FOR THE RECORD

Conformational flexibility and crystallization of tandemly linked type III modules of human fibronectin

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Abstract: Fibronectin is a large cell adhesion molecule that is composed of several functional domains. The cell-binding domain that binds to cell surface integrins consists of repeated homologous type III modules. In this study, recombinant fragments from the cell-binding domain of human fibronectin that participate in a newly characterized fibronectin–fibronectin interaction with FNIII₁ were crystallized. In each case, the crystals had more than one fibronectin fragment in the asymmetric unit. Crystals of FNIII_{10–11} grew in the space group *C*2 with *a* = 117.1 Å, *b* = 38.6 Å, *c* = 80.6 Å, β = 97.2°, and two molecules in the asymmetric unit. These crystals diffracted to 2.5 Å resolution. Fragment FNIII_{8–11} and a shorter fragment, FNIII_{8–10}, crystallized in hexagonal space groups with large unit cells and two to four molecules per asymmetric unit. Even very large crystals of these fragments did not diffract beyond 4 Å. The crystal packing for this collection of fibronectin fragments suggests conformational flexibility between linked type III modules. The functional relevance of this flexibility for elongated versus compact models of the cell-binding domain of fibronectin is discussed.

Keywords: cell adhesion; conformational flexibility; crystallization; fibronectin; integrins

Fibronectin, a common constituent of normal basal and connective tissue, plays a crucial role in cell adhesion, cell migration, cytoskeletal organization, thrombosis, embryogenic differentiation, wound repair, and phagocytosis. This versatile molecule is a dimeric glycoprotein, consisting of two 250-kDa monomers joined by a disulfide bond at the C terminus. Each polypeptide is composed of homologous repeats of 40–90 amino acids, each of which can be classified into one of three types of structural unit referred

to as type I, II, and III modules (Ruoslahti, 1988; Hynes, 1990). Fibronectin exerts its action through interactions with cells and other extracellular matrix molecules. Cell surface integrin receptors bind an Arg-Gly-Asp (RGD) sequence localized in the tenth type III module (Pierschbacher & Ruoslahti, 1984). The type III consensus sequence (FNIII) has been identified in many different proteins in species ranging from bacteria to humans (Doolittle & Bork, 1993), and is found in intracellular and extracellular proteins (Bork & Doolittle, 1992) as well as in a variety of membrane cytokine receptors, such as growth hormone receptors and adhesion molecules (Patthy, 1990). The tertiary structures of a number of FNIII modules have revealed a common molecular topology (DeVos et al., 1992; Leahy et al., 1992, 1996; Main et al., 1992; Dickinson et al., 1994; Huber et al., 1994): seven β strands that form a sandwich of two antiparallel β sheets, one containing three strands and the other four, enclosing a core of highly conserved hydrophobic residues. Although the RGD sequence in FNIII₁₀ has been identified as a key adhesive motif (Pierschbacher & Ruoslahti, 1984), other sites in the molecule are also necessary for full adhesive activity (Obara et al., 1988; Bowditch et al., 1994). Two distinct peptide regions in the eighth and ninth type III modules have been proposed as synergistic sites. Receptor binding of fibronectin may depend not only on the conformation and the accessibility of the RGD segment, but also on the relative orientation of secondary sites on other type III modules. Thus, the way in which strings of type III modules are joined together is of considerable interest.

The conformational arrangement of fibronectin modules is also important for self-assembly and matrix formation. Several regions of fibronectin have been implicated in the assembly process, including amino-terminal 26-kDa or 70-kDa domains (McKeown-Longo & Mosher, 1985; Peters & Mosher, 1987; Quade & McDonald, 1988; Schwarzbauer, 1991). The first type III module (FNIII₁) has been shown previously to play an important role in matrix assembly (Morla & Ruoslahti, 1992; Mosher et al., 1992). More recently, a new fibronectin–fibronectin binding site has been identified (Morla et al., 1994). Fibronectin exhibits enhanced ad-

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hesive properties when incubated with a fragment of the first type III module. This fragment (FNIII_{1-C}) binds to a site contained in the FNIII₁₀₋₁₁ modules (A. Morla et al., in prep.). The binding site may be composed of components from both of the tandemly linked modules, given that fragment FNIII_{1-C} does not bind to fibronectin fragments lacking module III₁₁. As a first step to understand the structural features and domain association in this new fibronectin self-association site, we crystallized multimodule fragments that contain the tandemly linked FNIII₁₀ and FNIII₁₁ modules and bind to FNIII_{1-C}. The goal is to provide new information of the three-dimensional organization of fibronectin. In this article we report the crystallization of FNIII₁₀₋₁₁ and FNIII₈₋₁₁.

Results: Characterization of purified fibronectin fragments: FNIII₁₀₋₁₁ and FNIII₈₋₁₁ fragments were purified to apparent homogeneity as evaluated by PAGE and isoelectric focusing. For comparison, another fragment lacking module III₁₁ (FNIII₈₋₁₀) was also purified for crystallization. The fibronectin fragments were further characterized by dynamic light scattering (DLS) to determine if soluble aggregates were present. Samples of each of the fibronectin fragments were monodisperse and therefore were good candidates for crystallization (Ferre-D'Amare & Burley, 1994).

Crystallization and data collection: FNIII₁₀₋₁₁ formed needle crystals readily at 4 °C or 22 °C when polyethyleneglycol (PEG) was used as the precipitant. However, the morphology of these crystals could not be improved. Finally, ammonium phosphate was found to be more promising as a precipitant for the crystallization of this fragment. Phosphate seemed to be the critical element because the fragment also crystallized with other phosphate salts. The addition of organic anions improved the quality of the crystals at concentrations below 0.2 M. However, the FNIII₁₀₋₁₁ crystals always grew as thin stacked plates and large single crystals were extremely difficult to obtain. Using 2.0 M ammonium phosphate, pH 4.6, and 0.2 M sodium propionate, a single crystal of the FNIII₁₀₋₁₁ was obtained by cutting the protruding portion of one large crystal from the stack. This crystal diffracted to high resolution. The space group and cell parameters were C2 with $a = 117.1$ Å, $b = 38.6$ Å, $c = 80.6$ Å, and $\beta = 97.2^\circ$, and two FNIII₁₀₋₁₁ molecules in the asymmetric unit ($V_m = 2.37$ Å³/Da). A full intensity data set was collected that is 80% complete to 2.5 Å resolution (Table 1).

For FNIII₈₋₁₁, one set of conditions from the initial screen formed small needles. The first crystals were very sensitive to pH, growing from small needles at pH 6.0 and gradually forming individual rods with increasing pH up to 8.0. These conditions were optimized to provide reproducible crystals suitable for X-ray diffraction analysis. Long hexagonal individual rods were obtained at pH 7.5 in Tris-HCl solutions with 1.2 M ammonium sulfate as precipitant in the presence of 10% glycerol at 22 °C. The time required for small crystals to appear was five days, with full growth occurring in about two weeks. PEG 4000 could be substituted for glycerol as the additive, forming the same rod-shaped crystals. Crystals formed in the space group $P6_122$ or $P6_522$, with unit cell parameters $a = b = 170.8$ Å and $c = 170.2$ Å and diffracted to 4 Å resolution. The volume of the unit cell suggested that there are four molecules per asymmetric unit with a solvent content of 46% with $V_m = 2.30$ Å³/Da.

Another crystal form of FNIII₈₋₁₁ was produced from a solution of 0.1 M MES, pH 6.5, and 1.6 M magnesium sulfate. Under these conditions, the fragment also formed hexagonal rods. The cell parameters for these crystals in a P hexagonal lattice were $a = b = 164.0$ Å and $c = 119.9$ Å. There are two FNIII₈₋₁₁ molecules per asymmetric unit ($V_m = 2.99$ Å³/Da). Although these crystals diffracted to 3.5 Å, the mosaicity was significant.

The FNIII₈₋₁₀ fragment formed an amorphous precipitate or showers of microcrystals with one of the crystal screen conditions. Refinement of these conditions produced small crystals after 7–10 days. The best crystals were produced at 22 °C at pH 5.5 from a 100 mM sodium acetate buffer with 10% PEG 200 as precipitant and 30 mM cadmium chloride. The concentration of cadmium chloride was critical to obtain single, well-formed crystals. Large crystals were obtained in 3 weeks as hexagonal diamonds and formed in the space group $P6_122$ or $P6_522$ with $a = b = 120.1$ Å and $c = 138.9$ Å. The crystals had two molecules per asymmetric unit with a solvent content of 49% ($V_m = 2.41$ Å³/Da) and diffracted only to 7 Å.

Discussion: Crystals of the FNIII₁₀₋₁₁ fragment are suitable for high-resolution studies. Numerous probe models have been tested in rotation function searches based on the atomic model (Dickinson et al., 1994) of FNIII₁₀ using the single module, as well as two domain models generated by linking one FNIII₁₀ model to a second module with the side chains of FNIII₁₁ substituted on the

Table 1. Summary of data collection statistics

Minimum resolution (Å)	Average intensity (I)	Average $I/\sigma(I)$	No. observations	No. reflections	R_{sym} ^a
6.03	4,780	13.7	662	968	0.048
4.79	5,183	13.1	658	906	0.062
4.18	5,381	12.5	690	912	0.069
3.80	4,250	10.5	702	906	0.085
3.53	3,162	9.2	697	896	0.107
3.32	2,503	8.2	713	911	0.116
3.15	1,933	6.9	709	886	0.135
3.02	1,345	5.4	740	909	0.164
2.90	987	4.6	708	873	0.223
2.80	765	2.4	718	874	0.261
Totals	2,983	9.4	6,997	9,041	0.091

^a $R_{sym} = \sum |I_h - \langle I \rangle| / \sum I_h$, where I_h is the intensity of an individual measurement and $\langle I \rangle$ is the mean value of its equivalent reflections.

FNIII₁₀ framework (R. Kodandapani et al., unpubl. results). Even when the search was performed with the linked model and the angle between the modules was varied widely, no distinct solution was apparent. Similarly, in studies of the FNIII₇₋₁₀ fragment (Leahy et al., 1996), attempts to use molecular replacement (MR) methods to solve the structure were not successful. It will probably be necessary to produce heavy-atom derivatives and to use the multiple isomorphous replacement method to calculate phases for the FNIII₁₀₋₁₁ fragment.

There are two molecules of FNIII₁₀₋₁₁ in the asymmetric unit, i.e., four type III modules. The fact that the rotation function searches were not straightforward suggests that the conformations of the two FNIII₁₀₋₁₁ molecules in the crystal are dissimilar and that the differences between the two fragments are probably significant, obscuring a clear MR solution. Modules in FNIII₇₋₁₀ are related by rotation angles varying from 43–112°, as well as by variable tilt angles (Leahy et al., 1996). It is possible that the two copies of the FNIII₁₀₋₁₁ molecule in the crystal differ in the torsional relationship between modules. This conformational flexibility could have important implications for self-assembly interactions with FNIII_{1-C} (A. Morla et al., in prep.). It has been suggested that fibronectin self-association may be influenced by integrin binding (Aguirre et al., 1994) and that conformational changes in fibronectin may disrupt or introduce intramolecular contacts (Litvinovich & Ingham, 1995). Such conformational adjustments could be mediated by changes in the torsional relationships between linked modules, possibly affecting FNIII_{1-C} binding. One of the two conformations in the FNIII₁₀₋₁₁ crystals could represent the conformation that makes the FNIII_{1-C} site available.

It is quite likely that the molecular flexibility of fibronectin affected the crystallization of the longer fragments in this study. For the three- and four-module fibronectin fragments, the crystals diffracted poorly and the limit of diffraction could not be improved significantly even when very large crystals (0.3 × 1.0 mm) were produced. A similar situation was reported for the crystallization of the molecular chaperone SecB (Vrielink et al., 1995). These authors used DLS to evaluate the molecular aggregation state of the SecB tetramer, and the results of the DLS analyses were consistent with the observation that SecB protein failed to form ordered crystals. However, in the case of the FNIII fragments, the samples were each monodisperse in solution prior to crystallization. The monodispersity of a protein solution can be used as an indicator to predict that a protein is likely to crystallize (Ferre-D'Amare & Burley, 1994). Apparently it is not a tendency to aggregate that prevents the longer FNIII fragments from forming highly ordered crystals.

Other multimodule FNIII fragments, FNIII₇₋₁₀ (Leahy et al., 1996), FNIII₁₂₋₁₄ (Pechik et al., 1993), or FNIII₁₂₋₁₅ (Pechik et al., 1993) crystallized with one molecule in the asymmetric unit. Similarly, the two-module neuroglyan (Huber et al., 1994) crystallizes as a single molecule in the asymmetric unit. In the neuroglyan structure, a cation binding site was located at the interface between modules and coordinated to side chains from each of the FNIII modules (Huber et al., 1994). The cation may stabilize the orientation between the modules. The addition of cations had no obvious effect on the quality of the FNIII₁₀₋₁₁ or FNIII₈₋₁₁ crystals in our study. Although the concentration of cadmium was critical for the growth of large crystals of FNIII₈₋₁₀, the addition of this cation to the crystallization mixtures did not stabilize the protein to a single conformation.

Three fragments of fibronectin have been crystallized in this study and in each case, the multimodule polypeptides assume more than one conformation in the crystal lattice. The presence of more than one molecule in the repeating unit in a crystal lattice indicates that there are conformational differences between the molecules in a single crystal. These differences can exist at the level of side-chain conformations or, in the case of multimodule fragments, may result from hinge flexibility relating linked modules. For example, the two domain fragment of the T-cell surface lymphocyte antigen CD2 crystallized with two molecules in the asymmetric unit (Jones et al., 1992). The relationship of modules in the two independent copies of CD2 differed by a hinge-bending of 7°, with an 18° rotation.

Electron microscopy studies have shown fibronectin as a long, thin flexible strand (Engel et al., 1981; Erickson et al., 1981). To explain these observations, based on the structure of the two-module neuroglyan fragment, a model was proposed for tandem FNIII repeats in intact neuroglyan and extracellular matrix proteins containing these repeats (Huber et al., 1994). This model, as well as another model based on the crystal structure of a tenascin FNIII module (Leahy et al., 1992), suggested a straight rod with "zig-zag repeats" of the type III modules. The crystal structure of FNIII₇₋₁₀ did not confirm the "zig-zag repeat," but the molecule was extended and rod-like (Leahy et al., 1996). This is one possible conformation for this region of fibronectin, repeated regularly in the crystal lattice. But other conformations of this fragment may be possible, as with other multimodule proteins. A variety of angles and rotation axes relate sequentially linked domains in T-cell surface molecules, such as CD2 (Jones et al., 1992) or CD4 (Ryu et al., 1994), and also in intact antibodies (Guddat et al., 1993). The flexible polypeptide linkage between Ig modules in antibody molecules permits a range of motion between domains and this flexibility relates directly to functional activity. It has been noted that FNIII and Ig modules have a similar overall structure (Main et al., 1992; Ely et al., 1995). It follows that the linkage patterns of FNIII and Ig modules may also be similar.

Conformational flexibility and variable linking orientations between linked modules (suggested by our fibronectin crystals) are consistent with another model proposing a globular arrangement of type III modules in fibronectin (Litvinovich & Ingham, 1995). In this compact model, based on results of scanning calorimetry and fluorescence spectroscopy, stabilizing interactions were proposed between modules III₇ and III₁₀ and between modules III₇ and III₉, suggesting that these pairs of modules interact to provide thermal stability to the cell-binding domain. It is possible that molecular flexibility in fibronectin permits a "family" of molecular shapes that may be functionally relevant for matrix interactions or receptor recognition. Verification of the existence of these molecular shapes awaits structural analyses of fibronectin complexes with other matrix proteins or integrins.

Materials and methods: *Protein expression:* Recombinant fibronectin fragments FNIII₁₀₋₁₁, FNIII₈₋₁₁, and FNIII₈₋₁₀ [corresponding to residues 1423–1691, 1240–1691, and 1240–1509 (without the EDA modules) in the human fibronectin sequence (Kornblihtt et al., 1985)] were cloned as fusion proteins fused to glutathione-S-transferase (GST) (pGEX-2T vector; Pharmacia). This expression system introduces a glycine-serine sequence at the amino-terminus of each fragment after removal of GST. Fragments were expressed in *Escherichia coli* strain XL1 blue harboring the fusion plasmids. Overnight cultures were used to inoculate 7 L of LB

media (Sambrook et al., 1989) with ampicillin 100 $\mu\text{g}/\text{mL}$ (LBA media) at a cell density of 0.05–0.1 at 600 nm. After overnight growth, 3 L of LBA medium were added before induction with 0.4 mM IPTG. After 3–5 h at 30 °C, cells were pelleted by centrifugation at $5,000 \times g$, 4 °C for 20 min and resuspended in 50 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 2 mM EDTA, 0.01% NaN_3 , and 1–5 $\mu\text{g}/\text{mL}$ protease inhibitors leupeptin and aprotinin. Cells were lysed by the addition of 0.1 mg/mL of lysozyme and then 2,000 units of DNAase I, 8 mM MgCl_2 , and 1.2 mg RNase A were added. Lysis proceeded for 1 h at 4 °C, then the lysate was centrifuged at 4 °C, $27,000 \times g$ for 1 h. Lysates were passed through a 21-gauge needle before centrifugation. Soluble fractions were cleared of cell debris and stored at –80 °C.

Protein purification: To purify the GST-fusion proteins, the soluble lysates were passed over a glutathione-agarose (Sigma) column that had been pre-equilibrated with 50 mM Tris-HCl, pH 8.0, 0.1 M NaCl, 0.01% NaN_3 ; after a wash with the same buffer, the fusion protein was eluted with 5 mM reduced glutathione. The fibronectin fragments were released from the GST fusion partner by thrombin digestion (~1–4 units/mg protein) overnight at room temperature for FNIII_{8–11} or FNIII_{8–10}, or at 37 °C for FNIII_{10–11}. The digest was stopped by the addition of 1 mM PMSF.

The FNIII_{8–11} fragment was separated from GST by anion-exchange chromatography at pH 7.5 (Q-Sepharose fast flow, Pharmacia) with a gradient of 0.1–0.5 M NaCl in 20 mM Tris-HCl, 0.01% NaN_3 . FNIII_{8–11} eluted at 0.25 M NaCl and was concentrated by precipitation with 40% saturated ammonium sulfate before molecular sizing on a Sephacryl S-100 HR (Pharmacia, Inc.) gel filtration matrix at pH 7.4 with 20 mM Tris-HCl, 0.25 M NaCl. The yield of purified FNIII_{8–11} was 15–20 mg of protein/L of bacterial culture. This protein was dialyzed at pH 7.5 in 20 mM Tris-HCl, 0.01% NaN_3 , and concentrated by ultrafiltration with Centrprep or Centricon devices (Amicon) to 20 mg/mL for crystallization trials.

FNIII_{8–10} fragment was separated from GST by the same anion exchange chromatography used for FNIII_{8–11}. FNIII_{8–10} eluted at 0.3 M NaCl. FNIII_{8–10} was finally purified to homogeneity by affinity purification with the glutathione-agarose column. The yield of purified FNIII_{8–10} was 18–20 mg of protein/L of bacterial culture. The purified protein was dialyzed against 10 mM Tris-HCl, pH 7.5, 0.01% NaN_3 and concentrated to 20 mg/mL prior to crystallization.

For FNIII_{10–11}, after thrombin digestion, GST and the uncleaved fusion protein were removed from the fibronectin fragment by passing the digest over a second glutathione-agarose column. Fractions containing FNIII_{10–11} were pooled, concentrated by precipitation with ammonium sulfate (75% saturation), and dialyzed against 25 mM histidine-HCl, pH 6.2, overnight before further purification on a PBE94 chromatofocusing column (Pharmacia, Inc.). Purification on the PBE94 column was conducted in a pH range of 6.2–4.0 using 25 mM histidine-HCl buffer, pH 6.2, and Polybuffer 74, pH 4.0 (Pharmacia). FNIII_{10–11} eluted at pH 5.0 and pooled fractions were then concentrated by ultrafiltration. Final purification to homogeneity was accomplished by gel filtration on a Sephacryl S-100HR (Pharmacia, Inc.) column in PBS buffer pH 7.4. The final yield of purified FNIII_{10–11} protein was 7–8 mg/L bacterial culture. The FNIII_{10–11} fragment was dialyzed at pH 7.5 against 5 mM Tris-HCl, 0.02% NaN_3 and concentrated to 15–20 mg/mL before crystallization.

DLS: Prior to initiating crystallization trials, FNIII samples were characterized by DLS to evaluate the solution properties and aggregation state of the proteins. Samples were tested in a Dynapro-801 Molecular Sizing Instrument (Protein Solutions, Inc. Charlottesville, Virginia).

Crystallization: Crystallization conditions were initially obtained using a sparse-matrix trial screen (Jancarik & Kim, 1991) (Crystal Screen™ kits, Hampton Research). The crystallization trials were performed at both 4 °C and 22 °C.

Fibronectin fragments were tested for crystallization in hanging drops by the vapor diffusion technique (McPherson, 1982). A volume of 1 mL of buffered reservoir solution was set in each well of a 24-well Linbro tissue culture plate (Flow Laboratory). A 2- μL volume of reservoir solution was mixed with a 2- μL drop of protein solution on a siliconized cover glass. The cover glass with the droplet was inverted and placed over the reservoir solution and the chamber was sealed with vacuum grease.

Data collection: For FNIII_{10–11} crystals, intensity data were collected at 4 °C using $\text{CuK}\alpha$ X rays from a Rigaku FR generator operating at 50 kV and 100 mA using a fine focus beam (0.2 $\times$ 0.2 mm) and a MAR image plate detector (University of California, San Diego). The cell parameters and space group were determined using the DENZO program (Otwinowski, 1993). Intensity data for FNIII_{8–11} or FNIII_{8–10} crystals were collected using a MAR image plate detector with $\text{CuK}\alpha$ X-rays from a Siemens rotating anode X-ray generator operating at 45 kV and 80 mA (Area Detector Systems Corp., Poway, California). Data were collected at room temperature. Data reduction was executed with DENZO software (Otwinowski, 1993) and space group and cell dimensions were determined with autoindexing software (LePage, 1982) and DENZO (Otwinowski, 1993).

Flash freezing of FNIII_{8–11} crystal: After soaking a FNIII_{8–11} crystal for 30 min in 40% PEG 200 as cryoprotectant, the crystal was mounted in a loop and was flash frozen with a nitrogen stream. X-ray data were collected at –140 °C using a cryocooling device and a liquid nitrogen-cooled gas stream (Molecular Structure, Corp.).

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