Amino acid polymorphisms in the fibronectin-binding repeats of fibronectin-binding protein A affect bond strength and fibronectin conformation

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The Staphylococcus aureus cell surface contains cell wall-anchored proteins such as fibronectin-binding protein A (FnBPA) that bind to host ligands (e.g. fibronectin; Fn) present in the extracellular matrix of tissue or coatings on cardiac implants. Recent clinical studies have found a correlation between cardiovasculare infections caused by S. aureus and nonsynonymous SNPs in FnBPA. Atomic force microscopy (AFM), surface plasmon resonance (SPR), and molecular simulations were used to investigate interactions between Fn and each of eight 20-mer peptide variants containing amino acids Ala, Asn, Gln, His, Ile, and Lys at positions equivalent to 782 and/or 786 in Fn-binding repeat-9 of FnBPA. Experimentally measured bond lifetimes (1/\(k_{\text{off}}\)) and dissociation constants (\(K_D = k_{\text{off}}/k_{\text{on}}\)) determined by mechanically dissociating the Fn-peptide complex at loading rates relevant to the cardiovascular system, varied from the lowest-affinity H782A/K786A peptide (0.011 s, 747 \(\mu\)M) to the highest-affinity H782Q/K786N peptide (0.192 s, 15.7 \(\mu\)M). These atomic force microscopy results tracked remarkably well to metadynamics simulations in which peptide detachment was defined solely by the free-energy landscape. Simulations and SPR experiments suggested that an Fn conformational change may enhance the stability of the binding complex for peptides with K786I or H782Q/K786I (\(K_{D}^{\text{pp}} = 0.2\)–0.5 \(\mu\)M, as determined by SPR) compared with the lowest-affinity double-alanine peptide (\(K_{D}^{\text{pp}} = 3.8\) \(\mu\)M). Together, these findings demonstrate that amino acid substitutions in Fn-binding repeat-9 can significantly affect bond strength and influence the conformation of Fn upon binding. They provide a mechanistic explanation for the observation of nonsynonymous SNPs in fnbA among clinical isolates of S. aureus that cause endovascular infections.

Staphylococcus aureus is a Gram-positive bacterium that has a symbiotic relationship with humans. Its primary reservoir is the nares (1), although other sites may be colonized, such as the throat, groin, and axillae (2, 3). This microorganism also is an opportunistic pathogen and can cause serious endovascular infections if bacteria access the bloodstream (4, 5). These kinds of infections have been linked, at least in part, to interactions between host fibronectin (Fn) and Fn-binding protein (e.g. FnBPA encoded by fnbA) anchored to the outer cell wall of S. aureus (6–9).

This particular ligand–receptor interaction involves contacts between several type I modules (F1) at the N terminus of Fn and Fn-binding repeats (FnBRs), each ~40 amino acids, within FnBPA. Six of the 11 repeats in FnBPA exhibit high affinity toward Fn (10, 11). Some of these high-affinity repeats, such as the FnBR-9 of fnbA, have been found to contain nonsynonymous single-nucleotide polymorphisms (SNPs) associated with infective endocarditis and cardiovascular device infections (12–15).

Here we used single molecule (atomic force microscopy; AFM) and ensemble (surface plasmon resonance; SPR) approaches to experimentally probe association and dissociation reactions between Fn and polymorphic peptides synthesized to resemble different variants of FnBR-9. Molecular dynamics (MD) were also performed to simulate the dissociation pathway of each peptide. AFM data and MD simulations reveal an energy landscape defined by multiple barriers. At high loading rates, amino acid substitutions H782Q and K786N sig-

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significantly alter bond strength and unstressed off-rate \( (k_{off}) \) upon dissociation from fibronectin. SPR data, along with metatodynamics and MD simulations, suggest that amino acid substitutions H782Q and K786I may impart conformational variability to the complex, which in turn affects affinity and detachment pathway. This information provides a mechanistic explanation for the correlation between SNPs in FnBPA and endovascular infections of implants or tissue by *S. aureus*.

### Results

**Peptides used in experiments and simulations**

A series of synthetic 20-mer peptides, synthesized to resemble FnBR-9, were used in AFM, SPR, and computational simulations of binding reactions with Fn (Table 1). Fig. 1A shows a schematic structure of Fn in complex with FnBR-9, based on X-ray diffraction (11). Herein, we focused on the portion of FnBR-9 that overlaps with the binding site in Fn, two F1 domains, \(^3\)F1 and \(^2\)F1 (Fig. 1B). Two residues within this region, corresponding to amino acids 782 and 786 in FnBPA in *S. aureus* 8325, were changed to match the nonsynonymous SNPs associated with endovascular disease in humans (Table 1). Negative controls were created using alanine as the replacement residue, an approach that has long been used to identify specific side chains that strongly modulate binding (16–18), including for bacterial FnBRs (19).

AFM utilizes an optical lever detection system to monitor the deflection (nm) of a flexible cantilever as a tip, on the end of the cantilever, approaches, touches, and is then retracted from a sample. Interaction forces are determined by multiplying the work of detachment, respectively, from immobilized Fn at one temperature. Several thousand approach-retraction curves were collected for each peptide. Unbinding or dissociation events (Fig. 2) were observed in < 30% of touches, indicating that most detected events were due to single bonds (20, 21).

The work of detachment was calculated by integrating force against distance (see shaded region in Fig. 2, inset). Unlike the force values, which include only the rupture of a bond, the determination of work includes contributions from breaking a bond as well as stretching of various elastic elements and unfolded segments in Fn. Still, we have decided to include work values because this parameter has been reported for other ligand-receptor pairs (e.g. see Refs. 22–26).

**Direct measure of Fn-FnBR-9 bond strength**

AFM was used to measure the force needed to break the bond between Fn immobilized on a slide and each of seven different peptides immobilized on an AFM tip. Force measurements were performed in phosphate-buffered saline (pH 7.4) at room temperature. Several thousand approach-retraction curves were collected for each peptide. Unbinding or dissociation events (Fig. 2) were observed in < 30% of touches, indicating that most detected events were due to single bonds (20, 21).

The work of detachment was calculated by integrating force with respect to distance (see shaded region in Fig. 2, inset). Unlike the force values, which include only the rupture of a bond, the determination of work includes contributions from breaking a bond as well as stretching of various elastic elements and unfolded segments in Fn. Still, we have decided to include work values because this parameter has been reported for other ligand-receptor pairs (e.g. see Refs. 22–26).

Fig. 3, A and B, are box-and-whisker plots of the force and work of detachment, respectively, from immobilized Fn at one retraction velocity. Bean plots were constructed to visualize the underlying data distribution (Fig. 3C). Similar shapes indicate a similar distribution for all peptides (27–29). The wild-type peptide serves as a reference with median force and energy of binding equal to 0.182 nN and 1.15 aJ, respectively. Non-overlapping notches, shown in Fig. 3, A and B, give 95% confidence that

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**Table 1**

| Peptide name | Amino acid sequence (positions 778-796 in *S. aureus* FnBPA) | associated clinical disease |
|--------------|---------------------------------------------------------------|-----------------------------|
| Wild type    | VPQHGQNKAGNSQFEEDTE\(^a\)                                    | reference                   |
| H782Q        | VPQHGQNKAGNSQFEEDTE                                          | CDI                         |
| K786N\(^b\)  | VPQHGQNGNQSFEEDTE                                            | CDI                         |
| H782Q/K786N  | VPQHGQNGNQSFEEDTE                                            | CDI                         |
| K786I        | VPQHGQNGNQSFEEDTE                                            | PB                          |
| H782Q/K786I  | VPQHGQNGNQSFEEDTE                                            | PB                          |
| H782A        | VPQHAGQNKAGNSQFEEDTE                                          | negative control            |
| H782A/K786A  | VPQHAGQNGNQSFEEDTE                                            | negative control            |

\(^a\) Only molecular dynamics simulations were performed on this peptide.

\(^b\) For AFM, a Cys residue was added to the C terminus so that the peptide could be linked to a gold-coated tip. For MD simulations, an Ala residue was placed at the C terminus.

\(^c\) CDI, cardiovascular device infection; PB, persistent bacteremia (no indwelling cardiac device).
two medians differ. The Mann-Whitney (Wilcoxon rank-sum) test and \( t \) test were used to determine \( p \) values relative to the wild-type peptide (supplemental Table S1).

Compared with the wild-type peptide, the single- and double-alanine mutants exhibit weaker binding in terms of both force and energy. All other amino acid replacements exhibit statistically stronger binding relative to the wild-type peptide, with the exception of the energy approximation for Fn H782Q. The peptides H782Q, K786I, H782Q/K786N, and H782Q/K786I each exhibit a statistically stronger force of binding, as determined by AFM relative to the two negative-control alanine peptides (\( p < 0.0001; \) Mann-Whitney test).

**AFM measure of off-rate binding constant \((k_{\text{off}})\)**

The above results describe the tensile bond strength at one speed (i.e. retraction velocity). Bond strength is actually dependent upon the loading rate (pN s\(^{-1}\)) on the bond. When an external force is applied slowly (low loading rate), then dissociation occurs at small force values, whereas large rupture forces occur when a binding complex is subjected to high load rates (21, 30).

The relationship between unbinding/rupture force \((f)\) and loading rate \((r)\) is described by the Bell model (31, 32),

\[
f = k_BT/x_\beta \times \ln (rx_\beta/k_{\text{off}}T),
\]

where \( r \) (pN s\(^{-1}\)) is the product of the retraction velocity (nm s\(^{-1}\)) and the slope of the force-distance curve at unbinding (pN nm\(^{-1}\)) (21, 23, 33), \( k_{\text{off}} \) (s\(^{-1}\)) is the dissociation rate constant in the absence of the applied force, \( x_\beta \) (nm) is the potential barrier position, and \( k_BT \) is 4.1 pN nm at room temperature. After plotting \( f \) versus \( \ln r \), the unstressed off-rate \( (k_{\text{off}})\) and the separation distance along the reaction coordinate \((x_\beta)\) can be determined from the intercept with the abscissa (at \( f = 0 \)) and the slope of the fitted line, respectively (34, 35).

To utilize the Bell model, we determined the force of unbinding for each peptide from \( 10^3 \) to \( 10^5 \) pN s\(^{-1}\) (Fig. 4 and supplemental Fig. S1). This range in loading rate was selected to

**Energy landscape for dissociation of Fn-FnBR-9 bond**

Figure 2. Example AFM force spectra between full-length Fn and a synthetic peptide that mimics a portion of repeat 9 from FnBPA (blue) versus nonspecific interactions between Fn and a glass surface (black). By convention, negative values indicate attractive force in nanonewtons (\( 10^{-9} \) N). The arrow points to the “rupture event,” force at bond dissociation, for this spectrum. The inset shows the energy/work of binding (i.e. integrated force with respect to distance).

Figure 3. Top panels, box-and-whisker plots of experimentally measured rupture forces (in nanonewtons) (A) and work of binding (in attojoules) (B) for immobilized Fn and each of the seven peptides synthesized to resemble a portion of the wild-type sequence of FnBR-9 (VPQIHGQNKGNQSFEEDTE) and the following polymorphisms: H782Q, H782Q/K786N, K786I, H782Q/K786I, H782A, and H782A/K786A. These data were collected at the same retraction velocity. Box ends represent the first and third quartiles. Whiskers extend to the 9th and 91st percentiles. Center lines show the medians, and crosses represent the means. The width of the boxes is proportional to the square root of the sample size (\( n = 501, 649, 721, 418, 485, 390, \) and 337 sample points). Box notches represent the 95% confidence interval for each median (defined as \( \pm 1.58 \times \) interquartile range/\( \sqrt{n} \) (86)). C, bean plots of the force data. Blue polygons represent the estimated density of the data. Green lines show each observation as a scatter plot. Black lines represent the medians. Bean plots are less statistically informative that the box-and-whisker plots but allow better visualization of the data distribution (29).
simulate physiologically relevant loads that may be experienced by adhesins on bacterial circulating within the cardiovascular system. For example, selectin molecules that mediate tethering or rolling of circulating leukocytes on vascular surfaces experience loading rates of $10^3$ to $10^6$ pN s$^{-1}$ (23, 24, 33, 36, 37). For the mammalian heart, the loading at valve closure computed from in vivo ventricular pressure recordings ranges from 100,000 to 400,000 N s$^{-1}$ m$^{-2}$ (38, 39). This corresponds to loading rates of up to $3 \times 10^5$ pN s$^{-1}$ for a bacterium (~500-nm radius). Similarly, Bustanji et al. (40) estimate that under physiological conditions, receptors on a bacterium experience loading rates from $3 \times 10^4$ to $6 \times 10^5$ pN s$^{-1}$ when moving in shear flow over a substrate.

The Bell parameters, namely the unstressed off-rate ($k_{\text{off}}$) and potential barrier position ($x_p$), were determined for each peptide by a least-squares fit to the linear region of the rupture force versus logarithm of the loading rate (Fig. 4A and supplemental Fig. S1). All peptides exhibited a similar $x_p$ value (0.05 nm) that is comparable with published $x_p$ values for adhesin molecules subjected to relatively high loading rates (20, 23, 24, 36, 41–46). Table 2 shows the tabulated $k_{\text{off}}$ values for each peptide dissociating from immobilized Fn. The double-alanine mutant exhibited the shortest bond lifetime ($1/k_{\text{off}} = 0.011$ s), whereas H782Q/K786N exhibited the longest bond lifetime (0.192 s).

### AFM-measured off-rate ($k_{\text{off}}$) for interactions with the N-terminal domain (NTD) of Fn

A primary focus of this work is the examination of binding reactions on immobilized, full-length Fn. This is due to the clinical pathogenesis of disease in vivo, where bacterial pathogens use Fn-binding proteins to interact with extracellular matrix and ligands adsorbed on implanted medical devices. To further explore the binding reactions between peptides and Fn, we determined the unstressed off-rate for interactions using only the NTD of Fn (NTD-Fn). These experiments were performed on the wild-type peptide and each of the double mutant peptides. We focused on the double rather than single mutants because multiple polymorphisms have been linked to disease in humans (12, 14, 15, 47). Supplemental Table S2 shows these $k_{\text{off}}$ values for NTD-Fn. Similar to the results for full-length Fn, the H782Q/K786N peptide exhibited the longest bond lifetime (0.029 s). Relative to interactions with Fn, the bond lifetimes for each peptide were shorter for interactions with the NTD-Fn. Norris et al. observed a similar decrease in bond lifetime (~30×) for peptide-binding reactions with the NTD-Fn relative to interactions with full-length Fn (19).

### AFM measure of on-rate binding constant ($k_{\text{on}}$)

AFM was also used to determine the on-rate for binding to immobilized Fn. This was accomplished by determining the binding probability as a function of the contact or dwell time for each peptide (21, 48). Longer dwell times result in higher probability of binding until reaching a plateau (Fig. 4B and Supplemental Fig. S2). Assuming first-order kinetics for the Fn-peptide interaction, $k_{\text{on}} = (\tau \times C_{\text{eff}})^{-1}$, where $\tau$ is the interaction time and $C_{\text{eff}}$ is the effective concentration (21, 49). The interaction time was calculated from the binding probability ($p$) at different dwell times by using $p = A(1 - \exp(-t - t_0)/\tau)$, with

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**Figure 4.** A, plot showing the dependence of unbinding forces (in piconewtons, $10^{-12}$ N) on the logarithm of the loading rate (pN s$^{-1}$). A linear regression was used to determine the unstressed off-rate ($k_{\text{off}}$). B, also shown is the binding probability as a function of interaction time, which was used to determine $k_{\text{on}}$. Plots for each peptide are shown in the supplemental material.

**Table 2**

| Construct             | $k_{\text{off}}$ | $k_m$ | $K_d$ | $\Delta G$ | $\Delta \Delta G$ versus AA (or A) |
|-----------------------|------------------|-------|------|------------|-----------------------------------|
| H782A/K786A           | 93.0 ± 11.0      | 1.3 ± 0.9 | 74.7 ± 54.5 | -4.26 | 0.00 |
| H782A                 | 25.6 ± 5.0       | 1.1 ± 0.6 | 240.4 ± 134.9 | -4.93 | (0.00) |
| K786I                 | 22.6 ± 1.9       | 1.0 ± 0.5 | 222.6 ± 100.8 | -4.98 | ND$^a$ |
| H782Q/K786I           | 19.9 ± 0.6       | 1.1 ± 1.0 | 189.1 ± 178.9 | -5.08 | 0.81 |
| Wild type             | 10.4 ± 0.2       | 1.8 ± 0.1 | 58.0 ± 3.7   | -5.78 | 1.51 (0.84) |
| H782Q                 | 5.9 ± 0.7        | 1.9 ± 1.0 | 31.2 ± 17.5  | -6.14 | (1.21) |
| H782Q/K786N           | 5.2 ± 1.2        | 3.3 ± 1.9 | 15.7 ± 9.9   | -6.55 | 2.29 |

$^a$ The S.D. of $K_d$ was estimated according to Chivers et al. (59) with the formula, $(A \pm a)/(B \pm b) = (C \pm c)$, where $c = C \times \sqrt{[(a/A)^2 + (b/B)^2]}$, in which $A$, $B$, and $C$ are $k_{\text{off}}$, $k_m$, and $K_d$ respectively, with lowercase letters representing the S.D. of each.

$^b$ Not determined for K786I because AFM data were not obtained for a single-mutant K786A peptide.
**Length scale of interaction for the Fn-FnBR-9 complexes**

In the AFM experiments, a peptide does not break free of Fn right at a distance of zero (see Fig. 2). This is due to the unraveling of Fn and/or peptide. Length scales of the bond rupture events for each peptide are shown in supplemental Fig. S3. Histograms for each peptide can be fit to two component peaks.

Distance values for these component peaks range from 52 to 160 nm (supplemental Fig. S3). Unraveling of Fn clearly contributes the most to the rupture distance, because the size of an amino acid is 0.38 nm, and the peptides on the AFM tip consist of only 20 residues. The wild-type and H782Q/K786N peptides have similar rupture distances and areas for each component peak (supplemental Fig. S3). Component peaks for the “single mutant” peptides H782Q and K786I are smaller than the others. The H782Q/K786I peptide stands apart as having the longest rupture distances for both component peaks (80 and 160 nm; see supplemental Fig. S3). This peptide also has the longest rupture distances for force measurements performed with the NTD-Fn (supplemental Fig. S4).

**SPR measure of kinetic parameters**

Whereas AFM can be used for single molecule measurements, ligand-receptor interactions are often probed with ensemble techniques. Here we used SPR to measure $k_{on}$ and $k_{off}$ values by injecting each of seven different peptides over a sensor chip supporting immobilized NTD-Fn. AFM experiments differed in that both reactants (peptide and ligand) were immobilized to substrates. SPR measurements were performed at 25 °C, pH 7.4, in PBS.

Fig. 5 shows the sensorgrams for the binding of each peptide to NTD-Fn. The SPR response data were corrected for bulk refractive index effects and fitted using a two-state binding model to calculate kinetic parameters (Table 3). This model was selected over the 1:1 Langmuir model based on the two-step sequential unbinding of NTD-Fn from FnBR-9, as suggested by the MD simulations (see below) and zipper type of binding that involves the conformational transition of the peptide from disordered to ordered. Accordingly, fitting the sensorgrams to the two-state model yielded a more accurate curve fit ($\chi^2 = 0.75$ for the two-step reaction (Fig. 5) versus 0.34–5.35 for Langmuir binding (supplemental Fig. S5)). The two-step binding model includes (i) the initial binding of peptide to the NTD-Fn (A + B $\rightarrow$ AB) followed by (ii) a conformational change to form a more stable complex (AB $\rightarrow$ AB*). It is worth mentioning that the 1:1 Langmuir model generated $\chi^2 < 0.85$ for H782A/K786A, H782A, and K782Q, which could be considered acceptable fitting values. Larger $\chi^2$ values (0.95–5.35) were obtained for H782Q/K786L, H782Q/K786N, wild type, and K786I with the 1:1 model (supplemental Fig. S5).

Compared with the AFM-measured kinetic parameters (Table 2), the SPR data showed wider variation in the association rate constant and comparatively less variation in the dissociation rate constant between the peptides (Table 3). For all peptides, progression of the second step is slower than the first unbinding step ($k_{d2}$ versus $k_{d1}$; Table 3), suggesting that a conformational change is expected to be rate-limiting for generation of a stable complex in the two-state model. The mutations in positions His-782 and Lys-786 associated with disease-causing SNPs result in up to 22-fold stronger FnBR-9 than peptides with alanine substitutions. For example, $K_{d}^{pp}$ is 0.17 μM for K786I compared with 3.84 μM for H782A/K786A (Table 3). The enhanced affinity of the Ala-free peptides is due to increased strength of the initial binding step ($K_{d1}^{pp}$) with higher on-rate and slower off-rate and also more favorable conformational change indicated by a smaller ratio of $k_{d1}/k_{d2}$ (Table 3). A steady-state analysis was also performed on the binding isotherms (supplemental Fig. S6). This model-independent analysis of the SPR data confirms that the K786I and H782Q/K786I peptides have the highest binding affinity for NTD-Fn as probed by SPR (see $K_{d}^{eq}$ values in supplemental Table S4).

**Simulations for decoupling Fn-FnBR-9 complexes under pathway-undetermined conditions**

A computational approach was used to simulate the dissociation of each peptide from F1 modules of the ligand (Fig. 1). Metadynamics simulations were performed by allowing peptides to freely detach in a manner defined only by the free energy landscape along a particular collective variable (CV). Fig. 6 illustrates the free energy surface landscape for the unbinding reaction of each peptide from Fn. The wild-type, H782Q/K786N, and H782Q/K786I variants exhibit two well-defined minima (Fig. 6, A, C, and E). Conversely, the alanine variants display a flatter and more frustrated energy landscape (Fig. 6, F and G). The other single mutations tend toward one well-defined binding minimum (e.g. Fig. 6, B, D, and H). These data were used to determine the binding free energy for each peptide variant.

Fig. 7A shows the free energy surface for fully detached peptides. The H782Q/K786N variant, which displayed a well-defined minimum among all free energy landscapes (Fig. 6), exhibits the strongest binding free energy. The alanine variants, with their more frustrated energy landscapes (Fig. 6, F and G), are the weakest (Fig. 7A). For these pathway-undetermined conditions, H782Q/K786I is stronger whereas K786I and H782Q/K786N show no increase in binding strength (Fig. 7A).

An interesting feature of Fig. 7A is that the total free energy of a double mutant variant (e.g. H782Q/K786N) does not equal the sum of the individual mutations (H782Q plus K786N polymorphism). This finding suggests that mutations in the peptide will affect the conformational variability of the complex, which in turn affects affinity and consequently the detachment path-
A similar phenomenon has been described previously for other systems, and it is well illustrated by the conformational variants of cell surface-associated class II MHC peptide complexes (51).

The free energy landscapes shown in Fig. 6 also provide snapshots of the unbinding trajectories for each peptide (stick model) and Fn (yellow ribbon) complex. Specifically, the simulations reveal how \( \beta \)-strand-1 and \( \beta \)-strand-2 segments of a peptide (see Fig. 6I) are predicted to detach from the F1 domains of Fn. For example, from right to left in Fig. 6A, the snapshots correspond to fully bound, unbinding of one \( \beta \)-strand of Fn, and complete detachment of the wild-type variant peptide.

These metadynamics simulations predict that the wild-type and H782Q/K786N variants unbind in a similar fashion that is different from the other peptides. For these two peptides, the \( \beta \)-strand-1 segment of the peptide (i.e. the segment containing the amino acid replacements) remains attached to Fn until the very end of the simulation (Fig. 6, A and C). For all other peptides, the \( \beta \)-strand-1 detaches first, followed by the \( \beta \)-strand-2 segment (see ribbon-and-stick models in each panel of Fig. 6). This corroborates the dependence of the detachment pathway on the conformational variability of the complex due to the mutations. The associated conformational change upon binding of FnBR-9 to NTD-Fn and the dual-step dissociation of \( \beta \)-strands are consistent with a two-state binding model (see supplemental Movies S1 and S2).

MD simulations of Fn-FnBR-9 complexes under pathway-determined conditions

The above computational approach was conducted by allowing the peptide to freely detach in a manner defined only by the free energy landscape along a particular CV. To assess whether the unbinding pathway may influence affinity, simulations were also performed by inducing the peptide to detach by applying a force on the C-terminal residue and then calculating the work necessary to pull the peptide out of binding with Fn (Fig. 8).
Like the prior metadynamics, the H782Q/K786N variant bound the strongest, and the double alanine peptide exhibited the weakest interactions. However, unlike the metadynamic simulations, when H782Q/K786I and K786I variants were forced to unbind through a well-defined pathway, they exhibited higher affinity (Fig. 8) relative to the pathway-undetermined condition (Fig. 7A). Observed differences in the pathway-undetermined versus pathway determined simulations mean that the chosen pathway to separate a binding pair may impact a variant’s affinity for its ligand.

**Discussion**

Adhesion of bacteria to host tissue or implanted devices (e.g. cardiovascular implants) is a critical event in the pathogenesis...
of *S. aureus* infections. Binding involves cell wall-anchored MSCRAMMs (microbial surface components recognizing adhesive matrix molecules) on *S. aureus* and host proteins present as extracellular matrix material or coatings on device materials. 

Fn is a major component of the extracellular matrix and the proteinaceous fibrin sheath that forms around cardiac implants. Fn monomers consist of multiple repeats of three types of modules: 12 type 1 (F1; each ~40 residues), two type 2 (each ~60 residues), and 15–17 type 3 (each ~90 residues) units (52). N-terminal, F1 domains of Fn have been identified as the main binding site for *S. aureus* (53, 54). *S. aureus* have MSCRAMMs called fibronectin-binding proteins (e.g., FnBPA and FnBPB) that bind to host Fn. Clinical studies and animal models have shown that Fn-FnBPA interactions are linked to disease (6–9).

Recently, we discovered nonsynonymous SNPs in high-affinity repeats of FnBPA, such as FnBR-9, that were associated with several distinct *S. aureus* syndromes in humans: H782Q/K786N with cardiac device infections (12, 13) and H782Q/K786I with persistent bacteremia and infective endocarditis (14). This correlation has been externally corroborated (15). Another report shows that these SNPs in FnBPA are associated specifically with endovascular infections subject to hemodynamic shear, as opposed to extravascular infections (e.g., arthroplasties) that exist within a “sanctuary space” (47).

In this study, we probe the bond that forms between Fn and peptides mimicking clinically relevant variants of FnBR-9 (Table 1). We are particularly interested in how decoupling occurs under more dynamic, fast loading rates because bacteria bind to cardiac implants under similar conditions *in vivo*. Alanine substitutions were placed at positions equivalent to 782 and 786 in these peptides to identify side chains that strongly modulate binding (16–19). Indeed, the alanine peptides exhibited the weakest interaction as determined by all experimental and computational approaches (Table 4).

The use of AFM to probe ligand-receptor interactions is ideal because this technique is capable of directly quantifying the strength of a bond (Fig. 2). As illustrated in Fig. 3 (see also supplemental Table S1), FnBR-9 has a significantly stronger force of interaction toward immobilized Fn when the repeat contains mutations in positions His-782 and Lys-786 that are associated with disease-causing SNPs. Specifically, single and double polymorphisms of H782Q, K786N, and K786I enhance binding relative to the wild-type sequence and the alanine substitutions (Fig. 3A). However, a binding complex is more susceptible to dissociation when acted upon by an external force. Conceptually, the energy landscape of the complex is tilted by...
the applied force, which lowers energy barriers, speeds dissociation, and decreases the likelihood of bond survival (55).

Therefore, we measured bond strength at different loading rates to determine the unstressed off-rate for each peptide-Fn complex. The range in measured \( k_{\text{off}} \) values varies by a factor of up to \( \sim 20 \) (compare H782A/K786A versus H782Q/K786N in Table 2). Four of the peptides exhibit slower off-rates (or longer bond lifetimes; \( 1/k_{\text{off}} \)) relative to the alanine reference peptides: H782Q/K786L, wild-type sequence, H782Q, and H782Q/K786N.

AFM was also used to determine the association rate constant for each peptide-Fn complex. The measured \( k_{\text{on}} \) values display a relatively narrow range (Table 2) in contrast to the \( k_{\text{off}} \) values. These results are similar to another AFM study of alanine point mutations of fluorescein-binding fragments where the range of off-rates varied much more than the on-rates (35). Likewise, another study of five Fn-binding polypeptides that differed by as few as two residues exhibited considerably more variation in off-rates than on-rates (54).

Based on these AFM results, polymorphisms in FnBR-9 have a greater impact on off-rates rather than on-rates for interactions with immobilized Fn at high loading rate. There is a remarkable similarity between experimentally measured bond lifetimes (\( 1/k_{\text{off}} \) Fig. 7B) and metadynamics simulations where peptide detachment is defined solely by the free energy landscape along a particular CV (Fig. 7A). Focusing on the observed off-rates, we see bond lifetimes for interactions with Fn ranging from 0.011 to 0.192 s (Table 2). For comparison, the interaction between immobilized Fn and a strain of *Lactococcus lactis* expressing the wild-type sequence of fnbA has a bond lifetime of 0.29 s, as determined from data presented previously (56).

Using SPR, a complementary ensemble approach, we observed bond lifetimes (\( 1/k_{\text{off}} \)) of 1.8–96.9 s for the reaction between immobilized NTD-Fn and the peptides (Table 3). Such differences between AFM and SPR measurements are not uncommon. For example, others have found that \( 1/k_{\text{off}} \) values can be 10–106 times longer for SPR versus AFM measurements conducted on the same ligand-receptor pair (22, 40, 57).

This difference in off-rates could indicate the existence of an additional energy barrier situated further out on the potential energy surface (35, 40, 46, 55). This outer barrier might be probed by loading at a very slow rate. Our AFM experiments were designed to expose hidden, inner energy barriers expected to be rate-determining at the high loading rate experienced by *S. aureus* receptors in a cardiac setting (see “Results”). Alternatively, the differences in AFM versus SPR off-rates may simply reflect the inherent differences in the techniques. With AFM, the relatively fast pulling rates put a lower limit on the estimated \( k_{\text{off}} \) value. On the other hand, the SPR measurements are challenged when the off-rates are faster than 1/s due to limitations with the flow and detector sampling rates. Thus, the regimes that are probed are different, and it may be impossible to measure the fast loading rates with the SPR method and vice versa.

The dissociation constant was determined according to \( K_d = k_{\text{off}} / k_{\text{on}} \) (21, 30, 50) for each Fn-peptide probed with AFM (Table 2). \( K_d \) values for immobilized Fn range from 15.7 to 747 \( \mu M \) for H782Q/K786N and H782A/K786A, respectively. SPR measurements with NTD-Fn yielded apparent \( K_d \) values from 0.2 to 4.7 \( \mu M \) (Table 3). For comparison, values of \( K_d \) as determined by another ensemble technique (fluorescence anisotropy), ranged from 3.1 to 24.4 \( \mu M \) (53) for fibronectin binding to synthetic peptides (18–38 residues in length) whose sequences overlap with portions of the wild-type sequence of FnBR-9 used herein. Another investigation using NMR chemical shift observed \( K_d \) of 0.5–9.4 \( \mu M \) for \( ^2F_1\)F1 binding to synthetic peptides (19–22 residues in length) that resembled FnBR-1 and FnBR-8 in FnBPA (58).

We used the method of Chivers et al. (59) to estimate the S.E. of the AFM-derived \( K_d \) values (Table 2). As described in the supplemental material, this is a stringent definition of error. When taking into account the Chivers estimate of error, three peptides clearly exhibit stronger affinity for immobilized Fn relative to the alanine reference peptides: H782Q/K786N, H782Q, and the wild-type peptide (Table 2). The \( K_d \) of H782Q/K786L indicates a relatively higher affinity too, but the S.D. value is greater for this peptide. When probed at high loading rates, double replacements in positions His-782 and Lys-786 associated with disease-causing SNPs result in 4–48-fold stronger binding to FnBR-9 than the alanine substitutions (\( K_d \) of 16 \( \mu M \) for QN, 58 \( \mu M \) for HK, 189 \( \mu M \) for QI, and 747 \( \mu M \) for AA).

For the AFM data, \( \Delta G \) was determined for each peptide using the alanine mutants as the references: \( RT \ln(K_d) \) for alanine peptide/\( K_d \) peptide of interest (Table 2). Single alanine mutations of Ala \( \rightarrow \) His (wild-type peptide) and Ala \( \rightarrow \) Gln (H782Q peptide) yield \( \Delta G \) of 0.84–1.21 kcal/mol, which falls within the > 1 to 1.5 kcal/mol threshold for defining a “hot spot” (60–62). These values are similar to another report that documented that 4 of 13 single alanine replacements in FnBRs contributed \( \approx 1.3 \) kcal/mol \( \Delta G \), as determined by SPR and isothermal titration calorimetry (19).

Double alanine mutations (18, 63) at positions that are separated by four residues, result in \( \Delta G \) of 1.51–2.29 kcal/mol for the AA \( \rightarrow \) HK and AA \( \rightarrow \) QN peptides, respectively (Table 2). This suggests that at high loading rates, multiple polymorphisms in FnBR-9 equivalent to A782Q/A786N might constitute a hot spot as defined by the more stringent threshold of \( \Delta G > 2 \) kcal/mol (19). Regardless of whether these polymorphisms represent an true hot spot, it is clear that the H782Q/K786N peptide binds to immobilized Fn stronger than either the double alanine or wild-type peptides (\( \Delta G \) 0.8–2.3 kcal/mol; Table 2).

H782Q/K786L exhibited higher affinity for immobilized Fn at fast loading rates versus the alanine peptide (\( \Delta G \) 0.81 kcal/mol; Table 2), but binding was weaker than for the wild-type and Gln/Asn peptides. This is not entirely unexpected because the Gln/Ile polymorphisms (unlike the Gln/Asn polymorphisms) are associated with persistent bacteremia in patients who do not necessarily have a cardiovascular implant (14). The Gln/Ile isolates are also associated with a host invasion motif (GIDFVDE) in FnBR-11 and an extra repeat of FnBR-9 giving these strains a total of 12 rather than 11 FnBRs (14, 15). Therefore, the clinical observation of H782Q/K786L may have more to do with invasion of host cells and subsequent persistence of infection.

Internationalization could be enhanced by extension or opening of Fn molecules, thereby exposing cryptic binding sites.
Energy landscape for dissociation of Fn-FnBR-9 bond

(64, 65), some of which (e.g. integrins) are involved in host cell invasion (66, 67). Both metadynamics and steered MD simulations reveal that mutations in the peptide affect the conformational variability of the complex, which in turn influences affinity and the detachment pathway (Figs. 6, 7A, and 8). AFM and SPR data offer support for this observation. For example, the H782Q/K786I peptide exhibited the longest length of interaction when pulled on with the AFM probe (supplemental Fig. S3). This extension was longer when Fn served as the ligand as opposed to the NTD-Fn (supplemental Fig. S4), indicating that under dynamic conditions, some extension originates from regions of Fn that are distant from the binding site (i.e. outside of the N-terminal F1 modules). We speculate that most of the unfolding of Fn originates from the type III modules on the Fn molecule, which contain the cryptic binding sites mentioned above.

SPR data also point to a possible conformational change in the binding complex. The SPR response profiles fit better to a two-state reaction model (Fig. 5 versus supplemental Fig. S5). SPR data show that the K786I and H782Q/K786I peptides bound to the NTD-Fn with a higher affinity than the wild-type or alanine peptides (Table 3). In terms of $K_d$ values, the peptides with K786I exhibited 23-fold higher affinity than the alanine peptides (Table 3). For NTD-Fn binding to H782Q/K786I or K786I, this corresponds to $\Delta \Delta G$ of up to 1.19–1.84 kcal/mol for the first and second steps, respectively, of the two-state reaction model. Enhanced affinity of the K786I peptides is due to increased strength of the initial binding step ($K_{1t1}$) with higher on-rate and slower off-rate and also more favorable conformational change, indicated by a smaller ratio of $k_{d1}/k_{u2}$ (Table 3). Interestingly, the wild type and H782Q/K786N also have relatively small $k_{d1}/k_{u2}$ ratios.

Collectively, the extension length data (provided by AFM) and the two-state binding process supported by the MD simulations and SPR data indicate that polymorphic changes in the FnBRs might impact conformational stability of the binding complex. In contrast, X-ray crystallography studies (11) show that the F1 module structures are very similar for the free and peptide-bound forms. However, these X-ray analyses offer only a static picture of each end of the process and therefore do not capture the binding dynamics. Rearrangement of the binding complex is here supported by three different techniques that probe the dynamics of binding/unbinding.

We have shown that amino acid changes at positions 782 and 786 in FnBPA can impact the formation, function, and/or regulation of the complex within specific pathological contexts (Table 4). Still, the wild-type variant exhibits a strong interaction with Fn (Tables 2 and 3 and Figs. 3 and 6A). The affinity of the wild-type sequence is due in part to conserved residues, such as glycine at position 783, which are part of key binding motifs in the FnBRs (11). The higher affinity observed for the H782Q/K786N peptide (versus the wild-type peptide) suggests that flanking residues (e.g. Gln at 782 and Asn at 786) can significantly enhance binding. From an evolutionary perspective, perhaps these “mutations” are selected during endovascular infections where hemodynamic flow is a factor and/or immobilized fibronectin molecules are the dominant host ligand. This provides a mechanistic explanation for the association of SNPs in S. aureus FnBPA from populations of patients with infected cardiac device implants (12, 13, 15) rather than patients with infected arthroplasties, where the abscess exists within a sanctuary space (47).

Finally, we have found that FnBRs dissociate from Fn along a free energy landscape (Fig. 6). In vivo, there are several attachment/detachment pathways that are influenced by local flow conditions, entropic effects, ionic strength, soluble versus bound ligand, etc. Furthermore, FnBPs on S. aureus located at different positions in the human body are exposed to very different external forces ranging from dynamic shear associated with endovascular environments (fast load rates) to more quiescent conditions in the anterior nares or an extravascular site (slow load rates). These different environments may select for isolates of S. aureus expressing adhesins with different binding and/or invasive attributes (12, 14). The intrinsic disorder of FnBRs may even facilitate this through the formation of a “fuzzy” protein complex upon binding to Fn (68). Although the ligand binding motifs of FnBR-9 are intact, changes in the fuzzy regions connecting the motifs might influence ligand binding by fine-tuning the kinetics and dynamics of the reaction.

Experimental procedures

Materials

Peptides were synthesized by United Biosystems (Herndon, VA). Sequence and purity (>96%) were confirmed with mass spectrometry and HPLC, respectively.

AFM

Each peptide was linked to an AFM tip through a strong, covalent bond between gold coating on the tip and the thiol group of a cysteine engineered at the C terminus of each peptide (69). At least four different functionalized tips were used for each peptide shown in Table 1. AFM measurements were performed with silicon nitride cantilevers (measured spring constant 0.060 ± 0.017 N m$^{-1}$) in PBS at retraction velocities from 0.5 to 18.8 µm/s, speeds at which hydrodynamic drag does not have an effect on cantilever deflection (70). Experiments utilized commercially available Fn-coated slides (BD Biosciences) and homemade slides coated with dilute solutions (<0.01%) of Fn that promote single-molecule binding events while avoiding the extremely low-density protein coverage that can lead to partial surface denaturation of Fn or conformationally inactive forms of Fn (45).

Single-molecule force measurements were performed according to published methods (e.g. see Refs.13, 21, 30, and 71). To favor single-molecule interactions, we coated tips with peptide concentrations that generated low frequency of binding (<30%) based on Poisson statistics (20, 21). As a further check, retraction profiles were fit to the wormlike chain model (56, 72) to confirm that all binding events involved less than two reaction pairs for all peptides analyzed with AFM. This was deemed appropriate because others have shown that a D motif in FnBPA binds one to two molecules of the NTD-Fn (53). To ensure specificity, only the final rupture peak (25, 34, 35, 73) was included in the analysis presented herein. Control experiments were performed with uncoated AFM tips on Fn-coated slides in PBS (74, 75). Negative controls were performed using...
peptides with single- or double-alanine mutants, a classic approach to identify specific side chains that modulate binding (16, 18, 63). Alanine was selected as the replacement residue because it is considered the least disruptive amino acid to the structure (76), and it deletes all interactions by atoms beyond the β-carbon, thereby revealing the contribution made by the removed side chain (60).

**SPR**

Experiments were performed at 25°C on a Biacore 3000 SPR system with CM3 sensor chips (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). The sensor surfaces were prepared using amine-coupling kits (GE Healthcare/Biacore AB) at a flow rate of 5 μl/min in PBS (8.06 mM Na₂HPO₄, 1.94 mM KH₂PO₄, 2.7 mM KCl, 137 mM NaCl, pH 7.4), which was used as running buffer for immobilization. Peptide concentration was determined using absorbance at 205 nm (77). The N-terminal domain of Fn (Sigma-Aldrich, product F9911) was dissolved and dialyzed in PBS before immobilization. The NTD-Fn surface was prepared using a standard procedure; the flow cell was activated with 35 μl of a 1:1 mixture of 0.4 M EDC and 0.1 M NHS, followed by a 35-μl injection of 20 μg/ml NTD-Fn (in 10 mM sodium acetate, pH 5.5), and the surface was deactivated with ethanolamine. The reference flow cells were prepared with activation and deactivation steps where no protein was coupled. Binding was performed in PBS containing 0.01% (v/v) Tween 20 at a flow rate of 50 μl/min. To regenerate the sensor surface, bound peptide was removed by flowing 10 mM glycine (pH 2.2) over the surface for 12 s. Reference-corrected SPR response curves (with buffer blank run further subtracted) were evaluated using BIAevaluation (version 4.1.1).

**MD simulations**

MD simulations were performed by starting from previously modeled coordinates for Fn complexed with the wild-type form of FnBR-9 (12). Residue replacement was carried out with wild-type complexes (GE Healthcare/Biacore AB) at a flow rate of 5 μl/min in PBS (8.06 mM Na₂HPO₄, 1.94 mM KH₂PO₄, 2.7 mM KCl, 137 mM NaCl, pH 7.4), which was used as running buffer for immobilization. Peptide concentration was determined using absorbance at 205 nm (77). The N-terminal domain of Fn (Sigma-Aldrich, product F9911) was dissolved and dialyzed in PBS before immobilization. The NTD-Fn surface was prepared using a standard procedure; the flow cell was activated with 35 μl of a 1:1 mixture of 0.4 M EDC and 0.1 M NHS, followed by a 35-μl injection of 20 μg/ml NTD-Fn (in 10 mM sodium acetate, pH 5.5), and the surface was deactivated with ethanolamine. The reference flow cells were prepared with activation and deactivation steps where no protein was coupled. Binding was performed in PBS containing 0.01% (v/v) Tween 20 at a flow rate of 50 μl/min. To regenerate the sensor surface, bound peptide was removed by flowing 10 mM glycine (pH 2.2) over the surface for 12 s. Reference-corrected SPR response curves (with buffer blank run further subtracted) were evaluated using BIAevaluation (version 4.1.1).

**Metadynamics simulations**

The final structures from the MD simulations were used as a starting point for metadynamics calculations using Plumed version 2.0.1 (85) within Gromacs version 4.6.7 (81). The polymorphic structures were transferred to a simulation box with dimensions 10 × 6 × 12 nm and solvated, and the ionic strength was adjusted to 0.15 M. The systems were heated from 0 to 300 K in 100-ps intervals with the solute atoms fixed in the initial position. Pressure and density of solution was adjusted after 2 ns of unrestrained simulation at NpT ensemble at constant pressure and temperature of 300 K and 1 bar, respectively. Unbinding was driven by two collective variables (CV; Fig. 1B). CV1 was defined as the distance between the center of mass (COM) of α-carbons (Cα) forming the first β-strand of FnBR-9 and Fn, whereas CV2 was defined between the COM of Cα forming the second β-strand of FnBR-9 with Fn. A potential bias in the form of a Gaussian with width and height equal to 0.05 nm and 0.05 kJ/mol, respectively, for both CVs was added along the z axis projection in 1-ps increments until complete unbinding. Positional restraints were used on the Cα atoms of the Fn β-strands using a force constant of 1000 kJ/mol to avoid artificial distortions of the protein structure during the unbinding process. Detachments for all simulations took place within 40 ns of simulation.

**Energy landscape for dissociation of Fn-FnBR-9 bond**

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**Author contributions**—S. K. L., R. D. L., and N. N. C.-I. designed the study and wrote the paper. N. N. C.-I. and A. C. D. performed the AFM experiments. X. L. and M. H. performed the SPR experiments. R. L. and C. H. B. C. performed the MD and metadynamics simulations. J. H., M. R. S.-H., and I. F. T. V. provided technical assistance on AFM and SPR. All authors reviewed the results and approved the final version of the manuscript.

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