 6), with I1–3 in a ratio of 3.1 (mean ± S.E. = 2.93 ± 0.15, n = 6), and with I1–2 in a ratio of 1.1 (mean ± S.E. = 1.22 ± 0.04, n = 6). The 4:1 stoichiometry of anastellin to I1–1–12 is the same as that for full-length FN.

Kinetics of SFN Formation—The aggregation process was monitored spectrophotometrically by measuring the turbidity of the solution at 550 nm (Fig. 7). The kinetic profiles for FN, I1–3, and I1–2 aggregation with anastellin were very similar to each other, although the kinetic profiles at low concentrations of I1–2 showed a lag phase that may indicate nucleation (Fig. 7C). The assembly curves appear to comprise an initial rise that is approximately exponential, followed by a slower rise that may be linear or a slow exponential. Because I1–3 fragments were smaller than FN, higher I1–3 concentrations were required to detect turbidity. Although I1–3 and I1–2 were similar in size, the turbidity measurements for I1–2 required still higher concentrations than I1–3, consistent with the above observation that I1–2 may aggregate with anastellin less efficiently than I1–3.

We attempted to fit the initial turbidity rise to a single exponential. The fit was not perfect but was reasonably good (Fig. 7, A–C). Remarkably, the reaction time for the fit was approximately the same, 500–700 s, for FN, I1–3 and I1–2, despite the different molecular structures and concentrations. Because anastellin was constant at 40 μM in all of these reactions, we next tested whether the kinetics were dependent on anastellin concentration. As seen in Fig. 7D, the kinetics for FN were the same when anastellin was increased from 40 to 160 μM. At lower concentrations of anastellin the kinetics are complicated by the lower rate and extent of reaction, and we did not investigate this range. We conclude that for (saturating) anastellin concentrations above 40 μM, the kinetics of assembly are determined by a first order reaction with a reaction time of ~600 s. The possible nature of this reaction will be addressed under “Discussion.”

Anastellin Alters Protease Sensitivity of FN-III Domains—We next used thermolysin to map anastellin-binding sites on FN. Limited ther-

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**FIGURE 6.** Ability of recombinant FN fragments to inhibit sFN formation (A) and aggregate with anastellin (B). For the pelleting assay, the indicated FN fragments at 10 μg were mixed with 20 μg anastellin, with (A) or without (B) 1 μg pFN. The samples were centrifuged after 16 h. I1–3 and I3–5 fragments dramatically reduced sFN aggregation. I1–9 and I1–2 co-precipitated with sFN without interfering with sFN formation. I1–3 and I1–2 fragments were able to form aggregates with anastellin in the absence of FN. A very small fraction of I1–9 co-precipitated with anastellin, probably because of minor FN contamination in the I1–9 preparation. S, supernatant; P, pellet. Std, BenchMark™ protein ladder (Invitrogen).

**TABLE ONE**

| Fragments | Inhibition of sFN formation | Aggregation with anastellin |
|-----------|-----------------------------|-----------------------------|
| I1–9      | −                          | −                           |
| I1–12     | +                          | +                           |
| I1–5      | +                          | +                           |
| I1–3      | +                          | +                           |
| I1–2      | −                          | +                           |
| I3        | −                          | −                           |
| I12–12    | +                          | −                           |
| I12–5     | +                          | −                           |
| I12–3     | +                          | −                           |
| I3–12     | +                          | −                           |
| I3–5      | +                          | −                           |
| I3        | +                          | −                           |
| I4–12     | +                          | −                           |
| I4–7      | −                          | −                           |
| I4–5      | −                          | −                           |
| I7–14     | +                          | −                           |
| I7–10     | −                          | −                           |
| I10–12    | +                          | −                           |
| I12–14    | −                          | −                           |
| I13–15    | −                          | −                           |

a 1–9 and I1–2 co-precipitated with anastellin and FN.
b I1–12, I1–5, and I1–3 inhibited sFN formation by competing for aggregation with anastellin.

A negligible amount of aggregation was formed with I3–2–5 and I4–4–12.
molysin digestion of FN is well characterized and often used for generating FN fragments (24, 37, 38). We initially wondered whether anastellin binding might mask some thermolysin-sensitive sites on FN. However, we found that anastellin binding enhanced the thermolysin sensitivity of FN. Thermolysin digestion of native FN in the presence of anastellin caused a loss of the two largest fragments of FN (III2–14 and III2–15) and the appearance of a new 80-kDa band (Fig. 8, first and second lanes). We then used the FN fragments to map the site(s) more precisely.

There were two major sites that became thermolysin-sensitive in the presence of anastellin. The first site appears to be in III3. III1–5, III2–5, and III3–5 were digested to identical sized fragments (~27 kDa) in the presence of anastellin (Fig. 8). The digestion at this site apparently produces the 80-kDa fragment in native FN (Fig. 8, second lane, arrow). III1–12, III2–12, and III3–12 were also digested to ~80-kDa fragments in the presence of anastellin (data not shown). No digestion at this site was observed in the absence of anastellin, suggesting that anastellin induces a major conformational change in III3, exposing a cryptic thermolysin cut site. The second anastellin-sensitive site was identified in recombinant III7–14, which was partially digested into bands the size of III7–10 and III12–14 (Fig. 8, last four lanes) in the absence of anastellin. Proteolysis of this site in III11 was substantially increased by binding anastellin.

**Engineered Disulfide Bond in III3 Inhibits Aggregation and Protease Digestion**—The enhanced thermolysin sensitivity of III3 suggested that the unfolding of domain III3 plays a role in aggregation. To test this hypothesis, we generated two disulfide mutants, S795C/S837C and A782C/S859C. S795C/S837C, which locks strand B to strand E within the three-stranded sheet, reduced III1–5 aggregation with anastellin and prevented thermolysin digestion in the presence of anastellin (Fig. 9A) and prevented thermolysin digestion in the presence of anastellin (Fig. 9B). When these experiments were performed under reducing conditions, S795C/S837C behaved just like wild type, confirming
that it is the disulfide bond that alters the properties of III3, not the mutations themselves. Surprisingly, A782C/S859C, which locks strands A and G across the two sheets, did not affect aggregation and thermolysin sensitivity (Fig. 9, A and B), suggesting that this disulfide bond did not stabilize III3. We also found that the thermolysin sensitivity of III3, not stabilize III3. We also found that the thermolysin sensitivity of III3, not stabilize III3, and these bonds may be involved in sFN aggregation. Our analysis identifies the segment III1–3 as the primary site of anastellin binding and aggregation. Approximately three molecules of anastellin bind to III1–3 and produce two effects. There is a conformational change in III3 that exposes a cryptic thermolysin cut site, and there are additional reactions in III1–2 that lead to aggregation.

In addition to the primary sites in III1–3, another molecule of anastellin appears to bind III11 and induce a conformational change that enhances thermolysin digestion in III11. A previous study found that binding of anastellin to FN in a cell culture matrix or FN immobilized on plastic caused the complete loss of an epitope in EDA (the alternatively spliced FN type III domain between III11 and III12), although it did not bind to EDA directly (20). This epitope loss was originally interpreted as remodeling of the matrix. However, our work suggests it may result from a conformational change passed from III11 to the adjacent EDA.

The FN-III domain is a sandwich of two β-sheets, three β-strands (A, B, and E) on one side and four β-strands (C, C', F, and G) on the other (Fig. 2) (29, 30). Anastellin is derived from the first FN-III domain but lacks the N-terminal β-strands A and B. Despite this disruption anastellin is a soluble, nonaggregated protein. When analyzed by NMR (44) anastellin showed few cross-peaks, suggesting a loose structure with rapid dynamics. However, an ordered structure was produced by adding the detergent CHAPS. This NMR structure showed that strand E is somewhat extended, whereas the other half of the β-sandwich (strands C, C', F, and G) forms a sheet virtually identical to that in the native FN-III domain (44). The hydrophobic surface that normally interacts with strands A, B, and E is therefore largely exposed and potentially capable of binding complementary hydrophobic surfaces. Briknarova et al. (44) also made the important point that anastellin has exposed β-sheet edges that are susceptible to amyloid-like association with other β-sheets. Interestingly, our disulfide mutant, S795C/S837C, which stabilized III3, locked strand B to strand E (Fig. 2A).

Protein domains in general spontaneously unfold and refold. For most FN-III and Ig domains the rate of spontaneous unfolding is 10^{-3}–10^{-4} s^{-1} (45–49). Measurements of specific domains from FN have reported even faster rates, 4 × 10^{-3} s^{-1} for III1–2, and 2 × 10^{-2} s^{-1} for III10 and III13 (50), but we will assume the more usual 10^{-3} s^{-1} for this discussion. Briknarova et al. (44) suggested that anastellin may bind FN-III domains following stretch-induced unfolding, but sFN aggregation occurs in solution, where there is no stretching force. We suggest that spontaneous unfolding is the primary mechanism for exposing anastellin-binding sites on FN. Stretching might increase the rate of unfolding if the force is large enough but is not necessary.

The pathway of spontaneous unfolding is not known. The initial step
might be separation of the two halves of the β-sandwich, or it might involve separation of a smaller unit. The engineered disulfide that stabilized III-5, between strands B and E (Fig. 2A), would not prevent separation of the two halves, nor would it block separation of any β-strand from the four-stranded sheet. The domain unfolding may thus begin on the three-stranded sheet. One model is that the pair of strands A and B may lift up and separate from strand E, breaking the hydrogen bonds to E and the hydrophobic contacts to the four-stranded sheet. The disulfide would prevent this by locking strands B and E together. Separation of any pair of strands would expose a large portion of the hydrophobic interface between the two halves and would expose new β-sheet edges. Other models, such as the flipping out of C and E, are also consistent with this disulfide lock.

We propose a three-step model for the formation of sFN aggregates. The first step is the spontaneous unfolding of FN-III domains, which is slow and rate-limiting. As stated above, the key step may be folding out a pair of β-strands, not a complete unfolding. The second step is the binding of anastellin to these unfolded domains, which stabilizes the unfolded conformation. This leads to the third step, the association of the unfolded FN-III domains to form aggregates. We will now elaborate these steps. When an FN-III domain spontaneously unfolds in the presence of a high concentration of anastellin (40 μM or more), anastellin rapidly binds some part of the partially unfolded domain and blocks refolding. This then leaves the remaining part of the domain with exposed hydrophobic patches and β-sheet edges. We propose that aggregation follows when the partially unfolded domains of one molecule of FN (or an FN fragment) binds to unfolded domains from another. Although anastellin may also participate in bridging FN-III domains, its major role may be to stabilize the partially unfolded FN-III domains.

This model could explain the first order kinetics we observed for aggregation. We suggest that 40 μM anastellin is saturating, and above this concentration every time a susceptible FN-III domain spontaneously unfolds; it binds anastellin much more rapidly than it can refold. The rate of aggregate formation is thus determined by the rate of spontaneous domain unfolding. The 500–700-s reaction time we observed is very similar to the 1,000-s time for spontaneous unfolding.

As described so far the anastellin binding might involve nonspecific binding of hydrophobic patches. However, we have found that anastellin binds specifically to III-1–3 and III-11. Furthermore, other truncations of FN-III domains similar to that of anastellin do not have an anastellin-like activity (16, 21). There is thus a substantial specificity to the binding of anastellin. This specificity is likely to involve both a steric complementarity of the hydrophobic surfaces and interactions between
β-strands. The extended E strand of anastellin is a likely candidate for specific binding because mutations there eliminated the aggregation activity (44).

An essential feature of aggregation is that each III1–3-anastellin complex must have at least three sites for binding other complexes. If there was only one site, the association would be limited to dimers, and two sites would produce only linear chains of molecules. The binding sites are presumably the unfolded parts of FN-III domains and anastellin, but it is premature to speculate on what they are and what the binding partners could be.

This mechanism can also be cast in terms of a domain swapping model (see Ref. 51 for review). Domain swapping in FN-III domains was originally suggested by Litvinovich et al. (52) to explain the ability of the isolated III9 domain to partially denature and then reassemble into amyloid fibrils. The relationship of domain swapping, amyloid formation, and sFN was also suggested and discussed by Briknarova et al. (44).

Anastellin is an artificially constructed fragment and probably does not exist in vivo; it is therefore unlikely to be involved in FN fibril assembly. Nevertheless, we believe that native matrix assembly may use a mechanism that is related to the aggregation of sFN. The interaction between unfolded and folded domains or two unfolded domains occurs rarely in dilute solution, but it may be enhanced by concentrating the FN molecules on the cell surface. Therefore, domain swapping between FN-III domains may play a key role in matrix assembly.

In addition to domain swapping, we would like to address the possibility of a tandem β-zipper interaction between 11–9 and unfolded FN-III domains during FN matrix assembly. The tandem β-zipper interaction was originally reported in the NMR structure of 1-2 bound to a peptide from a bacterial adhesin (53). In this structure, the bacterial peptide bound to 1-2 by forming β-strands that extended the β-sheet of FN-1 domains. A related mechanism may be involved in the binding of II1–2 to 11–9. Thus, when FN-III domains spontaneously unfold on the cell surface, where there is a high local concentration, unfolded domains may form isolated β-strands that can interact with 11–9 before refolding themselves. A β-zipper interaction between unfolded FN-III domains and 11–9 is an intriguing candidate for a mechanism of FN matrix assembly.

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REFERENCES

1. Hynes, R. O. (1990) Fibronectin, Springer-Verlag, New York
2. George, E. L., Georges-Labouesse, E. N., Patel-King, R. S., Rayburn, H., and Hynes, R. O. (1995) Development 119, 1079–1091
3. Sakai, T., Johnson, K. J., Murozono, M., Sakai, K., Magnuson, M. A., Wieloch, T., Cronberg, T., Ishikawa, A., Erickson, H. P., and Fassler, R. (2001) Nat. Med. 7, 324–330
4. Murozono, M., Sakai, K., Kojima, S., Matsumoto, K., Lin, D. C., Lin, S., Hahn, C., and Yamada, K. M. (2000) J. Cell Biol. 148, 149–160
5. Mosher, D. F. (1993) Curr. Opin. Struct. Biol. 3, 214–222
6. Wu, C. Y., Keightley, S. Y., Leung-Hagesteijn, C., Radeva, G., Coppolino, M., Goicoechea, S., McDonald, J. A., and Zardi, L. (1996) J. Cell Biol. 134, 155–164
7. Briknarova, K., Akerman, M. E., Hoyer, D. W., Ruoslahti, E., and Ely, K. R. (2003) J. Mol. Biol. 332, 205–215
8. Plaxco, K. W., Spitzfaden, C., Campbell, I. D., and Dobson, C. M. (1997) J. Mol. Biol. 270, 763–770
9. Clarke, J., Hamill, S. A., and Johnson, C. M. (1997) J. Mol. Biol. 270, 711–717
10. Carrion-Vazquez, M., Oberhauser, A. F., Fowler, S. B., Marx, P. E., Broedel, S. E., Clarke, J., and Fernandez, J. M. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3694–3699
11. Fong, S., Hamill, S. A., Proctor, M., Freund, S. M., Benian, G. M., Chothia, C., Bycroft, M., and Clarke, J. (1996) J. Mol. Biol. 264, 624–639
12. Briknarova, K., Akerman, M. E., Hoyer, D. W., Ruoslahti, E., and Ely, K. R. (2003) J. Mol. Biol. 332, 205–215
13. Plaxco, K. W., Spitzfaden, C., Campbell, I. D., and Dobson, C. M. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 10703–10706
14. Robinson-Bost, J., Orend, G., and Ruoslahti, E. (1998) J. Cell Sci. 111, 1463–1473
15. Ichihara-Tanaka, K., Maeda, T., Titani, K., and Sekiguchi, K. (1992) J. Biol. Chem. 267, 15473–15479
16. Mercuri, K. O., and Morla, A. O. (2001) BMC Cell Biol. 2, 18
17. McKeown-Longo, P. J., and Mosher, D. F. (1984) J. Biol. Chem. 259, 6595–6601
18. Peters, D. M. P., Chen, Y., Zardi, L., and Brummel, S. (1998) Microscopy Microanalysis 4, 385–396
19. McKeown-Longo, P. J., and Mosher, D. F. (1984) J. Biol. Chem. 259, 12210–12215
20. Eijj, O. S., Blunn, G. W., and Brown, R. A. (1993) Biomaterials 14, 743–748
21. Briknarova, K., Akerman, M. E., Hoyer, D. W., Ruoslahti, E., and Ely, K. R. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 8620–6244
22. Wei, M., and Ruoslahti, E. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 14351–14356
23. Vantie, T. (1986) J. Biol. Chem. 261, 9433–9437
24. Vartio, T. (1986) J. Biol. Chem. 261, 987–991
25. Pankov, R., Cukierman, E., Katz, B. Z., Matsumoto, K., Lin, D. C., Lin, S., Hahn, C., and Yamada, K. M. (2000) J. Cell Biol. 148, 1075–1090
26. Sechler, J. L., Rao, H., Cunisly, A. M., Vega-Colon, I., Smith, M. S., Murata, T., and Schwarzbauer, J. E. (2001) J. Cell Biol. 154, 1081–1088
27. Schwarz-Linek, U., Werner, J. M., Pickford, A. R., Gurusiddappa, S., Kim, J. H., Pilka, E. S., Briggs, J. A., Gough, T. S., Hook, M., Campbell, I. D., and Potts, J. R. (2003) Nature 423, 177–181