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Evidence for Steric Regulation of Fibrinogen Binding to Staphylococcus aureus Fibronectin-binding Protein A (FnBPA)*

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Background: Staphylococcus aureus fibronectin-binding protein A (FnBPA) binds fibronectin and fibrinogen at adjacent sites.

Results: The fibrinogen-binding mechanism is similar but not identical to homologous bacterial proteins. Ternary complex formation by intact fibronectin and fibrinogen on adjacent FnBPA sites could not be demonstrated.

Conclusion: Fibrinogen binding is sterically regulated by fibronectin binding.

Significance: Steric regulation might result in targeting of S. aureus to fibrin clots.

The adjacent fibrinogen (Fg)- and fibronectin (Fn)-binding sites on Fn-binding protein A (FnBPA), a cell surface protein from Staphylococcus aureus, are implicated in the initiation and persistence of infection. FnBPA contains a single Fg-binding site (that also binds elastin) and multiple Fn-binding sites. Here, we solved the structure of the N2N3 domains containing the Fg-binding site of FnBPA in the apo form and in complex with a Fg peptide. The Fg binding mechanism is similar to that of homologous bacterial proteins but without the requirement for “latch” strand residues. We show that the Fg-binding sites and the most N-terminal Fn-binding sites are nonoverlapping but in close proximity. Although Fg and a subdomain of Fn can form a ternary complex on an FnBPA protein construct containing a Fg-binding site and single Fn-binding site, binding of intact Fn appears to inhibit Fg binding, suggesting steric regulation. Given the concentrations of Fn and Fg in the plasma, this mechanism might result in targeting of S. aureus to fibrin-rich thrombi or elastin-rich tissues.

Staphylococcus aureus is a Gram-positive bacterium that can cause a variety of infections ranging from superficial skin infections to severe invasive infections, such as infective endocarditis (1); the emergence of antibiotic-resistant strains (2) presents significant therapeutic challenges. Infective endocarditis is an infection of the endocardium, often including the valves, and is a complication of S. aureus bacteremia (3). Infective endocarditis is associated with high morbidity and mortality (4, 5), and its development relies on interactions between S. aureus proteins and host proteins. The bacterial cell surface fibronectin (Fn)-binding protein A (FnBPA) has been implicated in this infection (6). The N-terminal region of FnBPA contains three subdomains N1–N3; N2N3 binds fibrinogen (Fg) and elastin (7, 8) (see Fig. 1A). The adjacent repetitive region of FnBPA contains multiple binding sites for Fn (Fig. 1A) (9, 10). Fn and Fg binding has been proposed to cooperate in infective endocarditis (6). FnBPA N2N3 has sequence homology with the N2N3 domains of the Fg-binding proteins ClfA (from S. aureus) (Fig. 1B) and SdrG (from Staphylococcus epidermidis); 24 and 22% of the residues are identical, respectively. ClfA has also been demonstrated to act as a virulence factor in infections such as septic arthritis (11) and experimental endocarditis (12). In ClfA (13) and SdrG (14), N2 and N3 both adopt an Ig-like fold; however, both proteins lack the C-terminal Fn-binding repeats found in FnBPA. The C terminus of SdrG contains two Cna_B (Pfam: PF05738) domains followed by a short serine-rich sequence, whereas ClfA lacks the Cna_B domains but has a longer serine-rich sequence (15). To date, there is no structural information for the N1 domain of ClfA, SdrG, or FnBPA.

Fg is a ~340-kDa glycoprotein comprising a dimer of heterotrimeric chains αA, βB, and γ (16). Fg is present in human blood at ~2–4 g/liter (17) and, when converted to fibrin (through the action of thrombin), plays a key structural role in the clot (17). Binding sites for integrins (18) and growth factors (19) within Fg are involved in hemostasis and wound healing. ClfA (20) and FnBPA (21) both bind near the C terminus of the most abundant fibrinogen γ-chain isoform γA; SdrG binds the γC domains of Fg (22). FnBPA binds to fibrin clots.

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† Author’s Choice—Final version full access.

The atomic coordinates and structure factors (codes 4B5Z and 4B60) have been deposited in the Protein Data Bank (http://wwpdb.org/).

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The abbreviations used are: Fn, fibronectin; Fg, fibrinogen; FnBPA, fibronectin-binding protein A; rFnBPA, recombinant fragment of FnBPA; NTD, N-terminal domain of fibronectin (proteolytic fragment); FgD, proteolytic fragment of Fg; FnBR, fibronectin (NTD)-binding repeat, FnBPA-1-11; FgC, synthetic peptide comprising 17 C-terminal residues of the Fg γA chain; AF1, recombinant FnBPA protein construct comprising the adjacent Fg and Fn-binding sites in FnBPA; r.m.s.d., root mean square deviation; ITC, isothermal titration calorimetry; SPR, surface plasmon resonance.
N terminus of the β chain (22). In binding to N2N3 of ClfA and SdrG, the different Fg peptides each form a β-strand conformation along the C-terminal strand of N3. Fg strand formation is parallel in ClfA binding to the γ-chain (23) and anti-parallel in SdrG binding (14) to the β-chain. In addition to strand formation, in the SdrG/Fg interaction, a C-terminal sequence beyond N3 binds back along N2 in a mechanism referred to as “dock, lock, latch,” with the N2-binding sequence being the “latch” (14). The ClfA-Fg interaction forms through a variation of this mechanism that includes latching strand formation along N2 (23).

Fn is a glycoprotein present in a soluble dimeric form (~450 kDa) in human blood plasma (0.3–0.4 g/liter) (24) or in the form of insoluble fibrils in extracellular matrices (25, 26). Fibronectin plays important roles in cell migration during development and wound healing (27) and thrombosis (28). FnBPA targets the N-terminal domain (NTD) of Fn, which is development and wound healing (27) and thrombosis (28).

We show that although the Fg-binding site and the most N-terminal Fn-binding site on FnBPA are in close proximity, the conformational change occurs in FnBPA on peptide binding. (a protolytic fragment of Fg) binding and that only a minimal FnBPA comprises 363 residues and contains eleven homologous, intrinsically unstructured repeats (Fn-binding repeats (FnBRs)), FnBPA-1 to FnBPA-11 (8, 29, 30) (Fig. 1A). At least six FnBRs are able to bind with nanomolar affinity to the Fn NTD (31). Each FnBR/NTD interaction occurs via an unusual tandem β-zipper mechanism in which the FnBR forms an additional anti-parallel β-strand along the triple-stranded β-sheets of sequential F1 modules (32, 33).

Although structural details of the Fg-FnBPA interaction have been unknown to date, based on the ClfA/Fg peptide structure (23) and the FnBPA-1/NTD structure (33), the Fg- and Fn-binding sites on FnBPA are in close proximity (Fig. 1B), suggesting that cooperativity (positive or negative) might be involved in ternary complex formation. Here we solve the structure of N2N3 from FnBPA in its apo and Fg peptide-bound forms. We show that the latch strand is not required for Fg peptide or FgD (a proteolytic fragment of Fg) binding and that only a minimal conformational change occurs in FnBPA on peptide binding. We show that although the Fg-binding site and the most N-terminal Fn-binding site on FnBPA are in close proximity, the residues involved are nonoverlapping. However, studies using intact Fn and Fg and their subdomains, and an FnBPA protein construct containing the adjacent Fg- and Fn-binding sites, provide evidence that Fg binding is sterically regulated by binding of Fn. This regulation is likely to have important implications for S. aureus interactions with host molecules in vivo.

EXPERIMENTAL PROCEDURES

Molecular Biology—Genes encoding the rFnBPA protein constructs (rFnBPA(189–511) and rFnBPA(189–505)) and AF1(rFnBPA(189–550)); Fig. 1A) were subcloned from a pQE-30 vector containing the cDNA of full-length FnBPA (GenBank™ accession number J04151.1, S. aureus 8325-4 strain) into pET-YSBLIC-3C (34). Sequences were confirmed by in-house DNA sequencing.

Expression and Purification of FnBPA constructs were expressed with an N-terminal His8 tag in Escherichia coli BL21 (DE3) Gold cells and purified using nickel affinity chromatography. The His tag was cleaved using HRV 3C protease, and after nickel affinity chromatography to remove the tag and uncleaved material, cleaved rFnBPA constructs were concentrated and further purified by size exclusion chromatography using a packed Superdex 75 16/65 HiLoad column (GE Healthcare). AF1 required additional anion exchange chromatography using HiTrap Q FF Sepharose (GE Healthcare) equilibrated in 20 mM Bis-Tris buffer, pH 6.2. The purity and molecular mass of final products were confirmed by SDS-PAGE and MS/ESI, respectively. Protein concentrations were determined from absorbance measurements at 280 nm.

Plasma Proteins and Peptides—Intact human plasma Fg (341 kDa, product no. 341576) and Fgd (a monomeric ~85-kDa proteolytic fragment of Fg containing the C-terminal regions of αα, ββ, and γ chains, product no. 341600) were purchased from Calbiochem-Merck-Millipore. Human plasma Fn (450 kDa, product no. F0895) and NTD (30-kDa proteolytic fragment of Fn, product no. F9911), which has been shown previously to bind FnBPA-1 (31), were purchased from Sigma-Aldrich. FgγC comprising the 17 C-terminal residues of the Fg γA chain (Ac-EGQQKLHGGAQAGDV-NH2) was purchased as a synthetic peptide from Severn Biotech Ltd. Fibronectin-free Fg was supplied by Enzyme Research Laboratories (Swansea, UK).

Isothermal Titration Calorimetry (ITC)—Experiments were performed using a Microcal VP-ITC calorimeter (GE Healthcare) in PBS (140 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 1.8 mM KH2PO4, pH 7.4) at 25 °C. Procedures were similar to those reported previously (35). Each titration started with one 2-μl injection followed by 27 × 10 μl injections at 0.5 μl/s using 6-min intervals. The stirring speed 307 rpm was used for all titrations except those involving Fg, where the stirring speed was increased to 321 rpm because of the higher viscosity of the Fg solution. Binding isotherms were fitted to a single-site binding model using nonlinear regression analysis in MicroCal-Origin 7.0 software.

Surface Plasmon Resonance (SPR)—Experiments were performed at 25 °C using a Biacore T100 system (GE Healthcare) upgraded to a T200 specification. Ligands in 10 mM sodium acetate (pH 5.5) were immobilized onto the experimental flow cell of a CM5 or C1 sensor chip (GE Healthcare) by amine coupling and subsequent blocking (36). The reference flow cell underwent identical but blank immobilization and blocking. Unless otherwise stated, low level immobilizations (50–150 response units) were used. The running buffer HBS-p + (10 mM HEPES, pH 7.4, 150 mM NaCl, 0.05% (v/v) polysorbate 20, GE Healthcare) was applied at 30 μl/min.

Analyte contact times were 60–300 s, dissociation times were 180–700 s, and stabilization times were 120–1500 s. Regenerations with low pH solutions were used only when needed and were optimized to minimize volume and harshness; pre- and postregeneration binding levels and curves were compared. Automated experiments were performed to measure dissociation constants (Kd values) by kinetic or equilibrium methods, and binding/inhibition. Five prior start-up cycles ensured stable baselines. For Kd determination, sensograms were measured for at least 10 sequential 2-fold analyte dilutions to cover the concentration range 0.1Kd–10Kd. Achievement of a steady-state response enabled Kd determination from the equilibrium binding (37). Alternatively, kinetic data series were

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TABLE 1
Crystallographic data collection and refinement statistics for rFnBPA(189–505) and the rFnBPA(189–505):FgγC complex
Data for the highest resolution shell are shown in parentheses where applicable.

| Parameters | rFnBPA(189–505) | rFnBPA(189–505):FgγC |
|------------|----------------|---------------------|
| Beamsline  | ID29          | 1041                |
| Space group| P2\_2\_2      | P1                  |
| Cell dimensions \(a, b, c\) (Å) | 62.6, 75.2, 85.5 | 37.5, 59.1, 73.5 |
| Cell dimensions \(\alpha, \beta, \gamma\) (°) | 90.0, 90.0, 90.0 | 91.8, 98.1, 97.9 |
| Wavelength (Å)    | 0.9763        | 0.9713              |
| Resolution (Å)    | 56.5–2.2      | 58.4–1.8            |
| \(R_{merge}\)    | 0.10 (0.52)   | 0.07 (0.36)         |
| \(I/\sigma(I)\)  | 8.6 (6.0)     | 9.1 (2.0)           |
| Completeness      | 99.2 (87.3)   | 93.6 (67.4)         |
| Redundancy        | 6.7 (3.6)     | 2.2 (2.2)           |
| No. of unique reflections | 21,217        | 51,268              |
| \(R_f\)          | 0.19          | 0.194               |
| \(R_{free}\)     | 0.24          | 0.24                |
| Mean B-factor (Å²) | 18.3          | 7.58                |
| r.m.s.d. bond lengths (Å²) | 0.02         | 0.02                |
| r.m.s.d. bond angles (°) | 1.89         | 1.95                |

fitted to a Langmuir 1:1 binding model. Response differences between sample and reference cells were analyzed using Evaluation Software (GE Healthcare). For \(K_d\) determination, an upper limit for analyte concentration for sensorgram analysis was determined to ensure reliable curve-fitting indicated by low \(\chi^2\) values. In the binding/inhibition experiments, either AF1 or the NTD was immobilized; pure or mixed proteins with known concentrations were the analytes.

Crystallography—Diffraction crystals of rFnBPA(189–505) grew in a Clear Strategy Screen II condition containing PEG 20000 (8%, w/v), PEG monomethyl ether 550 (8%, v/v), 0.2 M calcium acetate, with added 50 mM Tris-HCl, pH 7.4, 50 mM NaCl, at a protein concentration of 30 mg/ml using the sitting drop vapor diffusion method (38) combined with microseeding utilizing Seed Bead (Hampton Research). Clusters of protein crystals grew after 3 days at 18 °C. Clusters were transferred into cryo-protectant (reservoir solution with additional 10% (w/v) of PEG 20000 (8%, w/v), PEG monomethyl ether 550 (8%, v/v), 0.2 M NaCl, at a protein concentration of 30 mg/ml using the sitting drop vapor diffusion with the microseeding technique was set up as described above. The Ramachandran plot, 95.7% of residues are in the preferred region, 3.2% are in the allowed region, and 0.9% (three residues) are outliers. The rFnBPA(189–505):FgγC crystal diffracted to 1.83 Å, and the structure was solved by molecular replacement using the structure of rFnBPA(189–505) as a model. The refinement and validation was carried out as described above. The Ramachandran plot shows 96.5% of residues in the preferred region, 3.1% in the allowed region, and 0.3% (2 residues) as outliers. The atomic coordinates and structure factor amplitudes have been deposited in the Protein Data Bank under codes 4BSZ and 4B60.

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S. aureus Fn Binding Experiments—S. aureus expressing FnBPA constructs with differing numbers of FnBRs were produced, and binding experiments to immobilized Fn were performed, as previously described (47). Briefly, human plasma fibronectin (Sigma) was diluted in PBS and immobilized onto the plastic wells of an ELISA plate by incubation at 4 °C for 16 h. Unoccupied binding sites were blocked with 3% bovine serum albumin before the addition of ~10⁸ washed S. aureus cells expressing FnBPA constructs to each well. Experiments were done in the absence or presence of a range of concentrations of human fibrinogen. After incubation for 1 h at 37 °C, unbound bacteria were removed by three PBS washes. Adherent bacteria were quantified by crystal violet staining; bound dye was solubilized in 7% acetic acid, quantified by \(A_{595}\) measurements, and related to bacterial numbers by reference to standard plots (45).

Binding Site Occupancy Calculations—Relative occupancy of the Fg- and Fn-binding sites on AF1 was calculated by considering the binding as competitive. The apparent dissociation constant for Fg was determined according to the equation

\[ K_{d,Fg,	ext{app}} = K_{d,Fg}(1 + [Fg] / K_{d,Fn}) \]

The average number of Fg molecules bound per AF1 molecule (\(\alpha\)) was calculated using

\[ \alpha_{Fg} = (I_{Fg} / (K_{d,Fn} + [Fg])) \]

RESULTS

Structure of rFnBPA N2N3—Both rFnBPA(189–511) (the N2N3 domains including the latch strand) and rFnBPA(189–505) (without the majority of the equivalent strand-forming residues in the ClfA latch; Fig. 1) were used in crystallization trials. However, only rFnBPA(189–505) produced diffraction crystals. The structure of rFnBPA(189–505) comprises two distinct β-strand-dominated domains, N2 and N3, connected by an eight-residue linker (Fig. 2). The N-terminal N2 domain (residues 195–335) consists of nine β-strands arranged in a sandwich of four- and five-strand β-sheets. The N3 domain (residues 344–503) has a similar structure comprising two β-sheets, each formed by five β-strands. However, unlike N2, the two β-sheets within the N3 domain are linked by a short helix. The structures of N2 and N3 superimpose with a root mean square deviation (r.m.s.d.) of 3.17 Å. The structures of both domains resemble a distorted β-barrel rather than a typical β-sandwich. The N2 domain adopts the DE variant of a C-type IgG fold (DEv-IgG) identified...
in other proteins including ClfA (13). The asymmetric unit comprises a single copy of rFnBPA(189–505); no density was observed for the N- and C-terminal residues (residues 189–194 and 504–505, respectively). The N2-N3 interface buries 1024 Å² of surface area, and the main N2-N3 interdomain contacts originate from two regions: 1) interactions between two protruding loops that connect strands D1 and D1’ within the N3 domain and the E and F strands of the N2 domain and 2) interactions of the A strand of the N3 domain with a loop connecting the C and D strands in the N2 domain (Fig. 2).

The latch strand stabilizes Fg binding by only 2.5-fold—it was shown previously that the ClfA/Fg interactions are stabilized 10-fold by locking of the latch strand with a disulfide bond (23). To investigate potential stabilization provided by the FnBPA latch strand, the Kd values for interactions between FgD and rFnBPA(189–511) or rFnBPA(189–505) (Fig. 1A) were measured using SPR (Fig. 3, A and B). FgD was immobilized on the surface of a chip and subsequently exposed to increasing concentrations of rFnBPA(189–505) (Fig. 3A) or rFnBPA(189–511) (Fig. 3B). Kd values determined from equilibrium binding data were 3.7 ± 0.2 and 1.5 ± 0.1 μM for rFnBPA(189–505) and rFnBPA(189–511) respectively (Table 2). The similarity of these Kd values demonstrates that the presence of the putative latch strand only stabilizes the FnBPA-FgD interaction by ~2.5-fold. Hence, the rFnBPA(189–505)/FgγC peptide complex is a suitable minimal complex for crystallization.

Crystal Structure of the N2N3-FgγC Complex—The rFnBPA(189–505)/FgγC complex was solved using x-ray crystallography (Fig. 4A). The asymmetric unit comprises two copies

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TABLE 2
Summary of kinetic and thermodynamic parameters for specific interactions

| Interaction | Kd (μM) | ΔH (kcal/mol) | ΔS (cal/mol K) | n | Method |
|------------|---------|---------------|---------------|---|--------|
| rFnBPA(189–505)/FgD | 3.7 ± 0.1 | -6.1 ± 0.3 | -78.1 | 0.89 | SPR° |
| rFnBPA(189–511)/FgD | 1.5 ± 0.1 | -6.7 | 2.11 | 1TCA |
| AF1/Fg | 0.299 ± 0.001 | -38.5 | 6.7 | 0.1 | SPR° |
| AF1/FgD | 1.5 ± 0.1 | -6.1 | 2.11 | 1TCA |
| AF1/NTD | 0.391 ± 0.002 | -38.5 | 6.7 | 0.1 | SPR° |
| AF1/FgD | 0.7 ± 0.2 | -38.5 | 6.7 | 0.1 | SPR° |

*The data are shown in Fig. 3.
°The data are shown in Fig. 6.

Can AF1 Bind Fg and Fn Simultaneously?—The structure of the rFnBPA(189–505)/FgγC complex and of the previously determined structures of an FnBPA-1 (Fig. 1A) peptide in complex with 4F15F1 from the NTD of Fn (33) demonstrate that, although nonoverlapping, the Fg-binding site on FnBPA is in close proximity to the most N-terminal Fn-binding site (FnBPA-1). Given that Fg and Fn are both large proteins, the question of whether binding of one protein to FnBPA affects the binding of the other (either through conformational change or steric exclusion) arises (Fig. 5A). First, the ability of S. aureus expressing FnBPA with differing numbers of FnBRs to bind to Fn in the presence of increasing concentrations of Fg was tested. In support of the existence of negative cooperativity between the adjacent Fg- and Fn-binding sites on FnBPA, addition of excess Fg disrupted binding of bacteria to a Fn-coated plate (Fig. 5B). Importantly, at the lowest concentrations of Fg, significant inhibition of binding to Fn was only observed when S. aureus expressing an FnBPA construct with only a single
FnBR (FnBPA-1) was used. When more Fn-binding repeats were present, Fn binding plateaued at a higher level, suggesting, as might be expected, that only Fn binding to the FnBR adjacent to the Fg-binding site was inhibited by Fg. To investigate this potential negative cooperativity between Fg and Fn binding, AF1 (Fig. 1A) a recombinant FnBPA protein construct containing only the Fg (N2N3) binding site and the most N-terminal FnBR (FnBPA-1), was prepared. The first step was to measure the binding of the intact Fn and Fg and their subdomains to AF1.

The K_d values (Table 2) for the AF1-Fn (Fig. 6A; 0.36 ± 0.001 nM) and AF1/Fg interaction (Fig. 6B; 1.1 ± 0.1 μM) were measured using SPR kinetic analysis and ITC, respectively. The stoichiometry of 2:1 for the AF1/Fg interaction is consistent with the presence of two identical binding sites on intact dimeric Fg. The affinity of the AF1/FgD interaction (K_d 1.5 ± 0.1 μM) was determined using SPR equilibrium analysis (Fig. 6C). The K_d for the AF1/NTD interaction was determined using ITC (Fig. 6D) as 0.7 ± 0.2 nM. The steep transition of the binding curve resulted in a relatively high fit error (28%). Hence, this interaction was also measured using SPR kinetic analysis as 0.393 ± 0.002 nM (Fig. 6E) using increasing concentrations of the NTD as analyte and with AF1 immobilized onto a chip. Fig. 6 shows that both the Fg- and Fn-binding sites in AF1 are active. Because the K_d values for the AF1 interactions with Fg and FgD and with Fn and NTD are virtually identical, there appears to be no steric occlusion of the binding sites within AF1 when individual proteins are binding. The physiological relevance of AF1 was assessed by a plasma pulldown assay, which demonstrated that AF1 also binds Fg and Fn under the conditions of human plasma (data not shown). However, this experiment does not reveal whether Fg and Fn can bind simultaneously to the same AF1 molecule.

Fig. 7A shows, for the first time, that a subdomain of Fn (NTD), AF1, and intact Fg can form a ternary complex. Exposure of an NTD-coated chip to an AF1-Fg complex (0.5 μM) generated a response exceeding 650 response units, whereas separate injections of AF1 (0.5 μM) and Fg (0.5 μM) only produced responses of 50 and 0 response units, respectively. The formation of the ternary complex was blocked by addition of FgCy. The results clearly demonstrate that AF1 can bind both Fg and NTD simultaneously to form a ternary complex.

The binding of Fg and intact Fn to AF1 was then investigated using SPR (Fig. 7B). An AF1-coated surface was exposed to a solution containing either Fg (2.3, 23, and 90 μM) or Fn (23 and 230 nM); binding was observed in all cases, showing that both Fg- and Fn-binding sites were active on the chip surface. Next, Fg and Fn were flowed over the chip together at concentrations of 2.3 μM and 230 nM, respectively. This reflects the 10:1 Fg:Fn ratio present in human plasma (49, 50). The binding trace was virtually identical to that observed for Fn only (at 230 nM), suggesting that no Fg interacts when the AF1-coated surface is saturated with Fn. Next, Fg (90 μM) was added to a solution of Fn (23 nM) and injected over the surface. Now the trace was virtually identical to that given upon injection of Fg only at 90 μM. This suggested that the molar excess of Fg saturated the AF1-coated surface and blocked the AF1-Fn interaction. Thus when intact proteins are used, it appears that no ternary complex is formed. Note that when subsaturating concentrations of Fn (23 nM) and Fg (23 μM) are combined, there is an additive response in the SPR trace that likely reflects binding of Fg and Fn to separate AF1 molecules on the chip.

Relative Occupancy of the Fg- and Fn-Binding Sites of FnBPA in Plasma—When either Fg or Fn were present in large excess, SPR experiments suggested that the other ligand (Fn or Fg, respectively) could not bind (Fig. 7B) to AF1; that is, a ternary complex comprising AF1, Fn, and Fg was not observed. If only the affinities for the isolated binding sites in AF1 are considered, the reported concentrations of Fg (~3 g/liter; 9 μM) and Fn (0.3 g/liter; 0.7 μM) in plasma would lead to relative occupancies of 0.89 and 1.0 for the Fg- and Fn-binding sites, respectively. However, Fig. 7B suggests that Fg and Fn compete for binding to AF1. It is possible to calculate a reduced “apparent” K_d, for binding of one protein to FnBPA in the presence of a specific concentration of an inhibitor. When the concentration of the two proteins in the plasma is taken into account, the relative occupancies of the Fg- and Fn-binding sites at equilibrium are predicted to be 0.004 and 0.99, respectively. This sug-
suggests that the Fg-binding site on FnBPA is predominantly unoccupied during *S. aureus* bacteremia.

**DISCUSSION**

FnBPA was previously identified as a virulence factor in *S. aureus* infective endocarditis, with the Fg- and Fn-binding sites (21) synergistically promoting infection (6, 51). This work set out to characterize the FnBPA Fg-binding domain and its interaction with Fg and to test whether Fg and Fn can bind FnBPA simultaneously. Although the FnBPA/Fn interaction has been the subject of extensive structural characterization (31, 33, 52), no experimentally determined structure of FnBPA bound to its target site in Fg has previously been reported.

Previous studies of Fg binding to the bacterial proteins SdrG and ClfA revealed a mechanism of binding called *dock, lock, latch* (14). In SdrG, on binding of the Fg peptide the latch (a sequence at the C terminus of N3) forms a stabilizing strand/strand interaction along a *H*-strand in N2. In ClfA, the role of the latch is less clear (Protein Data Bank code 2VR3 (23)) because the deposited Fg peptide/ClfA structure contains an engineered disulfide bond between the latch and N2. In the apo-structure the latch sequence was either significantly truncated in the modeled structure, suggesting disorder (SdrG; Protein Data Bank code 2R19 (14)), or was stabilized by a short strand/strand interaction along N3 (ClfA; Protein Data Bank code 1N67 (13)). Sequence similarity between ClfA and FnBPA, the demonstration that the target site in Fg is the same, and that the affinity of the interaction is similar suggest both a similar mechanism of Fg binding for the two *S. aureus* proteins and a potentially puzzling level of redundancy.

In the present study, only a construct (FnBPA(189–505)) lacking the majority of the latch strand residues formed well diffracting crystals, and the affinity of the interaction of FgD with N2N3-containing constructs was reduced only ~2.5-fold by this truncation (Fig. 3). Further truncation (to FnBPA residue 498) has been previously shown to disrupt Fg and elastin bind-
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The structures of N2N3 (without latch) in isolation and in complex with FγC revealed, as expected, similarities in the apo-structures and in the mechanism of FγC binding between FnPBA and ClfA. Both N2 and N3 adopt Ig-like folds; N2 adopted the DEv-IgG variant described previously (13). The structure of the complex revealed parallel β-zipper binding by the peptide along the G' strand of N3 (as in ClfA) and within a cleft formed predominantly by residues with hydrophobic side chains, stretching between the N2 and N3 domains. Previous studies (53, 54) suggested the C terminus of the Fγ C chain is intrinsically disordered. Thus it is likely to undergo a disorder to order transition upon binding to FnBPA. It fits into the cleft, causing the N3-G' strand to translocate and wrap around the Fγ peptide C terminus, establishing a relatively large interface.

There are also important differences in the binding mechanism compared with ClfA. Clearly, the latch β-strand residues are absent in the FnBPA(189–505)FγC structure. Rather, the data suggest that only the first few residues of the putative latch region (Tyr-501 and Asn-503) might be required for Fγ binding. In a recent structural analysis of N2N3 from ClfB binding to a peptide from the Fγ (containing adjacent Fγ- and Fn-binding sites) represents an unusual opportunity to characterize formation of a ternary complex. Previous structural studies of FnBPA-1 in complex with the Fγ F1 module pair (51F15F1) and the structure of N2N3 in complex with an Fγ peptide presented here clearly show that the adjacent binding sites for Fγ and Fn in intact FnBPA are in close proximity but do not overlap. In particular, the lack of the requirement for latching strand residues in the
rFnBPA<sub>(189–505)/FgD</sub> interactions (Table 2) suggested larger potential separation in the primary sequence between residues in FnBPA directly involved in binding of Fg and those affected by binding of Fn to the most N-terminal FnBR (FnBPA-1). However, given that both the rFnBPA<sub>(189–505)/FgYc</sub> and FnBPA-1<sub>1–4F1/F1</sub> interactions involve strand formation, the potential for positive cooperativity through latch strand stabilization adjacent to the strand-forming residues of FnBPA-1 exists (Fig. 5A). For example, positive cooperativity was observed previously in NTD binding to adjacent FnBRs in a streptococcal protein (56). Alternatively, steric regulation of binding to the adjacent sites (because both intact Fg and intact Fn are large proteins; Fig. 5A) might occur. Fig. 5B provides the first evidence for steric regulation of the adjacent binding sites when FnBPA is expressed on the surface of <i>S. aureus</i>.

Investigations at the molecular level show that the adjacent Fg- and Fn-binding sites of FnBPA (when expressed recombinantly as AF1) can form a ternary complex with Fg and the NTD subdomain of Fn (Fig. 7A). However, in Fig. 7B in which intact Fg and Fn were used, ternary complex formation was not observed. These two results together argue in favor of negative cooperativity arising though steric exclusion. The linear, and nonoverlapping, nature of the Fg- and Fn-binding sites in AF1 suggests that this result can be extrapolated to intact FnBPA and provides an explanation for the inhibition observed in Fig. 5B.

Although Fg is at a 10-fold higher concentration than Fn in the plasma, occupancy analysis predicts that Fn binding predominates because of the higher affinity of the interaction. This makes sense because FnBPA, although having only one Fg-binding site, has multiple Fn-binding sites (31), so inhibition of binding of Fn (by Fg) to the most N-terminal site would have little overall affect because Fg could (presumably) bind at the other sites unaffected by Fg binding.

Intriguingly, the binding of ClfA to Fg has been shown previously also to be negatively regulated, in this case by Ca<sup>2+</sup>-binding (57). The Ca<sup>2+</sup>-binding site is not conserved in the N2N3 domains of FnBPA, so it is interesting that a different mechanism of negative regulation seems to present. The importance of Fg binding (by either FnBPA or ClfA) for the initiation of infection in infective endocarditis has been clearly demonstrated (6, 51). How might regulation of the Fg-binding site though steric exclusion (or Ca<sup>2+</sup> binding) aid initiation of infection? One possibility, suggested previously by O’Connell et al. (57) is that negative regulation of Fg binding might result in targeting of <i>S. aureus</i> to solid phase fibrinogen or fibrin clots. The C terminus of the γ chain of Fg is not only present in soluble Fg but also in cross-linked fibrin, and here it will have a higher local concentration and may thus be able to compete with Fn binding. <i>S. aureus</i> has previously been shown to bind more effectively to fibrin than fibrinogen (58). In the case of FnBPA where steric regulation appears to be operating, the accessibility of the binding site is also likely to play a role. Lastly, FnBPA also binds elastin (8) at a site that overlaps with the Fg peptide-binding site. Elastin binding to FnBPA was inhibited at lower concentrations of Fg peptide than Fg binding to FnBPA (8). Thus it is also possible that maintenance of a free binding site in N2N3 results in targeting of bacteria to elastin-rich tissues such as blood vessel walls during the initiation of infection.

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