High Affinity Streptococcal Binding to Human Fibronectin Requires Specific Recognition of Sequential F1 Modules*

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Fibronectin (Fn) binding by the Streptococcus pyogenes protein SfbI has been shown to trigger integrin-dependent internalization of this pathogen by human epithelial and endothelial cells. Here, using nuclear magnetic resonance spectroscopy and isothermal titration calorimetry in a dissection approach, the basis for the specificity and high affinity of the interaction between the N-terminal domain of Fn and SfbI is revealed. Each of the five Fn type 1 modules is directly involved in the interaction and is recognized by short consecutive motifs within the repeat region of SfbI. Crucially, these motifs must be combined in the correct order to form a high affinity ligand for the N-terminal domain of Fn.

Proteins that are natively unstructured (or intrinsically disordered) or those containing significant disordered regions are now recognized to account for a substantial fraction of all proteins (1). The function of most of these non-classical proteins appears to be molecular recognition in regulatory and assembly processes. Unfolded proteins can bind specifically and rapidly to their targets through large interfaces, thereby undergoing a significant disorder-order transition upon binding (2, 3). Another striking feature is the over-proportionate occurrence of tandem arrays of sequence repeats in intrinsically disordered proteins (4). Fibronectin (Fn)-binding proteins (FnBPs) that are expressed on the surface of pathogenic Gram-positive bacteria contain functional regions possessing all these properties.

FnBPs belong to a class of adhesins called MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) (5–7). The Fn binding activity of the FnBP SfbI from Streptococcus pyogenes and its allelic variant, PrtF1, was initially ascribed to the C-terminal third of the molecule where, depending on the bacterial strain, 1–6 Fn-binding repeats (FnBRs) of 37 amino acid residues are found (Fig. 1A) (8–10). The FnBRs of SfbI are homologous to tandem repeats in FnBPs of other streptococci and Staphylococcus aureus, and new boundaries for FnBRs of both streptococci and staphylococci have recently been suggested based on structural data (7, 11). FnBRs are intrinsically disordered and undergo a conformational change on binding to Fn (12–14).

Although S. pyogenes was considered previously to be an exclusively extracellular pathogen, it has been shown more recently both to adhere to and invade human epithelial and endothelial cells (15–17). The FnBP SfbI (18, 19) has been shown to efficiently mediate cellular internalization; in the uptake process Fn acts as a bridge between the pathogen and integrin receptors on the cell surface (20). Internalization may contribute to the persistence of S. pyogenes in antibiotic-treated individuals (21) and aid hematogenous dissemination and/or evasion of the host immune system.

Fn is present in a soluble form in human plasma and other body fluids and in an insoluble form in the extracellular matrix. Fn has a mosaic structure and is composed of independently folded Fn type 1, type 2 and type 3 (F1, F2, and F3) modules (22) (Fig. 1C). FnBRs of S. aureus, S. pyogenes, and Streptococcus dysgalactiae bind to the N-terminal domain (NTD) of Fn (8, 23, 24), which is composed largely of a string of five F1 modules (1–5F1). High resolution structures of subdomains of the NTD have been determined using nuclear magnetic resonance (NMR) spectroscopy (25–27). The consensus fold of the F1 module consists of a double-stranded anti-parallel β-sheet (strands A and B) folded over a triple-stranded anti-parallel β-sheet (strands C, D, and E).

The three-dimensional structure of an FnBP-derived peptide (B3 from S. dysgalactiae) in complex with 1F1F1Fn from the NTD of Fn was recently determined (11). The structure revealed an anti-parallel orientation of the binding partners with the peptide forming additional β-strands at the edge of the triple-stranded β-sheets of the two F1 modules. This novel binding mechanism was named a “tandem β-zipper” (11). Based on these structural data and sequence analyses, it was proposed that each FnBR forms an even longer tandem β-zipper when bound to intact NTD (11).

The main aim of this work is to address two key elements of the tandem β-zipper model of Fn/NTD interactions. First, we demonstrate that each of the five F1 modules of the NTD is specifically recognized by short, consecutive (i.e. tandemly arranged) amino acid sequences in FnBRs. Second, functional FnBRs are shown to be linear arrays of specific F1-binding motifs that need to be arranged in the correct order to bind to the NTD with high affinity. Data presented here also support the idea that bacterial peptides bind to all F1 modules of the NTD through a common mechanism, the β-zipper. This paper explains how a natively disordered functional domain of a bacterial protein forms a highly efficient ligand for a modular host protein.
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EXPERIMENTAL PROCEDURES

Expression and Purification of Recombinant Proteins—Unlabeled and U-15N-labeled module pairs 1F1,F1, 2F1, 3F1, 4F1, 5F1 (residues 17–109, 62–151, and 152–244 of mature human Fn) were expressed in Pichia pastoris and purified using procedures similar to those described previously (11, 27, 28). All expressions of 15N-labeled proteins were carried out in 500 ml of media containing 1% (w/v) (15NH4)2SO4. SfbI-4 and SfbI-5 were expressed as glutathione S-transferase fusion proteins as described previously (111). SfbI-1 and SfbI-4,5 were produced in a similar fashion using the following oligonucleotides: SfbI-1, 5’-gggctctttcgtatcttctgtc-3’; SfbI-4, 5’-gggctctttcgtatcttctgtc-3’; SfbI-4,5, 5’-gggctctttcgtatcttctgtc-3’. Recombinant SbiI peptides were purified by reverse-phase high performance liquid chromatography (HPLC) on a C4 silica column (10 µm, 300 Å, 25 x 25 cm) with an elution gradient of 8–60% acetonitrile containing 0.1% trifluoroacetic acid over 18 min at a flow rate of 3 ml/min.

Synthetic Peptides—PyOT5, PyOn5, and PyF05 were synthesized using standard N-(9-fluorenylmethyl)carbonyl chemistry and purified by reverse-phase HPLC. All other peptides were purchased from Alta Bioscience (Birmingham, UK) and purified by reverse-phase HPLC, if required, on a C18 silica column (10 µm, 90 Å, 25 x 1 cm) with an elution gradient of 10–20% acetonitrile in 5 mM ammonium carbonate buffer over 15 min at a flow rate of 3 ml/min. The N and C termini of all peptides were capped by acetylation and amidation, respectively.

Isothermal Titration Calorimetry (ITC) —The experiments were carried out with a VP-ITC microcalorimeter (MicroCal Inc., Northampton, MA). The method and equipment are identical to those described previously (11). Each module pair/peptide mixture was incubated at 25 °C for 45 min. The N and C termini of all peptides were capped by acetylation and amidation, respectively. All NMR experiments were performed on spectrometers equipped with Oxford Instruments superconducting magnets, 1H operating frequencies of 500, 600, and 750 MHz. The spectrometers are all equipped with Oxford Instruments superconducting magnets, OMEGA software and digital control equipment (Bruker Instruments), home-built triple-resonance pulsed-field-gradient probe-heads (30), and home-built linear amplifiers for 1H, 15N, and 13C nuclei. Spectra were recorded using standard pulse sequences previously (111). Spectra were assigned by 1H,15N HSQC spectroscopy (31) recorded on a C4 silica column (10 µm, 300 Å, 25 x 25 cm) with an elution gradient of 8–60% acetonitrile containing 0.1% trifluoroacetic acid over 18 min at a flow rate of 3 ml/min.

RESULTS

Dissection Approach —The interactions of three Fn type 1 module pairs 1F12F1, 2F13F1, 4F15F1 with peptides from FnBRs of SbiI were investigated by NMR spectroscopy and ITC (Fig. 1, Table I). Peptides from S. pyogenes FnBRs were chosen according to the tandem β-zipper model (11) to bind to either a specific F1 module or module pair of NTD. Series of 1H,15N HSQC spectra of 15N-labeled module pairs at increasing concentrations of peptide were acquired (HSQC titrations). Fn residues affected by peptide binding were identified on the basis of observed chemical shift changes of F1 module backbone amide resonances (chemical shift perturbation mapping (33)) (Fig. 2A and B). Kd values were derived directly from the chemical shift changes if the kinetics of binding were fast on the NMR time scale (34) (Fig. 2G). Other Kd values were determined using ITC (Fig. 2F). For some complexes, where it was impossible to track the resonances in the HSQC titrations, spectra of fully bound 15N-labeled module pairs were assigned using three-dimensional heteronuclear NMR spectroscopy.

1F1 and 2F1 Are Specifically Recognized by Short Consecutive Motifs of SbiI—The direct involvement of 1F1 and 2F1 in interactions with a bacterial peptide from S. dysgalactiae FnBB was demonstrated recently by the determination of the three-dimensional structure of 1F1F1 in complex with the B3 peptide (11). Here, the involvement of both modules in binding to the homologous peptide from SbiI is confirmed, and the specificity of F1-module recognition is demonstrated.

The peptide PyOT5 bound to 1F1F1 with a Kd of 0.45 μM (Fig. 2F) (11) similar to the Kd for binding to B3 (35, 36). In an NMR binding experiment significant chemical shift changes were induced in residues in both 1F1 and 2F1, indicating the direct involvement of both modules in binding to PyOT5 (Fig. 2C).

The tandem β-zipper model for NTD recognition is based on the existence of independent, consecutive motifs within FnBRs that interact with specific F1 modules. Therefore, PyOT5 was dissected into predicted single-module binding segments. PyOn5 and PyTw5, representing the C- and N-terminal halves...
of PyOT5, caused chemical shift changes exclusively in 1F1 (Fig. 2, A and D) or 2F1 (Fig. 2, B and E), respectively. The observed chemical shift differences resembled the perturbation patterns for each module on binding of PyOT5. The K_d values for binding to 1F12F1 were 159 and 63 nM for PyOn5 and PyTw5 (Fig. 2 G), respectively. We conclude that 1F1 and 2F1 are recognized specifically by separate motifs of SfbI, resulting in an anti-parallel attachment of the peptide to the module pair 1F12F1.

3F1 Is Involved in Binding of SfbI to the NTD—The direct involvement of 3F1 in FnBR binding has to date not been demonstrated. The K_d for binding to 1F12F1 was 159 and 63 nM for PyOn5 and PyTw5 (Fig. 2G), respectively. We conclude that 1F1 and 2F1 are recognized specifically by separate motifs of SfbI, resulting in an anti-parallel attachment of the peptide to the module pair 1F12F1.

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In NMR binding studies PyTT5 induced chemical shift changes in both 2F1 and 3F1 residues (Fig. 3A). To elucidate the role of 3F1, PyTT5 was dissected into single-module binding halves (PyTw5 and PyTh5). The 3F1-binding peptide, PyTh5, bound to the module pair with significantly lower affinity (K_d = 66 nM) than the two-module binding peptide. The pattern of chemical shift perturbations in 3F1 is very similar to that observed on binding of PyOT5 and PyTw5 to 1F12F1 (Fig. 2, C and E) and indicates specific recognition of 3F1 (Fig. 3B). Only one residue in 3F1, Gly-142, was significantly affected (using a 0.1 ppm cutoff) by PyTh5. Based on the previously determined structure of the highly homologous (48% identity) 4F15F1 module pair (26), this residue is in a loop in 3F1 that is expected to be in close proximity to 2F1 residues. Thus, it is likely that binding of PyTw5 to 3F1 causes limited chemical shift perturbations in 3F1 due to indirect (intermodular) interactions. Direct evidence for an intermodule interface awaits the determination of the structure of 2F13F1.

The putative 3F1-binding peptide, PyTh5, when added to 2F13F1 in excess, caused chemical shift changes that were confined to residues in 3F1 (Fig. 3C). It should be noted that although the chemical shift changes are very small, their significance is clear from their comparison with the even smaller changes observed for 2F1 residues. Moreover, the perturbation pattern induced by PyTh5 resembles the pattern for 3F1 residues upon binding of the two-module binding peptide, PyTT5. Thus, it could be shown that 3F1 is directly involved in interactions with FnBRs and is specifically recognized by the short peptide, PyTh5. The successful dissection of PyTT5 also further demonstrates the specificity of the 2F1-binding motif in SfbI in that it binds neither 1F1 nor 3F1. The PyTh5/2F13F1 interaction was too weak for a K_d to be measured. The 3F1-binding segment, however, contributes significantly to the affinity of the two-module binding peptide, PyTT5. This result is an example where NMR provides unique, residue-specific informa-
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Dissociation constants ($K_d$ values) for the interaction of a range of synthetic peptides derived from streptococcal FnBPs with three F1 module pairs from the NTD of human Fn

| Peptide residues | Name$^b$ | Predicted target | $K_d$ for the interaction with |
|------------------|----------|------------------|-------------------------------|
|                  |          |                  | $^{1}$F1 $^{2}$F1 | $^{1}$F1 $^{2}$F1 | $^{1}$F1 $^{2}$F1 |
| SfbI 587–591     | PyOT5    | $^{1}$F1        | $^{2}$F1           | 0.45 ± 0.10$^a$ | 32 ± 4 |
| SfbI 523–541     | PyTT5    | $^{2}$F1        | $^{3}$F1           | 0.41 ± 0.07$^c$ | 23 ± 0.6 |
| SfbI 560–577     | PyTT5    | $^{3}$F1        | $^{4}$F1           | 0.17 ± 0.05$^a$ | 17.5 ± 0.05 |
| SfbI 560–577     | PyTT5    | $^{4}$F1        | $^{5}$F1           | 0.17 ± 0.05$^a$ | 2.3 ± 0.4 |
| SfbI 540–561     | PyFT5    | $^{5}$F1        | $^{6}$F1           | 0.17 ± 0.05$^a$ | 0.17 ± 0.05 |
| SfbI 880–899     | SfbI-FF3 | $^{6}$F1        | $^{7}$F1           | 0.17 ± 0.05$^a$ | 0.17 ± 0.05 |
| SfbI 577–591     | PyOn5    | $^{7}$F1        | $^{8}$F1           | 0.17 ± 0.05$^a$ | 0.17 ± 0.05 |
| SfbI 505–514     | PyTw4    | $^{8}$F1        | $^{9}$F1           | 0.17 ± 0.05$^a$ | 0.17 ± 0.05 |
| SfbI 567–577     | PyTw5    | $^{9}$F1        | $^{10}$F1          | 0.17 ± 0.05$^a$ | 0.17 ± 0.05 |
| SfbI 521–531     | PyTb4    | $^{10}$F1       | $^{11}$F1          | 0.17 ± 0.05$^a$ | 0.17 ± 0.05 |
| SfbI 558–568     | PyTb5    | $^{11}$F1       | $^{12}$F1          | 0.17 ± 0.05$^a$ | 0.17 ± 0.05 |
| SfbI 548–561     | PyTb5    | $^{12}$F1       | $^{13}$F1          | 0.17 ± 0.05$^a$ | 0.17 ± 0.05 |
| SfbI 540–550     | PyFt5    | $^{13}$F1       | $^{14}$F1          | 0.17 ± 0.05$^a$ | 0.17 ± 0.05 |
| SfbI 540–550     | PyFt5    | $^{14}$F1       | $^{15}$F1          | 0.17 ± 0.05$^a$ | 0.17 ± 0.05 |

$^a$ The nomenclature used for the peptides is explained in the legend of Fig. 1. SfbI-FF3 is a peptide from SfbI of S. pyogenes.

$^b$ Values in italics were determined calorimetrically. Other values were determined using NMR spectroscopy. The errors for ITC experiments are S.D. of the fits, whereas for NMR titrations the values quoted are the mean ± the S.D. of $K_d$ values calculated for several (10–30) resonances in fast exchange.

$^c$ n/f indicates that although a peptide caused chemical shift changes, the data were not suitable for a fitting procedure; this was the case for very weak interactions.

$^d$ $K_d$ values > 1 mM could not be determined very accurately due to limited solubility of binding partners.

$^e$ For PyFi5, no binding could be observed.

Fig. 2. Specific recognition of $^{1}$F1 and $^{3}$F1 in $^{1}$F1$^{2}$F1 by short, consecutive fragments of SfbI. A and B, NMR titrations. Overlays of the same region of $^{1}$H, $^{15}$N HSQC spectra of 0.2 mM U-$^{15}$N-labeled $^{1}$F1$^{2}$F1 in the presence of increasing concentrations of PyOn5 (A) and PyTw5 (B). For clarity, not all titration steps are shown in the overlays. Black, cyan, orange, green, yellow, blue, and red spectra correspond to (uncorrected) concentrations of 0, 0.05, 0.1, 0.2, 0.4, 0.8, 1.4 mM PyOn5 or PyTw5. C–E, chemical shift perturbation maps for U-$^{15}$N-labeled $^{1}$F1$^{2}$F1 (Δ$\delta_{\text{bound}} - \delta_{\text{free}}$) upon binding to PyOT5 (C), PyOn5 (D), and PyTw5 (E). Red and blue bars represent the absolute differences in backbone amide $^{1}$H chemical shifts and $^{15}$N shifts, respectively. P stands for proline residues. The locations of $\beta$-strands and the intermodule linker (L) are indicated on top of the shift maps. F, ITC profile for the interaction of $^{1}$F1$^{2}$F1 with PyOT5. Top, heat changes obtained for 41 injections. Bottom, integrated curve with experimental points (□) and the best fit (―). Data were fitted using a one-site model, resulting in the following: stoichiometry, n = 1.003 ± 0.002; $K_d = 0.44 ± 0.02$ μM; Δ$H = -13980 ± 45$ cal mol$^{-1}$; ΔS = -17.79 cal mol$^{-1}$ K$^{-1}$. G, binding curves for the interaction of $^{15}$N-labeled $^{1}$F1$^{2}$F1 with PyTw5. The change in chemical shift at increasing concentrations of PyTw5 is shown and is normalized to 1 at saturation. Each data set was analyzed by non-linear regression, fitting to an equation relating the change in chemical shift to the peptide:protein ratio. The curve shown was derived from the averaged data of 14 resonances, only 6 of which are depicted here.

SfbI Contains $^{4}$F1- and $^{5}$F1-binding Motifs—Binding of the FnBR region of SfbI to $^{4}$F1$^{5}$F1 has been reported (11, 35), but the specific recognition of $^{4}$F1 and $^{5}$F1 has not yet been dem-
onstrated. Binding of the predicted 4F1-binding peptide (PyFF5) from SfbI-5 to 4F1 (Kd = 113 μM (11)) resulted in chemical shift changes in both F1 modules (Fig. 3D). On dissecting the peptide into potential 4F1- and 5F1-binding peptides, PyFo5, the 4F1-specific peptide, induced chemical shift changes primarily for residues in 4F1 (Fig. 3E). Several chemical shift changes in 5F1 could, however, be observed. These changes are in 5F1 residues in the A-B double-stranded β-sheet and the D-E interstrand loop that were previously shown to form part of the intermodule interface between 4F1 and 5F1 (26). The effects observed for these residues are, therefore, likely to be transmitted over this interface rather than to arise from direct interaction with the peptide (14). PyF55, predicted to interact with 5F1, did not cause any significant chemical shift changes when added to 4F1 (Fig. 3F). Another putative 4F1-binding peptide, PyFF1, lacks the EDT motif altogether. It was shown to bind to 4F1 (Table I) but with an even lower affinity than PyFF5 (Table I). PyF1 binding (Fig. 4A) perturbs chemical shifts of residues in both 4F1 and 5F1, including the E-strand of 5F1 (some distance from the interface with 4F1; Fig. 4B). This is an important finding because the N terminus of PyFF1 (which contains a predicted 5F1-binding motif) marks, according to the tandem β-zipper model, the N-terminal boundary of the FnBR region in SfbI (Fig. 1). In previous definitions PyFF1 was included in the adjacent functional region of SfbI, the UR segment (see “Discussion”) (39, 40).

Evidence for the Role of the EDT Sequence in 4F1 Binding—A sequence alignment of streptococcal and staphylococcal FnBRs reveals that the glutamic acid residue in an EDT motif, which is conserved in the predicted 4F1-binding motif in many FnBRs, is substituted with a lysine in FnBRs of SfbI (7). In a peptide from S. dysgalactiae FnBA, a Glu/Gln mutation in the EDT motif of the 4F1-binding region almost completely abrogated the ability of that peptide to inhibit FnBPA (from S. aureus) binding to Fn (37). The relatively weak affinities of PyFF5 and PyFo5 for 4F1 (Table I) also provide strong evidence for the important role of the glutamic acid residue in this motif. To support this finding, SfbII-FF3, a homologous peptide from S. pyogenes SfbII (38) but containing the EDT motif, was used in a binding study with 4F15F1. SfbII-FF3 bound 4F15F1 with significantly enhanced affinity (Kd = 22 μM).

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Further Evidence for a Common Structural Theme in F1 Recognition—The chemical shift perturbation maps in Figs. 2–4 provide information regarding bacterial peptide binding sites on each of the F1 modules. For example, a comparison of HSQC spectra of 1F15F1 in the presence and absence of PyOT5, PyOn5, and PyTw5 (Fig. 2C–E) shows a clustering of the most
As predicted from sequence analyses, a degree of "mismatch" binding to module pairs was observed for both \(^{3}\)F1- and \(^{2}\)F1-binding peptides from SfbI. That is, PyTw5 induced chemical shift changes in \(^{3}\)F1 residues (and \(^{1}\)F1 residues close to the intermodule interface) when added to \(^{4}\)F1:F1. PyFo5 induced chemical changes in \(^{3}\)F1 residues when added to \(^{2}\)F1:F1 or \(^{1}\)F1:F1. PyTw5 bound with a significantly higher affinity to its match module \(^{2}\)F1 (in \(^{1}\)F1:F1 or \(^{2}\)F1:F1) than its mismatch target \(^{4}\)F1 (in \(^{1}\)F1:F1; Table I). A quantitative analysis for match and mismatch binding by PyFo5 was hampered by difficulties in fitting binding data for the very weak binding to all module pairs (Table I). To allow further quantitative analysis, a similar approach to probing specificity was used for the higher affinity two-module binding peptides. PyTT5, PyFF5, and SbII-FF3 all bound to their mismatch target but with considerably lower affinity (Table I). It was also shown that \(^{4}\)F1:F1 efficiently competed with U-\(^{15}\)N-labeled \(^{2}\)F1:F1 for binding to the \(^{2}\)F1:F1-binding peptide SbII-FF3 (Fig. 6).

**Functional FnBRs: the Correct Order of F1-binding Motifs Is the Key to High Affinity**—To test the validity of the dissection approach and the functional relevance of the measured specificities of F1 module recognition, we investigated the interaction of recombinantly expressed full-length FnBRs with Fib1, a proteolytic fragment consisting primarily of the NTD of human Fn (Table II). As reported previously, the \(^{2}\)F1-binding FnBR SbII-5 and the \(^{1}\)–\(^{2}\)F1-binding FnBR SbII-5 both bound to Fib1 with relatively high affinities (\(K_d\) values of 62 and 2 nM, respectively) (11). SbII-1, the first FnBR from SbII, that is predicted to bind \(^{2}\)–\(^{3}\)F1, was found to have a somewhat lower affinity for Fib1 (\(K_d\) of 168 nM) than SbII-4. This reflects the presence of the weak \(^{2}\)F1:F1-binding segment PyFF1 (see above) in this repeat. All three binding processes were characterized by high exothermic enthalpies and unfavorable entropies (Table II), consistent with the considerable disorder-order conformational transition of FnBRs on Fn binding.

The boundaries for these three constructs were chosen based on the tandem \(\beta\)-zipper model for FnBR/NTD interactions to contain specific F1-binding motifs arranged in the correct order to bind \(^{2}\)–\(^{3}\)F1 (SbII-1 and SbII-4, Fig. 7A) or \(^{1}\)–\(^{2}\)F1 (SbII-5, Fig. 7C). Using different boundaries, however, it is possible to design a native peptide SbII-4.5 that contains the C terminus of SbII-4 and the N terminus of SbII-5 (Fig. 7B). Of course, like SbII-4, this construct contains four F1 module binding segments, but they are now arranged in the wrong order to bind \(^{2}\)–\(^{3}\)F1 in a single NTD molecule (Fig. 7B). Choosing conditions similar to the SbII-4 experiment, injections of SbII-4.5 into a Fib solution only gave rise to small exothermic responses. The titration curve was linear and, therefore, could not be deconvoluted (Fig. 7B). Although it is not possible to infer from the data if four or only two F1 modules of Fib1 take part in binding to SbII-4.5, the titration curve clearly demonstrates that SbII-4.5 binds Fib1 with much lower affinity than SbII-4 (Fig. 7, A and B).

**DISCUSSION**

The striking similarities between FnBRs of different streptococcal and staphylococcal FnBPs had been noticed and discussed extensively (6). However, the lack of structural information for FnBRs hampered the interpretation of sequence alignments in terms of the binding sites in Fn. In addition, sequence comparisons could not always predict or explain the binding properties of apparently homologous repeats. The cross-reactivity observed in inhibition studies with FnBRs from different species was also difficult to interpret (5, 35, 41).

The first structural information for FnBP-Fn interactions paved the way to a better understanding of the molecular basis of this important host-pathogen interaction, and a novel mech-
A strong test for the tandem beta-zipper model and the relevance of the results obtained with the dissection approach is the measurement of binding using intact FnBRs. Assembly of specific F1-binding segments in the correct order to match the E-strand of F1 modules, are consistent with the bacterial motifs binding the individual modules primarily by forming an additional beta-strand along the E-strand of each F1 module, as was observed previously for 4F1 binding to an FnBR peptide (11). Confirmation of this mechanism of binding awaits the determination of other F1 module-FnBR peptide complex structures. This work is currently under way.

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ments including variants of the 70-kDa fragment (comprising NTD and the gelatin-binding fragment (GBF)) with deleted F1 modules in the NTD were used. The data were interpreted in terms of parallel binding of streptococcal peptides to NTD. Parallel binding is not consistent with either the data presented here or previously (11). In the light of the tandem zipper model, where an array of binding sites in the correct sequence is crucial for activity, the use of gap constructs of the NTD (42) might explain the apparent discrepancy between the results.

Full Fn binding activity of SfbI (or PrtF1) requires both FnBR and a second, upstream Fn-binding region (UR, Fig. 1) as well as both the NTD and GBF from Fn (39). Upstream regions with differing boundaries have also been referred to as spacer (43), UFBD (44), or FUD (42). It was suggested that UR and FnBRs independently mediate adherence of bacteria to Fn, with UR acting as the high affinity Fn-binding site (39). However, the UR construct, as defined by Ozeri et al. (39), contains, according to our definition of FnBRs, an almost complete FnBR (SfbI-1) that would bind to the NTD. Because the N-terminal region of this UR construct contains a GBF-binding site (40), this might explain the high affinity binding of this construct to Fn. Talay and co-workers (40) suggest an anti-parallel binding mode of SfbI to Fn where the FnBR region interacts with the NTD and activates binding of UR to GBF. It will be interesting to examine how SfbI binding to the NTD extends into the GBF and whether any of the four F1 modules in GBF (6F1 and 7–9F1; Fig. 1C) is involved in binding to UR.

We have demonstrated with this paper that the FnBR/Fn interaction involves the binding of four or five short consecutive motifs in FnBRs to four or five sequential F1 modules in the NTD. As a result, bound FnBRs form a large intermolecular surface with their modular target. The very significant conformational change that natively unfolded FnBRs undergo upon binding is entropically highly unfavorable. The disordered nature of unbound FnBRs may be the perfect starting point for the adoption of an extended conformation. This is a striking example for the efficiency of intrinsically disordered proteins, which can present much larger interaction surfaces than a globular protein of the same size (45). Many mosaic proteins contain strings of modules with external β-strands, so it is tempting to suggest that the Fn/FnBR interaction is just the first example of this unusual mechanism of protein-protein interaction.

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**Table II**

| FnBR  | SfbI residues | c_{max} | c_{bind} | ΔH  | ΔS  | K_d  |
|-------|---------------|--------|----------|-----|-----|------|
| SfbI-1| 396–430       | 100    | 7        | -22 | -41 | 168 ± 16 |
| SfbI-4| 505–541       | 91     | 7        | -23 | -44 | 62 ± 6 |
| SfbI-4.5| 521–560      | 100    | 6 (exothermic) | n/f | n/f | n/f |
| SfbI-5| 542–591       | 29     | 2        | -44 | -102| 2 ± 0.2 |

a Values reported previously (11).

b n/f, non-fittable.

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**Fig. 7.** Isothermal titration calorimetry profile for the interaction of NTD with SfbI FnBRs. Binding of Fib1 (containing the NTD of Fn) to SfbI-4 (match binding) (A), SfbI-4.5 (mismatch binding) (B), and SfbI-5 (match binding) (C) is shown. Ways in which the two components in each titration are likely to interact are indicated in each panel.
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