Conformational Changes in the Fibronectin Binding MSCRAMMs Are Induced by Ligand Binding*

Karen House-Pompeo‡§, Yun Xu‡, Danny Joh‡, Pietro Speziale¶, and Magnus Höök‡

From the ‡Center for Extracellular Matrix Biology, Institute of Biosciences and Technology and Department of Biochemistry and Biophysics, Texas A & M University, Houston, Texas 77030 and the ¶University of Pavia, Department of Biochemistry, 27100 Pavia, Italy

Bacterial adherence to host tissue involves specific microbial surface adhesins of which a subfamily termed microbial surface components recognizing adhesive matrix molecules (MSCRAMMs) specifically recognize extracellular matrix components. We now report on the biophysical characterization of recombinant fibronectin binding MSCRAMMs originating from several different species of Gram-positive bacteria. The far-UV CD spectra (190–250 nm) of recombinant forms of the ligand binding domain of the MSCRAMMs, in a phosphate-buffered saline solution at neutral pH, were characteristic of a protein containing little or no regular secondary structure. The intrinsic viscosity of this domain was found to be the same in the presence or absence of 6 M guanidine hydrochloride, indicating that the native and denatured conformations are indistinguishable. On addition of fibronectin NH_2 terminus as ligand to the recombinant adhesin there is a large change in the resulting far-UV CD difference spectra. At a 4.9 M excess of the NH_2 terminus the difference spectra shifted to what was predominately a β-sheet conformation, as judged by comparison with model far-UV CD spectra. The fibronectin NH_2-terminal domain undergoes a minute but reproducible blue-shift of its intrinsic tryptophan fluorescence on addition of rFNBD-A, which contains no tryptophan residues. Since this result indicates that there is no large change in the environment of the tryptophan residues of the NH_2 terminus on binding, the large shift in secondary structure observed by CD analysis is attributed to induction of a predominately β-sheet secondary structure in the adhesin on binding to fibronectin NH_2 terminus.

Many pathogenic bacteria have been shown to specifically recognize and bind to various components of the extracellular matrix in an interaction which appears to represent a host tissue colonization mechanism. This adherence involves a group of bacterial proteins termed MSCRAMMs[1] (microbial surface components recognizing adhesive matrix molecules) (1, 2). A number of Gram-positive bacteria have been shown to express fibronectin (Fn) binding MSCRAMMs, and in some cases these proteins have been isolated and the corresponding genes cloned and characterized. The primary Fn binding sites in these MSCRAMMs have been localized to domains present in most Fn binding adhesins. This domain is composed of a unit of 37–48 amino acids, repeated three or four times (Fig. 1A).

The repeat regions have been overexpressed as recombinant fusion proteins in *Escherichia coli* where the recombinant Fn binding domains (rFNBD) are linked to a stretch of histidine residues which are utilized for affinity purification of the rFNBD proteins. These proteins have been designated as rFNBD-D, rFNBD-A, rFNBD-B, and rFNBD-P, respectively (Fig. 1A). The rFNBDs were found to exhibit similar binding kinetics and dissociation constants; for example, the dissociation constants of the four recombinant proteins binding to porcine Fn was determined by biosensor analysis to be in the low nM range with the dominant dissociation rates varying between 1×10^{-4} and 6×10^{-4} s^{-1}. Additionally, the recombinant proteins have been shown to have cross-species specificity and inhibit binding of Fn to many different bacterial cells (3).

The repeated units of the fibronectin binding domains of the different MSCRAMMs are strikingly similar (Fig. 1B) and appear to contain a consensus sequence (4). The repeat units have a high number of acidic residues, and there are conserved hydrophobic and acidic residues at certain positions. Overall there is a high degree of sequence similarity between repeated units in a specific MSCRAMM as well as between MSCRAMMs from different species. Our laboratory has determined that synthetic peptides, analogous to sequences shown in Fig. 1B, also bind Fn, and by amino acid substitution in these peptides it was determined that all conserved residues are not needed for Fn binding (4).

Fn is a disulfide-linked dimeric glycoprotein that is found in a soluble form in body fluids and a fibrillar form in the extracellular matrix. The primary biological function of Fn appears to be related to its ability to serve as a substratum for the adhesion of animal cells. This adhesion is mediated by a family of dimeric receptors which recognize and bind to specific sites in the central part of Fn. The primary binding site in Fn for MSCRAMMs from Gram-positive bacteria has been localized to the Fn NH_2-terminal domain (N29) (5, 6). This domain is composed of five type I modules (FI) which are about 45 amino acids in length. The structure of N29 is a series of anti-parallel β-sheets stabilized by several disulfide bonds interspersed at regular intervals in the sequence (7, 8).

Our laboratory is interested in the study of adhesin/host

§ To whom correspondence should be addressed. Tel.: 713-677-7557; Fax: 713-677-7576.

[1] The abbreviations used are: MSCRAMM, microbial surface components recognizing adhesive matrix molecules; Fn, Fibronectin; N29, fibronectin NH_2-terminal fragment; N31, NH_2-terminal tryptic digest fragment of Fn; rFNBD, recombinant fibronectin binding domain; FI, fibronectin type I repeat; PBS, phosphate-buffered saline; CD, circular dichroism; GdnHCl, guanidine hydrochloride; ANS, 8-anilino-1-naph- thalenesulfonic acid; LIBS, ligand-induced binding site; bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2]-(hydroxymethyl)-propane-1,3-diol.

interactions with the ultimate goal to develop agents capable of blocking adherence of bacteria to the host as potential antibacterial therapeutics. The present study was initiated to gain insight into how the MSCRAMM interacts with fibronectin N29 at the molecular level. The conformational state of the recombinant ligand binding domain before and after N29 binding was explored by biophysical means.

EXPERIMENTAL PROCEDURES

Recombinant Fibronectin Binding Domain Proteins—rFNBD proteins were expressed and purified according to procedures outlined in Joh *et al.* (3) and Speziale *et al.* (9).

Isolation and Purification of the Fibronectin NH₂-terminal Fragment—Bovine plasma (with EDTA as an anticoagulating agent) was purchased from Pel-Freez Biologicals (Rogers, AR) or was a generous gift from Dr. Albert Guidry (Milk Secretions and Mastitis Lab, USDA-ARS, Beltsville, MD). The plasma was passed through a gelatin-Sepharose column and the fibronectin was eluted with 2.5 M urea in 0.5 mM EDTA and PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂HPO₄, 1.4 mM KH₂PO₄, pH 7.4) (10). N29 was generated and purified essentially as described by McGavin *et al.* (11). After dialysis into digest buffer (25 mM Tris-HCl, 150 mM sodium chloride, 2.5 mM calcium chloride, 0.5 mM EDTA, pH 7.6) the fibronectin (~1 mg/ml) was digested with 5 μg/ml thermolysin for each milligram of fibronectin. After 2 h at room temperature with end-over-end mixing, the digestion was stopped by addition of EDTA to 5 mM. The N29 fragment was then affinity purified on a column of rFNBD-B coupled to CNBr-activated Sepharose 4B (Pharmacia LKB, Uppsala Sweden). Guanidine hydrochloride (1.5 M) in digest buffer was applied to elute the bound protein. The eluate was dialyzed against 10 mM bis-Tris (pH 6.5), 150 mM NaCl. After this purification step, two proteins were present by SDS-polyacrylamide gel electrophoresis analysis, the desired N29 fragment and an additional 80-kDa fragment. A Q-Sepharose column was used to further purify the N29. The Q-Sepharose non-binding fraction, which contained N29, was concentrated to ~1 mg/ml and dialyzed into PBS with a Micro-ProDi-Con Dialysis/Concentration system (Spectrum, Houston, TX). The molecular mass of N29 (26,000 Da) was determined by electrospray mass spectrometry by the Analytical Chemistry Center at the University of Texas Medical School at Houston as described previously (3).

Concentration Determination of Proteins—The concentration of recombinant proteins and N29 was determined by amino acid analysis, the modified Lowry method (Sigma), or spectrophotometrically using extinction coefficients calculated by the method of Gill and Hippel (12). For the proteins that contained tyrosine or tryptophan residues the spectrophotometric method was assumed to be the most accurate. For proteins that did not contain these residues, concentration values obtained from amino acid analysis were taken as the most accurate and a standard correction factor (0.57) was determined by multiple comparison of amino acid analysis and the modified Lowry method. The dye assay was then used routinely to determine the concentrations of these proteins and the result was multiplied by the appropriate correction factor. The concentration of bovine Fn was determined spectrophotometrically ($\epsilon_{280} = 581,818$ cm^{-1} M^{-1}).

Circular Dichroism (CD)—The secondary structure of selected proteins was monitored by CD spectroscopy on a Jasco J720 spectropolarimeter calibrated with a 0.06% (w/v) 10-*d*-camphorsulfonic acid ammonium salt solution. CD spectra were measured at room temperature (24 ± 2 °C) in a 0.2-mm path length quartz cell. To monitor changes in the far-UV CD with variable temperature, samples were placed in a water-jacketed 1-cm quartz cell. The sample temperature was monitored at the exit port of the water jacket and controlled electronically. All far-UV (250 to 190 nm) spectra were acquired with a time constant of 1 s, a scan rate of 20 nm/min, and 4 scans accumulated and then averaged. The molar ellipticity ([Θ]) was expressed in deg·cm²/dmol. For rFNBD/N29 binding experiments the spectrum of the corresponding concentration of N29 in isolation was subtracted before [Θ] for the rFNBD was calculated. As controls, the far-UV CD of N29 or rFNBD-A in the presence and absence of an equimolar concentration of bovine serum albumin were examined as well as the far-UV CD of a 2-fold molar excess of N29 in the presence of a control protein; a 17-kDa recombinant form of the *S. aureus* collagen binding MSCRAMM which appears to lack a significant amount of secondary structure (28).

Gel Permeation Chromatography—The denaturation profile of 1.4 mg/ml rFNBD-B in increasing concentrations of GdnHCl was monitored using a Superdex-75 column (Pharmacia) attached to a Pharmacia FPLC (V_o = 7.9 ml, V_t = 24 ml). The denaturation profile of 2 mg/ml ribonuclease A (13) was obtained for reference. A standard curve of log

protein molecular mass *versus* K_{av} in 0 and 7 M GdnHCl was determined using the low molecular weight gel filtration calibration kit (Pharmacia). The standard proteins were ribonuclease A (13,700 Da), chymotrypsinogen (25,000 Da), ovalbumin (43,000 Da), and albumin (67,000 Da).

Intrinsic Viscosity—The viscosity of the rFNBD proteins was measured using a Cannon-Ubbelohde Semi-Micro Viscometer (Cannon Instrument Co., State College, PA) with viscometer constant 0.004127 mm²/s². The temperature was maintained at 25.0 ± 0.1° C, and densities were determined gravimetrically. Prior to viscosity measurements all proteins were dialyzed overnight into the appropriate buffer and this buffer was used as the reference solution. The specific viscosity (η_{sp}) was determined according to methods described in Tanford *et al.* (14). The intrinsic viscosity [η] was determined by fitting the data to the least squares expression,

$$\eta_{sp}/c = [\eta] + k[\eta]^2 c \qquad \text{(Eq. 1)}$$

where c is concentration and k is a dimensionless constant.

Fluorescence—Fluorescence data was accumulated using a SLM 4800C calibrated before each use. Cells were quartz with a maximum sample volume of 3 ml and a 1-cm path length. Excitation and emission slits were routinely set to 4 nm. The excitation wavelength to evaluate intrinsic tryptophan fluorescence was 295 nm, and the excitation wavelength to evaluate 8-anilino-1-naphthalenesulfonic acid (ANS) fluorescence was set at 353 nm. The rFNBD-A contains no Trp; therefore, for rFNBD-A/N29 binding experiments the spectrum of the corresponding concentration of rFNBD-A in isolation was essentially background and was subtracted from the spectrum for the mixture.

RESULTS

Conformation of the rFNBD Proteins—The far-UV CD spectra of rFNBD-A, rFNBD-B, rFNBD-D, and rFNBD-P were very similar and were dominated by a large minimum at ~200 nm (Fig. 2). This type of spectra is characteristic of proteins that are predominately composed of random coil and γ-turns and contain a minimum amount of α-helix or β-sheet conformation (15, 16). A larger version of the FnbA Fn binding segment, PAQ8 (9), contains A1, A2, and A3 as well as an upstream sequence designated Au (Fig. 1*A*). This sequence is similar to the other Fn binding motifs (Fig. 1*B*) and has been shown to have Fn binding activity. PAQ8, when analyzed by far-UV CD, appears to have no significant increase in secondary structure over the smaller proteins (Fig. 2).

Both rFNBD-A and rFNBD-D were tested to determine if the proteins exhibited some degree of regular secondary structure elements that could be disrupted on thermal denaturation. For each protein sample the temperature was raised from 24 to 80 °C and far-UV CD spectra were accumulated at several different temperatures (data not shown). No conspicuous differences were seen in any of the spectra, indicating that there was not a significant amount of secondary structure present that was unfolded during heating.

Because of the apparent lack of stabilizing secondary structure elements, the possibility that the rFNBD proteins had a highly fluctuating tertiary structure in buffers approximating physiological conditions was investigated. One method of observing the stability of tertiary structure is to monitor the cooperativity of a protein unfolding transition. The term cooperativity describes the process in which weak intramolecular interactions cooperate so that the interacting groups have very high effective concentrations, thus stabilizing a particular folded conformation. As intramolecular interactions are disrupted the protein unfolds completely within a limited range of condition changes, and the abruptness of the unfolding is indicative of a cooperative transition (17). By monitoring the elution volume (V_e) of rFNBD-B using gel-permeation chromatography as the concentration of GdnHCl was increased from 0 to 6 M, it was determined that rFNBD-B does not follow a cooperative unfolding model (Fig. 3). The cooperative unfolding transition of a globular protein of comparable molecular mass,
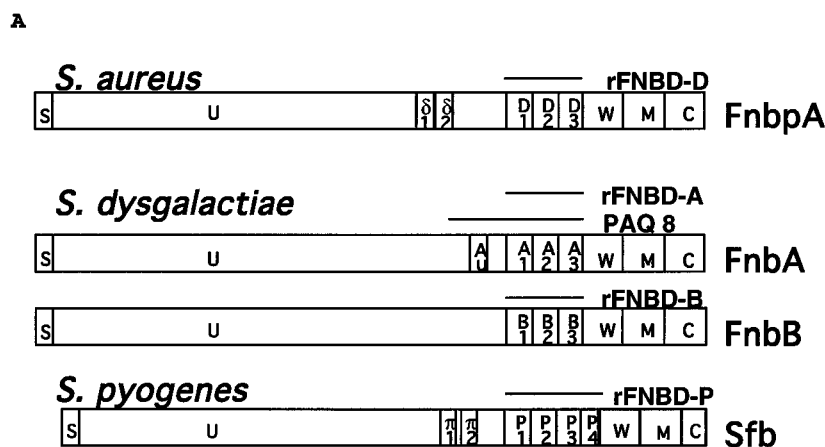
A

B

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Au          EGPTEGENNLGGQSEEITITEDSQSGMSGQNPG
A1:         VVEDTQTSQED-IVLGGPGQVIDFTEDSQPGMSGNNSHT
A2:        ITEDSKPSQEDEVIIGGGQGQVIDFTEDTQSGMSGDNSHTDGTV
A3:        LEEDSKPSQEDEVIIGGGQGQVIDFTEDTQSGMSGAGQVESP
P1,3:      VETEDTKE--P-EVLMGGQSESVEFTKDTQTGMSGQTTPQ
P2:        VETEDTKE--P-GVLMGGQSESVEFTKDTQTGMSGQTTPQ
P4:        VETEDTKE--P-GVLMGGQSESVEFTKDTQTGMSG
D1:        QNSGNQSFEEDTEEDKP-KYEQGGNIVDIDFDSVPQIHG
D2:        QNKGNQSFEEDTEKDKP-KYEHGGNIIDIDFDSVPHIHG
D3:        FNKHTEIIEEDTNKDKP-SYQFGGHN-SVDFEEDTLPKV
B1:  EETLPTEQGQSGSTTEVEDTKG--P-EVIIGGQGEIVDIEENL
B2:  EENLPTEQGQSGSTTEVEDTKG--P-EVIIGGQGEVVDI
B3:  EESLPTEQGQSGSTTEVEDSKPKLSIHFDNEWPKED
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FIG. 1. *A*, domain organization of fibronectin receptors from *Staphylococcus aureus*, *Streptococcus dysgalactiae*, and *Streptococcus pyogenes*. Fn-binding repeats are represented by *A, B, D,* and *P. S*, signal sequences; *U*, sequence unique to the Fn receptor; *W*, cell wall spanning region; *M*, membrane-spanning region; *C*, intracellular sequence. The recombinant proteins correspond to the regions indicated by rFNBD-D, rFNBD-A, PAQ8, rFNBD-B, and rFNBD-P. *B*, aligned sequences of the Fn-binding repeat units indicated by *A, B, D,* and *P* in *A*. Regions of very high similarity are shown in *bold type*.
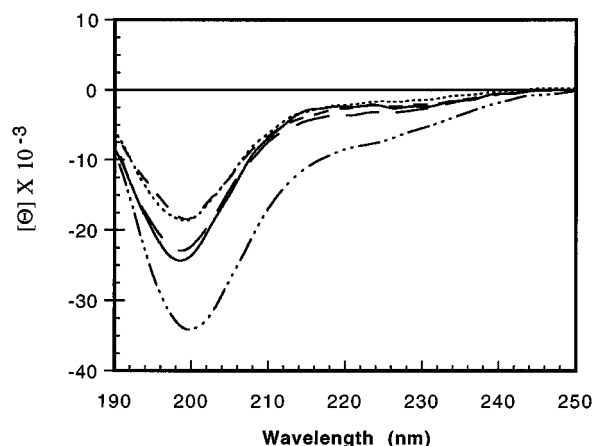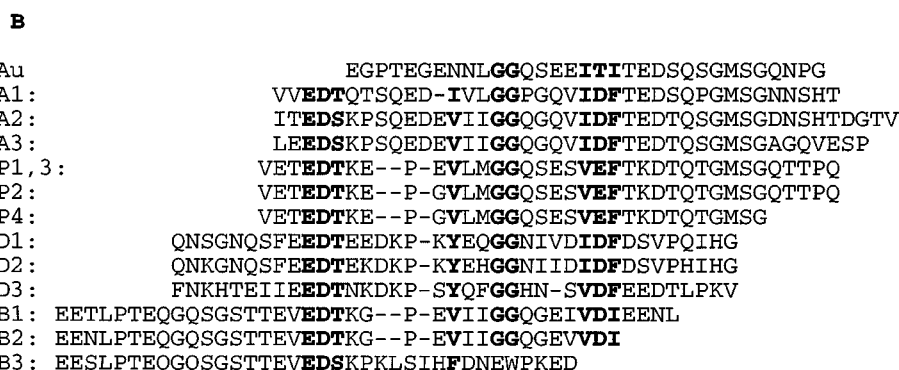

FIG. 2. **Far-UV CD of (from bottom to top at 200 nm) PAQ8, rFNBD-P, rFNBD-A, rFNBD-B, and rFNBD-D.**

FIG. 3. **Denaturation of rFNBD-B in increasing concentrations of GdnHCl (*open circles*).** The denaturation curve of ribonuclease A (*closed circles*) (13), a globular protein of comparable molecular mass, was also monitored for comparison.

ribonuclease A (13.7 kDa), is shown in Fig. 3 for comparison. It is readily apparent that ribonuclease A undergoes a distinct shift in V_e during its unfolding transition between 2 and 4 M GdnHCl as previously shown by Greene and Pace (13).

The V_e (9.6 ml) of rFNBD-B at 0 M GdnHCl corresponds to a molecular mass of ~57,000 Da, when compared to a curve developed using globular protein standards (ribonuclease A, chymotrypsinogen, ovalbumin, and albumin). Because of the abnormally low V_e, there is the possibility that rFNBD-B is aggregated under these conditions, may not possess a compact globular structure, or potentially some combination of the two possibilities. When compared to a curve developed from the same set of four standard proteins denatured in 7 M GdnHCl, the experimentally determined molecular mass of rFNBD-B (13.3 kDa) in the absence of GdnHCl corresponded reasonably
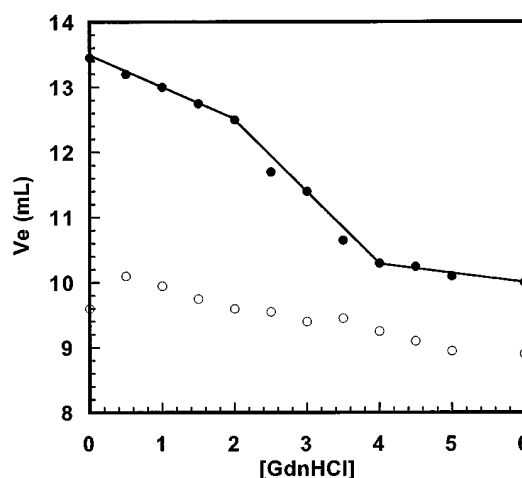
well to the actual molecular mass of rFNBD-B (14.6 kDa) (data not shown). This supports that rFNBD-B is an unfolded protein in PBS.

To further ensure that aggregation was not the cause of the abnormally low V_e of rFNBD-B in the gel-permeation chromatography experiment, the intrinsic viscosity of rFNBD-B and rFNBD-A was determined in the absence and presence of 6 M GdnHCl (Fig. 4). The intrinsic viscosity ([η]) is a measure of the effective specific volume of the domain of a macromolecule in solution (14). For folded globular proteins, [η] is small and independent of molecular mass, while [η] for denatured proteins is considerably larger and increases roughly as the mo-
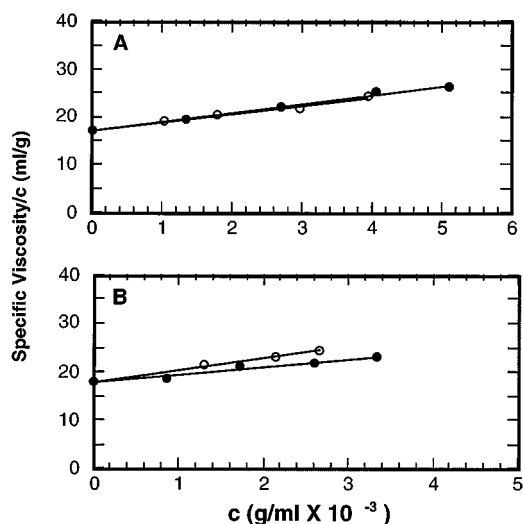
FIG. 4. **Viscosity data for (*A*) rFNBD-A in PBS (*open circles*) and 6 M GdnHCl (*closed circles*) and (*B*) rFNBD-B in PBS (*open circles*) and 6 M GdnHCl (*closed circles*).**

FIG. 5. **Intrinsic tryptophan fluorescence of 3.5 μM N29 in the absence (*solid line*) and presence of a 2.5 excess molar ratio of rFNBD-A (*dashed line*).**

lecular mass of the monomer unit increases. The specific viscosity (η_{sp}) of rFNBD-A and rFNBD-B at concentrations of 0 and 6 M GdnHCl was measured. By plotting η_{sp}/c *versus c* (Equation 1), it was determined that the [η] was the same for both rFNBD-A and rFNBD-B in the absence or presence of GdnHCl (Fig. 4). This result demonstrates that both rFNBD-A and rFNBD-B are monomers at 25 °C. Additionally, it is further evidence that both rFNBD-A and rFNBD-B do not significantly change conformation by changing from 0 to 6 M GdnHCl. That the proteins are in fact monomeric in PBS at 18–20 °C has subsequently been confirmed by both velocity and equilibrium sedimentation analysis using a Beckman XL-A analytical ultracentrifuge.[2]

One of the characteristics of partially folded proteins is the presence of pockets of hydrophobic side chains that are sequestered away from the highly polar solvent. Intrinsic tryptophan (Trp) fluorescence of rFNBD-B in PBS indicates that the single Trp residue is not in a hydrophobic region and on addition of 6 M GdnHCl there is no change in the wavelength or intensity of the maximum fluorescence for this Trp (data not shown). A convenient probe for monitoring the presence of hydrophobic pockets in partially folded proteins is ANS. ANS is a fluorophore that emits a large amount of fluorescence on binding to partially exposed hydrophobic regions. These regions are not normally present in tightly packed native proteins because their hydrophobic clusters are buried in the interior and are not accessible to solvent. Likewise, denatured proteins are known not to bind ANS (18). It was determined that ANS did not bind to rFNBD-A or rFNBD-B under any conditions tested, indicating that the proteins do not appear to be capable of forming localized, stable regions of hydrophobic side chains (data not shown). From the experimental evidence presented above we conclude that the rFNBDs do not have the characteristics of folded or partially folded (*i.e.* a molten globule) proteins, instead the structure of the repeat regions is highly dynamic and appears to be thermodynamically indistinct from the denatured state.

Conformational Changes in Recombinant MSCRAMMs on Ligand Binding—To experimentally measure possible conformation changes in the N29 fragment on binding of a rFNBD intrinsic Trp fluorescence of N29 was used. N29 contains seven Trp residues interspersed throughout the sequence. The intrin-
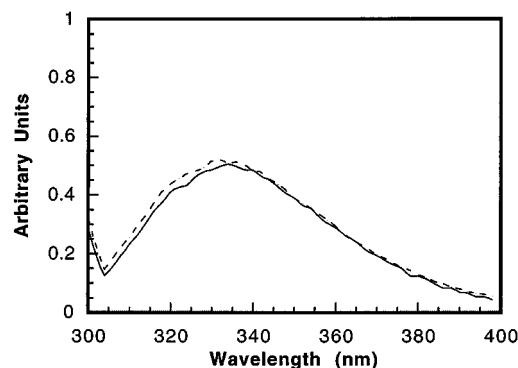
sic fluorescence of Trp side chains is very sensitive to minute changes in conformation and orientation, and due to the presence of multiple residues in N29, a change in intrinsic Trp fluorescence on binding of rFNBD-A is an excellent monitor for local and/or global conformational rearrangements. The rFNBD-A contains no Trp residues so its contribution to the fluorescence between 300 and 400 nm is negligible. As can be seen from Fig. 5, in the absence of rFNBD-A these N29 Trps are highly quenched and in non-solvent accessible environments. As increasing molar ratios of rFNBD-A were added to the N29 there was a small (~2 nm) blue-shift in the wavelength of maximum fluorescence but no change in the intensity (Fig. 5). From this it appears that the N29 intrinsic Trp fluorescence is not significantly affected by the binding of rFNBD-A and there is no evidence for a major conformational rearrangement in N29 on complex formation.

The far-UV CD of the N29 fragment is shown in Fig. 6*A* and it agrees well with previously published spectra (22). The N29 fragment exhibits an unusually low intensity far-UV CD and the observed positive band at ~230 nm has been indicated to arise predominately from the optical activity of tyrosine side chains in N29 (23, 24). A comparison of Figs. 2 and 6*B* indicates that the maximum intensity of the [Θ] values (190–250 nm) of N29 are significantly smaller than those for the rFNBD proteins.

Far-UV CD has been used on a variety of systems to gain information about secondary structure changes that take place in a protein on binding to various biological molecules (see for example, Refs. 19–21). On addition of N29 to rFNBD-A at ratios ranging from 0:1 to 4.9:1 there is a marked change in the appearance of the far-UV CD spectra. The mean residue ellipticity ([Θ]) of rFNBD-A at the 0:1 ratio is −23,500 deg·cm²/dmol at 200 nm. The Fn N29 is then added and a new spectrum is acquired. After the N29 spectrum in isolation is subtracted from the mixture spectrum, [Θ] is calculated for the subtracted spectrum using the mean residue molar concentration for rFNBD-A only (Fig. 6*B*). Treating the N29 spectrum as a constant and subtracting it from the mixture spectrum is justified by the presence of ordered secondary structure in N29 (22, 25, 26) as well as the lack of evidence for a significant conformation rearrangement in N29 on binding of rFNBD-A, as judged by fluorescence spectroscopy.

At a ratio of 4.9:1 the resulting spectrum now has a maximum [Θ] of +17000 deg·cm²/dmol at 200 nm and a minimum [Θ] of −6000 deg·cm²/dmol at 230 nm. The same changes in the far-UV CD spectra of rFNBD-D and PAQ8 was seen when each was titrated with N29 (data not shown). The rFNBDs appear to change structure from a predominately random coil conformation in an unoccupied form to a predominately β-sheet second-
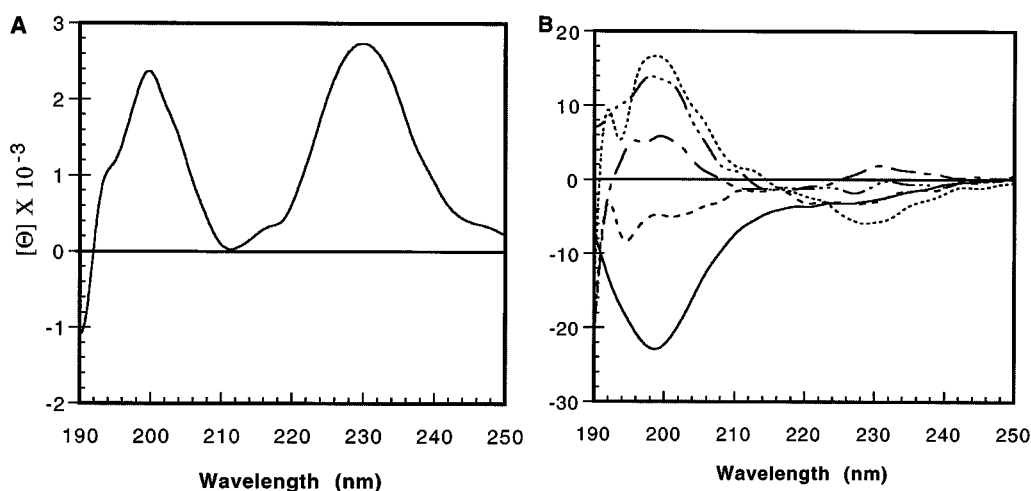
FIG. 6. *A*, far-UV CD of N29 (10.5 μM). *B*, far-UV CD of the titration of 8.6 μM rFNBD-A with increasing concentrations of Fn N29. The N29 concentrations are (*from bottom to top at 200 nm*) 0, 10.5, 21, 32, and 42 μM.

ary structure conformation in the MSCRAMM ligand complex.

Several controls were examined to ensure the changes seen in Fig. 6*B* were due to a binding event and not nonspecific hydrophobic interactions, due to rFNBD-A existing in solution as an unfolded protein. The far-UV CD spectra of either rFNBD-A or N29 in the presence of bovine serum albumin were identical to those acquired in the absence of bovine serum albumin. Moreover, when a 2-fold molar excess of N29 was combined with a relatively unstructured recombinant segment of the collagen-binding MSCRAMM (28) there was no evidence of any type of interaction as measured via this assay.

DISCUSSION

The rFNBD proteins appear to have little or no stable secondary structure in PBS. A distinction between native and denatured tertiary conformations was not measurable using intrinsic viscosity measurements. Tanford and co-workers (14) have shown that there is a linear relationship between the intrinsic viscosity [η] of a denatured protein and the number of amino acids in its polypeptide chain (*n*) as shown by Equation 2:

$$[\eta] = 0.716 n^{0.66} \tag{Eq. 2}$$

From Equation 2 the [η] of denatured rFNBD-A and rFNBD-B is calculated to be 18.8 and 18.2 ml/g respectively. This value is in agreement with the experimental [η] values extrapolated from Fig. 4 (16.1 and 17.1 ml/g for rFNBD-A, and 17.8 and 18.0 ml/g for rFNBD-B) under native and denaturing conditions, respectively. This result would suggest that the rFNBD proteins have the same highly dynamic structure in either solvent. Furthermore, these proteins do not aggregate and are essentially monomeric at 25 °C in PBS. The absence of fixed long-distance intramolecular interactions is confirmed by the results of unfolding experiments monitored by gel-permeation chromatography. For rFNBD-B in increasing concentrations of GdnHCl any structural changes occur in a gradual, linear, fashion without a clearly identifiable transition region, as seen for the control, ribonuclease A.

The solution structure of two of the five type I (FI) repeats present in the N29 portion of Fn has been determined in isolation and as a pair using multidimensional nuclear magnetic resonance spectroscopy (25, 26). The repeats are made up of a series of anti-parallel β-sheets where a single FI module consists of an amino terminus leading into a short double-stranded sheet and folded onto a larger triple-stranded sheet to enclose a hydrophobic core. Each FI module is further con-

strained by two conserved cysteine residues that link in a pattern Cys[1] to Cys[3] and Cys[2] to Cys[4]. Solution structure information indicates that the fourth FI module docks onto the fifth FI module via a hydrophobic interface. The four β-sheets stack on top of each other to form a fairly inflexible elongated rod-like structure that has only a limited clockwise twist around the long axis from the NH_2- to the COOH-terminal. From electron microscopy of rotary shadowed specimens it was also deduced that the entire Fn NH_2-terminal domain is most likely a rigid rod-like structure (22). Because of the defined, stable, secondary and tertiary structure in N29, the minute change in the intrinsic Trp fluorescence in N29 on binding of rFNBD-A, and the apparent lack of stable ordered conformation in the rFNBD proteins, the large conformational shift seen in the far-UV CD on recombinant adhesin binding to N29 is largely attributed to the rFNBD proteins assuming a predominately β-sheet structure on binding to N29.

To what extent might conformational changes in the N29 be contributing to the changes on binding seen in Fig. 6*B*? Previously far-UV CD has been used to monitor structural changes in a 31-kDa NH_2-terminal tryptic fragment of Fn (N31) (21). They recorded reproducible changes in the secondary structure of N31 when the polysaccharide heparin was added to the protein. On binding of heparin, the signal at 228 nm in the far-UV CD of N29 (shown in Fig. 6*A*) is slightly red-shifted and attenuated. Additionally, a minimum centered at 212 nm is accentuated. These effects are of much less magnitude and opposite direction when compared to Fig. 6*B*. We cannot discard the possibility of changes in the far-UV CD of N29 on binding to the rFNBD proteins. Since the secondary structure of the N29 repeat motifs is well ordered, the most likely place for a significant conformational change to occur is the segment of the polypeptide chain connecting the repeats. If there is rearrangement in this region on binding of the adhesin we believe that it would still not be sufficiently large to cause the shift from a random-coil to a predominately β-sheet secondary structure seen in Fig. 6*B*.

Several pieces of evidence combine to suggest that the binding site between these proteins may be a series of multiple contact points along one or both of the repeat regions. The binding sites on both Fn and the adhesin consist of repeated sequences. Inhibition studies have shown that synthetic peptides analogous to some of the single repeat regions (Fig. 1*B*) retain the ability to bind Fn (11). Additionally, repeats D1–3 expressed as a glutathione *S*-transferase fusion protein (D1–3) have recently been fluorescein-labeled and assayed for changes

in the fluorescence anisotropy of this tag on addition of N29 (27). They determined that 1.9 mol of N29 bound per mol of D1–3 with a dissociation constant of 1.5 nM, indicating that there is at least two distinct binding sites on the D1–3 repeat region capable of binding N29. Finally, the overwhelming conformational shift shown in Fig. 6*B* also indicates that binding affects the entire recombinant adhesin domain and is not localized to a single area.

The conformational variability of the rFNBD proteins and PAQ8 as well as the observed change in conformation for the rFNBD proteins on binding of N29 does not appear to be the result of expressing this portion of the adhesin as a recombinant protein. A monoclonal antibody, 3A10, has been raised against the full-length MSCRAMM FnbA and characterized (9). The epitope for this antibody has been mapped to the Au region of FnbA, which has also been shown to be most similar to the A2 sequence of FnbA and the P repeat motif, from *S. pyogenes*, and to bind Fn. However, 3A10 will only recognize and bind to the epitope in both full-length and truncated MSCRAMM protein when both components are in the presence of Fn. Additionally, monoclonal antibody 3A10 significantly enhances the binding of a corresponding epitope, present on the surface of *S. pyogenes* cells, to Fn (9). Combined, these observations indicate that structural changes reported in this paper could mimic the molecular basis for the emergence of the epitope recognized by 3A10 in the intact MSCRAMM. The microbe may have developed the capability to circumvent the effects of blocking antibodies produced by the host by presenting the biologically relevant conformation only when the MSCRAMM is already bound to its ligand of choice. The ligand induced binding site could then be of significant advantage for the microbe in avoiding host defense mechanisms because the host is unable to mount a response that includes blocking attachment of the organism to Fn and establishing colonization.

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Conformational Changes in the Fibronectin Binding MSCRAMMs Are Induced by Ligand Binding

Karen House-Pompeo, Yun Xu, Danny Joh, Pietro Speziale and Magnus Höök