Conformational switch of the bacterial adhesin FimH in the absence of the regulatory domain: Engineering a minimalistic allosteric system

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For many biological processes such as ligand binding, enzymatic catalysis, or protein folding, allosteric regulation of protein conformation and dynamics is fundamentally important. One example is the bacterial adhesin FimH, where the C-terminal pilin domain exerts negative allosteric control over binding of the N-terminal lectin domain to mannosylated ligands on host cells. When the lectin and pilin domains are separated under shear stress, the FimH–ligand interaction switches in a so-called catch-bond mechanism from the low- to high-affinity state. So far, it has been assumed that the pilin domain is essential for the allosteric propagation within the lectin domain that would otherwise be conformationally rigid. To test this hypothesis, we generated mutants of the isolated FimH lectin domain and characterized their thermodynamic, kinetic, and structural properties using isothermal titration calorimetry, surface plasmon resonance, nuclear magnetic resonance, and X-ray techniques. Intriguingly, some of the mutants mimicked the conformational and kinetic behaviors of the full-length protein and, even in absence of the pilin domain, conducted the cross-talk between allosteric sites and the mannoside-binding pocket. Thus, these mutants represent a minimalistic allosteric system of FimH, useful for further mechanistic studies and antagonist design.

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The key step in urinary tract infections (UTI) is the adhesion of uropathogenic Escherichia coli (UPEC) to urothelial cells of the host (1, 2). This initial step enables the bacteria to invade and colonize host cells but also to withstand clearance by the bulk flow of urine. Adherence to the urothelial surface is mediated by the adhesin FimH located at the tip of bacterial type 1 pili (3, 4). Full-length FimH (FimHFL) is composed of two domains: the N-terminal lectin domain (FimHLD) which is connected to the C-terminal pilin domain (FimHPD) by a short linker (5). FimHLD contains the carbohydrate recognition domain, which is responsible for binding to the highly mannosylated uroplakin 1a (UP1a) on the urothelial cell surface (6). FimHPD is anchored to the core of the pilus via a donor strand of the subsequent FimG subunit, a process termed donor strand complementation (7, 8).

In the bladder, under static conditions, FimH exhibits only weak interactions with the urothelial surface, which is highly beneficial for bacterial motility and host cell invasion (9). However, when shear forces originating from urine flow occur, a significant enhancement of the strength of the interaction (5, 10) due to a catch bond mechanism was observed (11, 12). Catch bonds play pivotal roles in the regulation and fine-tuning of cellular adhesion events, e.g. upon leukocyte recruitment by selectins (13) and integrins (14), by cadherins controlling tissue integrity (15, 16), in the epithelial adhesion of cancer cells (17), or in T cell receptor interaction with peptide-bound major histocompatibility complexes on antigen-presenting cells (18, 19).

Conformation and ligand-binding properties of FimHLD are under the allosteric control of FimHPD (5, 20, 21). Recent X-ray data of a recombinant FimHFL has provided structural evidence for the different conformational states (22, 23). Under static conditions, the interaction of the two domains of FimHFL stabilizes the lectin domain in the low-affinity state, which is characterized by a shallow binding pocket (Fig. 1). Binding to a mannoside ligand induces a conformational change leading to the medium-affinity state, in particular by the displacement of the so-called clamp loop toward the binding pocket. This medium-affinity state, where lectin and pilin domain are still in close contact, is characterized by micromolar affinities and fast off-rates (5, 23). However, upon shear stress, FimHLD and FimHPD separate, inducing the high-affinity state with an up to 300-fold enhanced affinity (20). Using donor−strand complemented FimHFL and combining X-ray and small angle x-ray scattering analyses, it was suggested that FimH is present in a two-state conformational ensemble of low- and high-affinity states in solution (22). This equilibrium can be influenced by sequence...
variations, thereby modulating the apparent binding affinity as well as the binding mechanism (induced fit or conformational selection), and thus influencing the infective potential of UPEC.

Because of the absence of negative allosteric regulation exerted by the pilin domain, isolated FimHLD is locked in the high-affinity state (24, 25). Mutations in the interdomain region altering the interactions to the pilin domain clearly demonstrated its contribution to the conformational change within the lectin domain (20). Interestingly, the medium-affinity state (domains interacting) and the high-affinity state (domains separated) exhibit nearly identical mannose-binding pockets, yet possess a strikingly 10^5-fold difference in the off-rate for mannosides (23). This structural information in combination with MD simulations and kinetics experiments (23, 26) led to the concept of dynamic allosteric regulation, i.e., the allosteric signal of the pilin domain is transmitted to the proximal regions of the lectin domain (27).

The isolated, recombinant FimHLD, which is locked in the high-affinity state, exhibits high temporal and thermal stability (24, 28, 29), and consequently, it was mostly used to generate affinity data from antagonist screening and structural data from X-ray crystallography (30–35). However, recent data on the conformational and dynamic regulation of FimHFL upon mannoside binding raised doubt on the applicability of the conformationally locked FimHLD for mimicking urinary tract infections in vivo (36, 37).

In a comparison of the low- and high-affinity states of FimHFL, Le Trong et al. (5) identified four regions undergoing conformational changes upon ligand binding, namely the pocket zipper/clamp loop (Phe-1–Ile-11/Gly-8–Gly-16), the /H9252-bulge (Gln-59–Ser-63), the /H9251-switch (Tyr-64–Phe-71), and the interdomain swing, insertion, and linker loops (Ala-25–Leu-34, Pro-111–Ala-119, Asn-152–Thr-158) (Fig. 2). Later, Rodriguez et al. (38) used RosettaDesign and MODIPDesign (computer-based tools for protein structural analysis) to predict specific point mutations within the aforementioned regions, causing the largest difference in energy between the low- and high-affinity conformations and hence should stabilize one of the affinity states.

The mutation A10P was predicted to favor the low-affinity conformation through perturbation of the backbone hydrogen bond of residue Ile-11 in the pocket zipper. In the /H9251-bulge region, the point mutation R60P was predicted to prevent the transition of the portion of Gln-59–Ser-63 from a small loop to a /H9251-helix and thus destabilize the high-affinity conformation. In the /H9251-switch (Tyr-64–Phe-71), the mutation V67K was expected to prevent the conversion of this region to a /H9251-helix and thus lock FimH in low-affinity conformation. The point mutation A119L and the double mutation V27C/L34C at the proximal end of the lectin domain were expected to preserve a conformation of the interdomain loops that allows interaction with the pilin domain and hence stabilizes the low-affinity conformation (5).
The five FimH variants selected upon this analysis (A10P, R60P, V67K, A119L, V27C/L34C) were expressed in *E. coli* in the context of the entire pilus, and their affinity state was evaluated based on binding to mono- and trimannoside ligands as well as their recognition by the conformation-specific monoclonal antibody mAb21 (38). This antibody recognizes an epitope located in the interdomain region of the isolated WT FimH [LD](21, 39). Therefore, binding of mAb21 indicates that FimH is in the high-affinity state, because only then is the interdomain accessible. Furthermore, bacterial adhesion is enhanced in the presence of mAb21, which stabilizes the high-affinity state (21). In contrast, a monoclonal blocking antibody directed against the FimH [LD](mutant V27C/L34C, presumably locked in the low-affinity state, protected mice from bacterial infection, thus demonstrating the relevance of considering all conformational states for the design of therapeutic FimH antagonists (40). Despite the intensive studies of the FimH variants mentioned above, structural, thermodynamic, and kinetic data regarding the low-affinity conformation were not reported so far.

To further elucidate the degree of conformational heterogeneity and the allosteric effects of mutants in the context of the whole pilus (38), we recombinantly expressed five selected variants (A10P, R60P, V67K, V27C/L34C, and A119L) of isolated FimH [LD] predicted to be stabilized in the low-affinity state, and we analyzed their thermodynamic and kinetic binding properties, as well as their structural characteristics by NMR and X-ray crystallography with the aim to reveal the allosteric crosstalk between the regulatory regions within FimH [LD]. Furthermore, FimH [LD] mutants could also serve as easy-accessible mimics of FimH [FL] for mechanistic studies as well as for the development of FimH antagonists applicable for the anti-adhesive therapy of UTI.

**Results**

Five FimH [LD] variants were generated with single- or double-point mutations within the four allosteric regions assumed to stabilize or even lock FimH in the low-affinity state (5, 38, 40). The A10P mutation is located in the pocket zipper/clamp loop; R60P and V67K are part of the β-bulge and α-switch regions, respectively; and A119L and V27C/L34C are located within the interdomain loops (Fig. 2). The purity and the monomeric state of the WT and all variants were confirmed by native PAGE analysis (data not shown).

**Folding and stability of FimH [LD] mutants**

The impact of these mutations on protein folding and stability was evaluated by thermal shift-based differential-scanning fluorimetry (42). In this assay, the fluorescence intensity of SYPRO orange dye was recorded during heat denaturation of the protein (42). The melting temperatures (T_m), which indicate the thermal unfolding event, were in the same region for WT FimH [LD] and the variants A10P, A119L, and R60P (Fig. S1 and Table S1) and were reduced by less than 8 °C for the mutants V67K and V27C/L34C. However, for two mutants, R60P and V27C/L34C, the extent of the thermal shift was significantly reduced, suggesting a lower degree of stabilization, i.e. less efficient binding than for WT FimH [LD] (Table S1).

**Correlation of structural and affinity changes of FimH [LD] mutants**

To examine the secondary and tertiary structures of WT FimH [LD] and the five variants in the absence and presence of mannose, circular dichroism (CD) spectra were recorded...
The far-UV (190–250 nm) CD spectrum of WT FimHLD is characteristic for a protein with a high β-sheet content (Fig. 3A) (43). Furthermore, upon addition of antagonist 1, the CD spectrum remained unchanged, indicating a stable fold. Similar conformations are suggested for the mutants A119L and A10P because their CD spectra in the absence and presence of antagonist 1 were almost identical to the WT (Fig. 3B and Fig. S2A). In contrast, the CD spectra of the mutants V27C/L34C, R60P, and V67K in the absence of ligand were clearly altered with an overall lower molar ellipticity, indicating significant changes in the global protein conformation and a reduction of the β-sheet proportion (Fig. 3C and Fig. S2). Surprisingly, the addition of mannose 1 to these three mutants re-established CD spectra nearly identical to WT FimHLD.

Next, the geometry of aromatic side chains was analyzed with near-UV CD (260–320 nm) (Fig. 3D–F, and Fig. S3). Considering the abundance of phenylalanine, tyrosine, and tryptophan residues, many of them in close vicinity of the mannose-binding pocket (Fig. S3, D–F), even small changes in the local protein conformation should be detectable (44). Because near-UV CD spectra of WT FimHLD, in the absence and presence of mannose 1 were again superimposable, it can be assumed that the protein conformation in the neighborhood of the aromatic residues is highly conserved (Fig. 3D). For the mutants A10P and A119L, the general shape and the curve maxima of the spectra were similar to WT FimHLD, and the spectra of the mutants V27C/L34C (Fig. 3F), R60P (Fig. S3B), and V67K (Fig. S3C) were significantly altered with shifted maxima and partly inverted signs of the molar ellipticity. Again, addition of antagonist 1 to these three mutants restored the near-UV CD spectra compared with WT FimHLD to a large extent. These results clearly indicate that mannose binding induces a significant conformational change toward the high-affinity conformation of the so far uncharacterized apo-forms of R60P, V67K, and V27C/L34C.

The dissociation constant \( K_D \) of mannose 1 binding to WT FimHLD and the five mutants were determined with a fluorescence polarization (FP) assay (32). While WT FimHLD and the mutants A119L and A10P exhibited affinities in the low nanomolar range, the mutants R60P and V27C/L34C show reduced affinity, namely by factors of 17 and 35, respectively (Table 1). Finally, despite its altered CD spectra, the mutant V67K showed only a 2-fold reduction in binding affinity. Thus, at this point it cannot be speculated that the conformational changes observed in the CD spectra result in an entropy penalty.

**Distinct thermodynamic profiles of conformationally different mutants**

To assess the impact of the observed conformational changes upon ligand binding on the thermodynamic fingerprint of the mutants, isothermal titration calorimetry (ITC) experiments were performed with mannose 1 (Table 2) leading to \( K_D \) values in the same range (within a factor of 2) as those obtained...
with the FP assay (Table 1). All mutants showed an enthalpy-driven binding profile. Compared with WT FimHLD, they exhibit a more unfavorable entropy term. In respect to enthalpy, the mutants A119L and V67K benefit from an improvement of up to 2.8 kJ/mol, whereas R60P and V27C/L34C display penalties in a comparable magnitude (Fig. S4).

We hypothesized that the conformational changes of the mutants R60P and V27C/L34C induced by ligand binding are responsible for unfavorable conformational entropy terms. Therefore, the entropy term obtained for R60P was dissected into solvation, conformational and mixing entropy (Equation 1) (Table 3).

$$
\Delta S^0_{\text{obs}} = \Delta S^0_{\text{solv}} + \Delta S^0_{\text{conf}} + \Delta S^0_{\text{mix}}\
$$

(Eq. 1)

The change in solvation entropy ($\Delta S^0_{\text{solv}}$) reflecting the loss in translational and rotational degrees of freedom of both interaction partners upon binding is equal for WT FimHLD and the R60P mutant (Equation 2, where $R$ is the universal gas constant and 55.6 is the molarity of water in molar).

$$
\Delta S^0_{\text{mix}} = R \ln\left(\frac{1}{55.6}\right)\
$$

(Eq. 2)

The change in solvation entropy ($\Delta S^0_{\text{solv}}$) was calculated based on the change in heat capacity ($\Delta C_p$, Equation 3),

$$
\Delta C_p = \left(\frac{\partial H^0_{\text{obs}}}{\partial T}\right)\
$$

(Eq. 3)

which was determined by measuring the change of enthalpy ($\Delta H$) as a function of temperature (Fig. 4).

$$
\Delta S^0_{\text{solv}, 298.15 K} = \Delta C_p \ln\left(\frac{298.15 K}{385 K}\right)\
$$

(Eq. 4)

By inserting the experimentally determined value for $\Delta C_p$ into Equation 4 at 385 K as the temperature of zero hydration ($T_r$), the change in solvation entropy ($\Delta S^0_{\text{solv}}$) at room temperature was obtained (47–49). Interestingly, the change of solvation entropy for the interaction of R60P with mannoside 1 was less favorable compared with WT FimHLD ($-T\Delta S^0_{\text{solv}} = 15.9$ kJ/mol) (Table 3). Considering that their ligand-bound structures are highly similar, a possible explanation may be a lower degree of ordering of interfacial water molecules in the apo-R60P, which presumably exhibits a broad and flexible binding pocket. In contrast, the well-defined and narrow binding pocket of WT FimHLD can establish a highly structured hydrogen bond network of water molecules supporting a more beneficial solvation entropy upon ligand binding. Surprisingly, the

### Table 1
Affinity of mannoside 1 for WT FimHLD and five mutants, as well as for FimHL obtained from the FP assay

| Variants of FimHLD | $K_D$ (nM)$^a$ | Relative $K_D$ |
|--------------------|---------------|---------------|
| WT                 | 19 ± 2        | 1             |
| A119L              | 5.2 ± 0.5     | 0.27          |
| A10P               | 16.5 ± 1.5    | 0.87          |
| V67K               | 37 ± 3        | 1.9           |
| R60P               | 322 ± 52      | 17            |
| V27C/L34C          | 661 ± 56      | 35            |
| Full-length FimH   | 2300 ± 100    | 121           |

$^a$ The assay was performed twice in duplicate measurements.

$^b$ Full-length WT FimH stabilized by FimG donor strand (23).

### Table 2
Thermodynamic profiles of WT FimHLD and mutants thereof from ITC measurements with mannoside 1 at 298 K

The ITC assay was performed twice in duplicate measurements.

| Variant | $K_D$ | $\Delta G^0$ | $\Delta H^0$ | $-T\Delta S^0$ | $c$-value | $N$ |
|---------|-------|--------------|--------------|----------------|-----------|-----|
|         | nM   | kJ/mol       | kJ/mol       | kJ/mol         |           |     |
| WT      | 28.9 ± 4.6 | −43.0 ± 0.4 | −50.3 ± 0.35 | 7.3 ± 0.5 | 320 | 1.00 |
| A119L   | 24.2 ± 10.53 | −43.3 ± 1.0 | −55.4 ± 1.5 | 8.9 ± 1.8 | 441 | 1.01 |
| V67K    | 76.8 ± 16.82 | −40.6 ± 0.5 | −53.1 ± 0.98 | 12.5 ± 1.1 | 131 | 1.03 |
| R60P    | 583 ± 122.8 | −35.6 ± 0.5 | −46.2 ± 2.05 | 10.6 ± 2.1 | 171 | 1.05 |
| V27C/L34C | 1117 ± 271.03 | −34.0 ± 0.6 | −45.3 ± 2.54 | 11.3 ± 2.6 | 13.4 | 0.93 |

### Table 3
Entropy dissection of the binding of mannoside 1 to WT FimHLD and the mutant R60P

| Variant | $\Delta C_p$ | $-T\Delta S^0_{\text{solv}}$ | $-T\Delta S^0_{\text{conf}}$ | $-T\Delta S^0_{\text{mix}}$ | $-T\Delta S^0_{\text{obs}}$ |
|---------|--------------|-----------------------------|-------------------------------|-----------------------------|--------------------------|
|         | kJ/K         | kJ/mol                      | kJ/mol                        | kJ/mol                      | kJ/mol                   |
| WT FimHLD | −0.907 ± 0.016 | −69.1 ± 1.22                | 66.4 ± 1.24                   | 10.0                        | 7.27 ± 0.245             |
| R60P    | −0.698 ± 0.098 | −53.2 ± 7.47                | 53.8 ± 7.51                   | 10.0                        | 10.6 ± 0.724             |

$^a$ $\Delta S^0_{\text{obs}}$ quantifies the loss in translational and rotational degrees of freedom upon complex formation: calculated as $\Delta S^0_{\text{obs}} = R \ln(1/55.6)$, with $R$ being the universal gas constant and 55.6 being the molarity of water in molar (47, 49). The assay was performed twice in duplicate measurements.
conformational entropy $T \Delta S_{\text{conf}}$ of R60P was less unfavorable compared with WT FimHLD ($-T \Delta S_{\text{conf}} = -12.6$ kJ/mol). This counter-intuitive observation remains to be investigated and may require NMR dynamics studies that report on the different time scales on which ligand binding and protein conformational changes can occur.

**Similar binding kinetics for V27C/L34C mutant and WT FimHFL**

To gain a detailed insight into the binding kinetics of the interaction of FimH with mannoside ligands, surface plasmon resonance (SPR) measurements were performed (Table 4). The carboxyl-functionalized mannoside 5 (see supporting information for structure and synthesis) was immobilized via an ethylenediamine linker on a CM4 chip. WT FimHLD, mutants, and FimHFL were injected in the mobile phase, and their association and dissociation were monitored (Fig. 5 and Fig. S5). WT FimHLD and mutants could be fitted with a one-state binding model (Equation 5).

\[
A + B \rightarrow AB \quad \text{(Eq. 5)}
\]

Compared with other carbohydrate–lectin interactions (50), the on-rate constant $k_{\text{on}}$ for WT FimHLD is in the typical range of $10^3$–$10^4$ M$^{-1}$ s$^{-1}$, whereas the off-rate constant $k_{\text{off}}$ is rather low, resulting in a complex half-life $t_{1/2}$ of 83.6 min. The binding kinetic of the mutants A10P and A119L was similar to the WT FimHLD (Table 4). For V67K, the $k_{\text{on}}$ was comparable with the WT FimHLD, whereas the $k_{\text{off}}$ was 7-fold increased. In case of FimHFL, $k_{\text{on}}$ was slightly increased, whereas the $k_{\text{off}}$ was drastically enhanced by a factor of 3700 resulting in a $t_{1/2}$ of only 1.4 s. The association and dissociation of FimHFL were characterized by a multiphasic behavior (Fig. 5B) requiring fitting to a two-state binding model (Equation 6).

\[
A + B \rightarrow AB \rightarrow A \cdot B \quad \text{(Eq. 6)}
\]

In this model, the analyte (FimH, A) binds to the ligand (mannoside, B) to form an initial complex (AB) and then undergoes subsequent binding or conformational change to form a more stable complex (A$\cdot$B). When the multicycle kinetics measurement was repeated on a chip surface with less densely immobilized ligand, the multiphasic behavior could be confirmed and steric hindrance as a possible artifact excluded (data not shown). Similar findings have recently been reported for FimH within the fully assembled type I pili (51). A two-state binding process of FimHFL featuring a second binding kinetic with a slow off-rate in the range of WT FimHLD (Table 4) likely represents the slow conversion of full-length FimH to the high-affinity state by separation of the lectin and pilin domains.

For the R60P mutant, $k_{\text{on}}$ was comparable with WT FimHLD, whereas $k_{\text{off}}$ was increased 29-fold leading to a $t_{1/2}$ of 2.9 min and placing its kinetic profile between WT FimHLD and FimHFL. Notably, the kinetic profile of the double-cysteine mutant V27C/L34C was very similar to full-length FimHFL, except that only a monophasic binding behavior was observed (Fig. 5A). In comparison with WT FimHLD, the $k_{\text{on}}$ of V27C/L34C was increased by a factor of 19 and $k_{\text{off}}$ by a factor of almost 2900, resulting in a $t_{1/2}$ of 1.7 s, i.e. very close to that of WT FimHFL. In summary, the loss in affinity for R60P and especially for V27C/L34C mainly originates from enhanced $k_{\text{off}}$ values, which is in agreement with the concept of a dynamic allostery, i.e. the binding pockets in the medium- and high-affinity states of FimH have nearly identical geometries, although the off-rates are modulated by the flexibility of the binding pocket and the clamp loop (23, 26). Consequently, we focused in the following sections on the two most interesting variants (R60P and V27C/L34C) and the mutant A119L for comparison with an unaffected variant.

**Conformational fingerprints of selected FimHLD variants by NMR**

$^1$H,$^1$H HSQC experiments of uniformly $^{15}$N-labeled WT FimHLD and the mutants R60P, V27C/L34C, and A119L were measured in the absence and presence of mannoside 1 to assess structural differences on a residue-based level. The backbone assignment of WT FimHLD was available from previous studies (25, 45). The $^1$H,$^1$H HSQC spectrum of WT FimHLD showed an excellent signal dispersion with numerous clearly isolated peaks, especially those of the binding pocket residues (Fig. 6A). Addition of mannoside 1 led to selective signal shifts of residues around the binding pocket (Fig. 6B and Fig. S6) as expected from direct ligand interactions (hydrogen bond formation) and from indirect effects (small changes in the binding pocket geometry, e.g. a conformational switch of the Tyr-48 side chain when interacting with the n-heptyl glycoside) (45). Pronounced
global conformational changes or allosteric effects upon mannoside binding could be excluded in agreement with the X-ray structural data (24, 25, 41, 45) and CD experiments (Fig. 3).

The $^1$H,${}^{15}$N HSQC spectra of the FimH<sub>LD</sub> mutant A119L in the absence and presence of mannoside were almost superimposable to that of the WT with only some deviations around the point of mutation (Fig. S7) and a certain degree of peak heterogeneity in the binding pocket region in the absence of ligand (Fig. S8, see “Discussion”).

In contrast, significant changes in the $^1$H,${}^{15}$N HSQC fingerprint spectra were observed for the R60P and V27C/L34C variants in comparison with the WT, suggesting drastically altered apo conformations (Fig. 6A). In particular, both mutants showed no peaks in the near vicinity of isolated signals characteristic for the binding pocket in the high-affinity state (Fig. 6A, lower panel). As we observed, roughly the expected number of peaks in the NMR spectra of the two mutants, this is unlikely to be the result of extensive line broadening due to chemical...


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exchange processes. Overlapping peaks in isolated spectral regions suggest that the two mutants have similar apo conformations (Fig. 59).

Strikingly, for both mutants, most signals of the binding pocket were recovered upon addition of mannoside 1 (Fig. 6B). Chemical shift maps for R60P and V27C/L34C, showing the shifts of the peaks relative to WT FimH_{LD} in the mannoside 1-bound form, indicate nearly identical chemical shifts in the binding pocket (Fig. 7). This suggests similar conformations for residues in direct contact with the mannosyl moiety (Fig. 7E: backbone signals of Gly-14, Asp-47, Asp-54, and Asp-140 and side chain signals of Gin-133 and Asn-135) as well as for the two loops encompassing Tyr-48 and Tyr-137 involved in hydrophobic interactions with the n-heptyl glycoside of mannoside 1 (Fig. 7F). In chemical shift perturbation (CSP) experiments with R60P and three different mannoside ligands (1-3, Fig. S10), characteristic peak positions observed in the WT FimH_{LD}, with each ligand could be restored, indicating identical ligand recognition. As a note, all mannoside complexes of FimH_{LD}, WT and mutants were in the slow or slow-intermediate regime as would also be expected from the off-rate constants measured with SPR (Table 4). Furthermore, for R60P and V27C/L34C, peak positions of the N-terminal pocket zipper and clamp-loop region (residues 2–16) and parts of the β-sheets were also recovered upon addition of mannoside 1. However, both mutants exhibit significant chemical shift differences compared with WT FimH_{LD}, encompassing large parts of the β-sheets distal from the binding pocket, the α-switch, and β-bulge regions as well as the interdomain loops (Fig. 7, C and D).

The 1H,15N HSQC spectrum of the V27C/L34C mutant upon treatment with the reducing agent DTT closely resembled that of WT FimH_{LD}, in the absence or presence of mannoside 1 (Fig. S11). Thus, in agreement with previous reports (5, 46), we confirmed that the additional disulfide bridge Cys-27–Cys-34 in the interdomain region of the double mutant V27C/L34C promotes the low-affinity conformation of FimH_{LD}.

Low-affinity conformation of mutant R60P results from a slightly altered clamp loop

X-ray crystallography was attempted with the FimH_{LD} mutants R60P and V27C/L34C to characterize their conformation on an atomic level. Although we did not succeed in growing crystals of the double mutant V27C/L34C, we obtained the X-ray crystal structure of R60P at a resolution of 1.6 Å (PDB code 5MCX) (Fig. 8 and Table S2). Whereas the structure was only poorly superimposable (r.m.s.d. of 2.2 Å) (52) with WT FimH_{LD} (PDB code 3WNJ) (41), it resembles the low-affinity state of FimH_{FL} (r.m.s.d. of 0.6 Å) as found in the crystal structure of the assembled fimbrial tip (PDB code 3WNJ) (5). In both structures, a twist in the β-sandwich fold of the lectin domain leads to a more compressed overall shape and, by a move of the clamp loop by ~3 Å, to a wider binding pocket in comparison with the high-affinity conformation present in WT FimH_{LD} (Fig. 8A). In more detail, the conformation of the clamp loop of the mutant R60P is located between the closed conformation present in WT FimH_{LD} and the completely open conformation of the full-length FimH in the assembled fimbrial tip (Fig. 8B). According to MD simulations of FimH_{FL}, the clamp loop is flexible in the absence of ligand (23), suggesting that its deviation in R60P from the low-affinity structure of FimH of the assembled fimbrial tip (PDB code 3WNJ) might be incidental or results from crystallization conditions.

Discussion

The catch bond behavior of the bacterial adhesin FimH represents a fascinating example of the allosteric regulation of protein’s conformational and ligand-binding properties (53, 54). Although the structural basis for this regulation has been elucidated (5, 23, 55), there is still a lack of experimental data on the transmission of the allosteric signal from the interdomain region to the distal binding pocket within FimH_{LD}. Four regions of FimH_{LD} exhibit significant structural differences in their low- and high-affinity conformation (Fig. 2) and are considered to play an active role in this signal transmission (5). Based on this information, the impact of site-specific mutations on the conformation of the FimH lectin domain has been previously investigated (38). Our own interest was initially focused on mutations expected to stabilize the lectin domain in the low-affinity state, because such mutants would allow a further evaluation of already available FimH antagonists until now mainly tested against the high-affinity state (30–33). To obtain such mutants and to further unravel the allosteric regulation mechanism, we expressed and analyzed the FimH_{LD} variants A10P, R60P, V67K, A119L, and V27C/L34C (Fig. 2), and compared their properties to WT FimH_{LD} and WT FimH_{LD}.

Our investigations show that not all point mutations were effecting protein conformation and binding properties. For instance, the mutations A10P (pocket zipper region) and A119L (insertion loop of the interdomain region) had no influence on affinity and kinetics when compared with WT FimH_{LD} (Tables 1 and 4). In a previous study using full-length FimH within the assembled pli of isogenic E. coli (38), these two mutants stabilized the low-affinity state, indicated by a low mono- versus trimannoside binding ratio. However, they retained the ability to adopt the high-affinity state similar to the WT variant as demonstrated by binding to the monoclonal antibody mAb21 in the presence of mannone. Therefore, for both mutants, these regions were assumed to show only weak allosteric coupling (38). In our study with A10P and A119L, CD analysis (Fig. 3 and Figs. S2 and S3) did not show any evidence of a conformational switch, but instead they strongly supported a high-affinity state similar to WT FimH_{LD} both in the absence and presence of mannoside 1. In the 1H,15N HSQC spectrum of apo-A119L (Fig. S7), double or triple peaks of binding pocket residues very close to the position of the WT signals were observed, indicating conformational flexibility. Upon addition of mannoside 1, single peaks at positions identical to WT signals were observed, suggesting a rigidification of the binding pocket (Fig. S8). In line with these results, ITC experiments revealed an entropic penalty for A119L in comparison with WT FimH_{LD}, which, however, was compensated by a more favorable enthalpy term (Table 2). Despite this altered thermodynamic profile, the binding kinetics were not affected (Fig. 9 and Table 4). To sum up, similar to previous reports (38), we observed only a weak coupling between residue Ala-119 of the
Figure 7. Chemical shift differences between WT FimHLD and the mutants R60P (A and C) and V27C/L34C (B, D, E, and F) in the mannoside 1-bound forms. A and B, CSP maps of the mutants relative to WT FimHLD in the presence of 1; red bars indicate lower limits according to tentative assignments to the closest unassigned peak of the mutants; CSP effects of WT FimHLD upon addition of mannoside 1 are shown for reference to indicate the location of the binding pocket (gray diamonds); secondary structure elements are indicated above; the positions of the mutations are marked with asterisks. C–F, X-ray structure of WT FimHLD with 1 (PDB code 4BUQ) (35) colored according to CSP effects between the 1-bound forms of WT FimHLD and R60P (C) or WT FimHLD and V27C/L34C (D–F).
interdomain region with the binding pocket that, however, does not affect the affinity or kinetics of the interaction.

CD analysis for V67K, R60P, and V27C/L34C (Fig. 3 and Figs. S2 and S3) and NMR spectra for R60P and V27C/L34C mutants (Figs. 6 and 7) revealed an altered protein conformation in the ligand-free state that was converted to a conformation similar to WT FimHLD upon mannoside addition. In contrast to previously suggested lock in the low-affinity conformation of these mutants (5, 38, 40), this demonstrates for the first time a considerable conformational change of the isolated FimHLD in the absence of the regulatory pilin domain. For the mutant V67K, designed to destabilize the helical α-switch region of the high-affinity FimHLD-binding affinity and kinetics were only mildly affected, suggesting solely a weak coupling between the α-switch region and the binding pocket. However, in comparison with WT FimHLD, R60P (β-bulge) and V27C/L34C (interdomain region) showed drastically reduced binding affinities (Table 1) as well as altered conformational (Figs. 6 and 8) and kinetic properties (Table 4). The high resolution X-ray structure of R60P in the absence of ligand (PDB code 5MCA) clearly showed that the mutant adopts a low-affinity conformation similar to full-length FimH as present in the assembled pilus (5). In particular, the clamp loop was not folded over the mannose-binding pocket as observed in WT FimHLD (e.g. PDB code 4AUU) but was instead located several angstroms away similar to the full-length structure (PDB code 3JWN, and Fig. 8).

1H,15N HSQC NMR spectra of R60P and V27C/L34C confirmed the drastically altered apo conformations, because nearly all peaks were shifted relative to WT FimHLD (Fig. 6A). However, addition of mannoside led to a complete recovery of NMR signals of the binding pocket, the clamp loop/pocket zipper, and large parts of the β-sheets, whereas the signals of the α-switch, β-bulge, and interdomain regions remained altered (Fig. 7). Interestingly, such a conformation accurately resembles the medium-affinity state observed in the crystal structure of the mannoside-bound FimHFL (23). Indirectly, this is evidenced by the observation that an inhibitory antibody raised against the FimHLD mutant V27C/L34C, with an epitope in the binding pocket, strongly binds to FimH in the high-affinity state (40), which displays a nearly identical geometry of the binding pocket compared with the medium-affinity state (23). However, addition of mannoside I led to a complete recovery of NMR signals of the binding pocket, the clamp loop/pocket zipper, and large parts of the β-sheets, whereas the signals of the α-switch, β-bulge, and interdomain regions remained altered (Fig. 7). Interestingly, such a conformation accurately resembles the medium-affinity state observed in the crystal structure of the mannoside-bound FimHFL (23). Indirectly, this is evidenced by the observation that an inhibitory antibody raised against the FimHLD mutant V27C/L34C, with an epitope in the binding pocket, strongly binds to FimH in the high-affinity state (40), which displays a nearly identical geometry of the binding pocket compared with the medium-affinity state (23). However, the exact conformation of V27C/L34C and R60P in the bound state remains to be confirmed, e.g. by X-ray structures of the mutants in complex with mannoside or comparative NMR studies with 15N-labeled FimHFL.

The similarity of the FimHLD mutant V27C/L34C with FimHFL was further substantiated by their almost identical kinetic profiles (Fig. 9). Finally, the reduction of the disulfide bond in the interdomain region of the V27C/L34C mutant restores a high-affinity state similar to WT FimHLD (Fig. S11),...
thus confirming the involvement of the interdomain region in the allosteric regulation of mannose binding (5).

Our data for the three mutants V67K, R60P, and V27C/L34C are in good agreement with MD simulations of FimHLD, indicating a weak coupling of the binding pocket to the α-switch region (V67K) but a strong coupling to the β-bulge (R60P) and to the interdomain regions (V27C/L34C) (26). These couplings are supposed to regulate the dynamics in the clamp loop near the binding pocket and to cause the renowned catch-bond behavior of FimH. A higher flexibility of the clamp loop in the medium-affinity state could be responsible for the dramatically enhanced off-rates for mannoses relative to the high-affinity state, despite nearly identical conformation of the binding pocket (23, 26).

In future work, NMR dynamics experiments (56, 57) could be used to address the higher flexibility of the clamp loop in the medium-affinity state. However, in the case of labeled FimHLD, the required expression in minimal media is hampered by the cumbersome expression and purification procedure (23) as well as the low yield. Therefore, the characterized mutants represent useful surrogates of full-length FimH as they can be readily expressed with uniform 15N and 13C labeling in preparative amounts (4–8 mg/liter) and benefit from their smaller size in terms of spectral dispersion and relaxation properties.

Based on the global conformational changes in the mutants of FimHLD, V67K, R60P, and V27C/L34C, we expected drastic entropic consequences. Although all three mutants displayed entropic penalties relative to WT FimHLD, large enthalpic penalties were the major reason for the loss of affinity of R60P and the medium-affinity state. Obviously, an interpretation of the thermodynamics of WT FimHLD and mutants thereof is highly speculative in view of the many contributing factors. Thus, the introduction of mutations may lead to local thermodynamic strain within the protein and to unexpected entropic effects of coordinated water molecules as a result of altered hydrophobic regions. Consequently, the entropic penalty caused by the conformational changes may be partially compensated.

In summary, our data clearly demonstrate that by a single mutation in the β-bulge (R60P) or by a double-point mutation introducing an additional disulfide bridge in the interdomain region (V27C/L34C), the isolated FimHLD can be converted from the high- to the low-affinity state and moreover can retain the same intricate allosteric regulation as observed in the full-length FimH. In particular, the kinetic profile of full-length FimH as the hallmark of the catch bond behavior was almost perfectly reproduced by the FimHLD mutant V27C/L34C (Fig. 9), thus clearly demonstrating the cross-talk between allosteric sites within the lectin domain in the absence of the pilin domain. Neither mutant was conformationally locked in the low-affinity state, but instead a switch to the medium-affinity state upon mannose binding similar to the full-length protein could be demonstrated. It remains to be seen whether a combination of different point mutations would lead to a complete lock of FimHLD in the low-affinity state, even in the presence of a mannoside ligand.

The mutants characterized herein therefore represent an easily accessible mimic of full-length FimH and may serve as valuable tools for further mechanistic investigations of the allosteric regulation. Ultimately, this knowledge will be required for the design of effective anti-adhesive drugs and antibody-based therapeutics for the treatment of UTI.

**Experimental procedures**

**Cloning of FimHLD mutants**

The FimHLD construct linked to a thrombin cleavage site (Th) and a His6 tag on the C terminus was generated as described (28). The mutants A10P, R60P, V67K, A119L, and V27C/L34C were generated by the overlap extension PCR method (58) using the WT-encoding plasmid as template. The inserts were digested with HindIII and XbaI restriction enzymes, gel-purified, and subsequently ligated into the corresponding cloning site of pDsbA3 expression vector (59). The vectors were then transformed separately into E. coli DH5α chemo-competent cells (Novagen, Lucerne, Switzerland). After plasmid isolation and restriction control, the correctness of the constructs was confirmed by DNA sequencing (Microsynth, Balgach, Switzerland). Finally, the vectors were transformed into the protease-deficient E. coli strain HM125 (60) for protein expression and export into the periplasmic space.

**Protein expression and purification**

Bacterial clones were grown at 30 °C with vigorous shaking (300 rpm) in M9 minimal medium (61) supplemented with 2 mM MgSO4, 0.1 mM CaCl2, 20% glucose, 10 μM of each amino acid, basal medium Eagle vitamin mix (Sigma, Buchs, Switzerland), and 100 μg/ml ampicillin. When an A600 of 0.8 was reached, the cells were induced with 1 M isopropyl-1-thio-β-D-galactopyranoside (Applichem, Germany) and further cultivated for 16 h at 30 °C and 300 rpm. Then the cells were cooled on ice for 5 min and harvested by centrifugation at 5000 rpm for 20 min at 4 °C. The pellet was suspended in a cold solution of 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 5 mM EDTA, 1 mg/ml polymyxin B sulfate (lysis buffer) and stirred for 2 h at 4 °C. After centrifugation at 11,000 rpm for 20 min at 4 °C, the supernatant (periplasmic extract) was dialyzed overnight against 50 mM NaH2PO4, 300 mM NaCl, and 10 mM imidazole, pH 8 (binding buffer), and applied to a nickel-nitrilotriacetic acid column (Sigma, Buchs, Switzerland) attached to a BioLogic fast protein liquid chromatography system (Bio-Rad, Reinach BL, Switzerland). The column was washed with binding buffer and afterward eluted with 50 mM NaH2PO4, 300 mM NaCl, and 250 mM imidazole, pH 8 (elution buffer). The fractions containing FimHLD-Th-His6 were pooled and dialyzed against 20 mM HEPES buffer, pH 7.4, with 150 mM NaCl and 1 mM CaCl2 (assay buffer). The purity of the protein was verified by SDS-PAGE analysis, and the monomeric state was confirmed by native PAGE. The amount (4–10 mg/liter of each of the five mutants) was determined by direct absorption at 280 nm on an ND-1000 nanodrop spectrophotometer (Thermo Fisher Scientific). Aliquots of the proteins could be stored for up to 3 months at 4 °C. For long-term storage, the proteins were frozen at −80 °C without additives. For protein crystallization, FimHLD constructs (amino acids 1–158), lacking the His6 tag, were

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generated following the same procedure described above and purified by ion-exchange chromatography (29). FimH<sub>EL</sub> was expressed and purified as described (23).

**Differential scanning fluorimetry**

Differential scanning fluorimetry was performed using a Rotor-Gene Q 6plex quantitative PCR instrument according to the procedure described previously (42). SYPRO Orange dye was diluted 1:50 in the final samples and was used to monitor the protein-denaturing profiles. The assay was performed in assay buffer with 5 μM protein and n-heptyl-α-d-mannopyranoside (1) (45, 62) at a final concentration of 5 mM.

**Circular dichroism analysis**

CD spectra of WT FimH<sub>LD</sub> and mutants were recorded in the absence and presence of 1 mM mannoside 1 (63, 64). Far-UV (180–260 nm) and near-UV CD spectra (250–350 nm) were recorded with a Chirascan CD spectrometer (Applied Photophysics, Leatherhead, UK). For far-UV analysis, the proteins were diluted to a concentration of 5 μM in 10 mM sodium phosphate buffer at pH 7.4 and measured at 20 °C using a thermostat-controlled cell with 0.1-cm path length. The CD spectra were recorded between 190 and 250 nm with 2 nm bandwidth and 10 scans per sample. For near-UV analysis, all samples were measured at a protein concentration of 1 mg/ml using a thermostat-controlled cell with a path length of 1 cm and between 260 and 320 nm. Mannoside 1 was added to a final concentration of 1 mM for both near- and far-UV CD analysis. CD spectra were corrected by buffer subtraction, and the CD signal was converted to molar ellipticity ([θ]/degree cm<sup>2</sup> dmol<sup>−1</sup>)

**FP assay**

The fluorescence polarization assay was performed as described previously (32). Briefly, a serial dilution of each FimH<sub>LD</sub> mutant and WT (0.1 nM to 2.6 μM) was mixed with a constant concentration of 1 (5 nM with WT and mutants V67K and A119L; 10 nM with mutants V27C/L34C and R60P) in a final reaction volume of 100 μl in 20 mM HEPES, pH 7.4, with 150 mM NaCl, and 50 μg/ml BSA. The mixture was incubated at room temperature in black, flat bottom, non-binding surface 96-well microtiter plates (Corning) for at least 24 h. Binding of the tracer ligand 4 (3′-chloro-N-(2-(3-(3′,6′-dihydroxy-3-oxo-3H-spiro-[isobenzofuran-1,9′-xanthan]-5-yl)-thioureido)-ethyl)-4′-(α-L-mannopyranosyl oxo)-biphenyl-4-carboxamide), synthesized as described previously (32) to each FimH<sub>LD</sub> mutant was monitored via an increase in FP using a Synergy H1 hybrid microplate reader (BioTek). Samples were excited with polarized light at 485 nm and emission was measured at 528 nm. FP data were fitted to a single-site binding model accounting for signal change (65) to determine the K<sub>D</sub> value of 4 for each FimH<sub>LD</sub> variant. The K<sub>D</sub> value for 1 was determined competitively via the displacement of 4 by 1 and the associated decrease in FP. For these competition assays, a serial dilution of competitor 1 (7.6 nM to 500.0 μM) was titrated into constant concentrations of FimH<sub>LD</sub> and 4 (10 nM). The concentration of each mutant was fixed such that it would be above K<sub>D</sub> and the competitor concentration at 50% signal change would be in the same approximate range for all mutants. Specifically, it was fixed at 200 nM for V27C/L34C, 100 nM for R60P and V67K, and 50 nM for A119L and WT. FP was measured as described above, and data were fitted to an equilibrium competition binding function (66). Data fitting and analysis were performed using Prism (GraphPad Software).

**Isothermal titration calorimetry (ITC)**

Prior to analysis, the proteins were dialyzed against 20 mM HEPES buffer, pH 7.4, with 150 mM NaCl. ITC experiments were performed at 15, 25, 31, and 37 °C using a VP-ITC microcalorimeter (Malvern Instruments, UK). Volumes ranging from 6 to 8 μl of 150 μM compound 1 were injected at 600-s intervals into a reaction cell containing 1.45 ml of 10–15 μM FimH<sub>LD</sub> WT or mutant. Baseline correction and peak integration were completed using Origin (OriginLab). The resulting binding isotherm was imported into SEDPHAT (National Institutes of Health) (69) for baseline subtraction and data fitting. The initial 2-μl injection was not included for data analysis. Data were fit to a single-site binding model to derive K<sub>D</sub> and ΔH (change in binding enthalpy) values from the binding isotherm. ΔS<sub>obs</sub> (change in entropy) was calculated from the known thermodynamic quantities and further dissected into conformational, mixing, and solvation entropy according to the methodology as described in Ref. 70. A concentration correction factor was included as fitting parameter to account for the uncertainty in determining FimH<sub>LD</sub> concentration. Statistical analysis to determine best-fit values and 95% confidence intervals was performed with SEDPHAT (69).

**SPR**

Kinetic data were obtained by SPR-based measurements using a Biacore T200 system (GE Healthcare, Uppsala, Sweden). Compound 5 (see supporting information for synthesis and structure) was immobilized on a carboxy-dextran-coated CM4 sensor chip (GE Healthcare) in a two-step process. In the first step, 1,2-diamino-ethane (0.1 M in borate buffer, pH 8.5) was covalently coupled to the chip matrix through NHS-ester chemistry using the amine coupling kit (GE Healthcare). The carboxy-functionalized compound 5 was activated by NHS/EDC. After raising the pH to 8.5 with 0.1 M borate buffer, the derivatized ligand was injected into the system (0.5 mM for 30 s) and reacted with the free amine groups of 1,2-diamino-ethane. Ethanolamine at a 1 M concentration was used to block any remaining activity on the surface. A reference cell was prepared by immobilizing 8-hydroxyoctanoic acid using the amine coupling kit (GE Healthcare) followed by blocking with 1 M ethanolamine.

All SPR measurements were performed at 25 °C using HBS-EP (0.01 M HEPES, pH 7.4, 0.15 M NaCl, 3 mM EDTA, 0.005% surfactant P20, from GE Healthcare) as running buffer at a flow rate of 30 μl min<sup>−1</sup>. Because of baseline drifts and incomplete chemical regeneration (50 mM NaOH, 180 s), complete dissociation of the protein (analyte) was achieved by sufficiently long dissociation times. Because of the resulting long measurement times for multicycle kinetic (MCK) assays, MCK was only performed if the k<sub>off</sub> was above 10<sup>−3</sup> s<sup>−1</sup>. Otherwise, the single cycle kinetics mode was used that includes a single
dissociation phase to obtain kinetic information. Data processing and kinetic evaluations were performed with the Biacore T200 control software and the Biacore T200 evaluation software (both version 1.0), respectively. The Langmuir 1:1 model of interaction was used to determine $k_{on}$, $k_{off}$, and $K_D$ values. For FimH$_{LD}$, data were fitted using a two-state reaction model. The bulk effect on the refractive index was set to zero because of the reference cell subtraction.

**NMR spectroscopy**

All experiments were performed at 298 K on a Bruker Avance III 600 MHz NMR spectrometer equipped with a 5-mm TXI room temperature probe head. Samples contained 120–240 $\mu$M $^{15}$N-labeled WT FimH$_{LD}$ or mutant in 20 mM phosphate buffer, pH 7.4, with 7% D$_2$O. Mannosides 1, O-methyl-$\alpha$-$\alpha$-mannopyranoside (2), and $\alpha$-heptyl 4-deoxy-4-fluoro-$\alpha$-$\alpha$-mannopyranoside (3) (synthesized as described before (45, 62), see Fig. S10 for structures) were solved in D$_2$O and added stepwise to the protein up to a 10-fold molar excess. The mutant V27C/L34C (150 $\mu$M) was additionally measured in the presence of DTT (5 mM) in the absence or presence of compound 1 (2 mM). $^1$H, $^15$N HSQC experiments were acquired and processed with Topspin 3.2 (Bruker BioSpin, Switzerland) and analyzed with CcpNmr Analysis (version 2.2) (67). The backbone assignment of FimH$_{LD}$ was available from previous studies (25, 45). Partial assignments of mutant proteins were done on the basis of chemical shift proximity. Where applicable, combined chemical shift differences, $\Delta \delta_{AV}$, between the signals of WT and mutant were calculated (68) as shown in Equation 7,

$$
\Delta \delta_{AV} = \sqrt{(\Delta \delta^{1H})^2 + (0.2 \Delta \delta^{15N})^2}
$$

(Eq. 7)

As all tested compounds bound to all FimH$_{LD}$ variants in the slow to intermediate–slow exchange regime on the NMR time scale, partial assignments of the bound states could not be easily transferred to the free states. For residues that cannot be assigned unambiguously in the spectra of the mutants, a lower limit of the $\Delta \delta_{AVG}$ value was determined that corresponds to the chemical shift difference between the WT peak and the nearest unassigned peak in the mutant spectrum (chemical shift maps in Fig. 7).

**Protein crystallization and structure determination**

FimH$_{LD}$ R60P crystals were grown (10 mg/ml in 20 mM HEPES, pH 7.4) in 1.5 M (NH$_4$)$_2$SO$_4$, 0.1 M BisTris, pH 6.5, at 20 °C. Crystals were cryopreserved by a quick soak in 2.5 M Li$_2$SO$_4$ (71) and flash-cooled in liquid nitrogen. Data were collected with synchrotron radiation at the X06DA beamline at the Swiss Light Source (Paul Scherrer Institut, Switzerland). Data were indexed, integrated, and scaled with the XDS package (72). The structure was solved by molecular replacement with PHASER (73) using the FimH$_{LD}$–biphenyl $\alpha$-$\alpha$-mannopyranoside complex (PDB code 4X50) as a search model (45). The structure was iteratively built using the COOT software (74), refined with the PHENIX software (75), and validated using MolProbity (76). The structure has been deposited in the Protein Data Bank under the accession code 5MCA.

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**Author contributions**—S. R. performed cloning, protein production, stability testing, and CD measurements. B. F. conducted and analyzed NMR experiments. D. E. and M. S. performed ITC and SPR measurements. R. P. J. and T. M. obtained the X-ray structure of mutant R60P. G. N. synthesized compounds. S. R., B. F., and B. E. designed the work and wrote the manuscript, and all other authors provided helpful discussions.

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