Single-Molecule Observation of Ligand Binding and Conformational Changes in FeuA

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ABSTRACT  The specific binding of ligands by proteins and the coupling of this process to conformational changes is fundamental to protein function. We designed a fluorescence-based single-molecule assay and data analysis procedure that allows the simultaneous real-time observation of ligand binding and conformational changes in FeuA. The substrate-binding protein FeuA binds the ligand ferri-bacillibactin and delivers it to the ATP-binding cassette importer FeuBC, which is involved in bacterial iron uptake. The conformational dynamics of FeuA was assessed via Förster resonance energy transfer, whereas the presence of the ligand was probed by fluorophore quenching. We reveal that ligand binding shifts the conformational equilibrium of FeuA from an open to a closed conformation. Ligand binding occurs via an induced-fit mechanism, i.e., the ligand binds to the open state and subsequently triggers a rapid closing of the protein. However, FeuA also rarely samples the closed conformation without the involvement of the ligand. This shows that ligand interactions are not required for conformational changes in FeuA. However, ligand interactions accelerate the conformational change 10,000-fold and temporally stabilize the formed conformation 250-fold.

INTRODUCTION

The non-covalent and specific interactions between ligands and proteins underlies almost all biological processes. The coupling of these binding events to conformational changes allows proteins to act as highly efficient enzymes, signal transducers, motors, switches, or pumps (1). Two basic models that describe the coupling between protein conformational changes and ligand binding are the induced-fit (2) and conformational selection mechanism (3). In the induced-fit mechanism, ligand interactions trigger a conformational change, whereas in the conformational selection mechanism, ligand interactions selectively stabilize a subset of conformations that already pre-exist in the unliganded protein (Fig. 1 A). Both mechanisms require intermediate states that are formed during the ligand-binding process. For example, when a protein switches between two conformational states, such as an open or a closed conformation (Fig. 1 A), an open-liganded state in the induced-fit mechanism and a closed-unliganded state in the conformational selection mechanism are essential intermediate states. However, the study of such transient and thermodynamically unstable states remains experimentally challenging.

Primarily driven by high-resolution structural analysis of proteins adopting different conformations when free and in complex with ligand, an induced-fit mechanism
was hypothesized for many proteins. However, development in single-molecule spectroscopy (4–7), NMR (8–10), and other spectroscopic methods (11) revealed that proteins are highly dynamic and can undergo large conformational changes intrinsically, that is, in the absence of ligand. Examples are substrate-binding proteins of type I ABC importers (4), ABC exporters (7), adenylate kinase (9), RNase A (10), dihydrofolate reductase (12), ubiquitin (13), and DNA polymerase (14,15). Because of the occurrence of intrinsic conformational changes, a conformational selection mechanism has been proposed to be the ligand-binding mechanism of many protein systems (3). However, the unambiguous determination of the binding mechanism requires simultaneous monitoring of ligand binding and the protein conformation. Moreover, the intrinsic conformational ensemble can, in principle, be investigated by studying the protein in the absence of ligand. However, trace contaminations of ligand can make this assessment experimentally difficult, especially under single-molecule conditions. To bypass these problems, we here established a fluorescence-based single-molecule assay and data analysis procedure that allows the simultaneous real-time observation of conformational changes and ligand binding in FeuA.

The substrate-binding protein FeuA is associated with the ATP-binding cassette (ABC) transporter FeuBC from *Bacillus subtilis* (16). This type II ABC importer is involved in the uptake of Fe³⁺ ions by an ATP-driven active transport of the siderophore bacillibacitbin (BB) in complex with Fe³⁺ (FeBB) (16). FeuA and other structurally related substrate-binding proteins (SBPs) (17) or domains (18) represent the primary receptors of bacterial ABC importers (19), tripartite ATP-independent periplasmic transporters (20), and others (17). These proteins capture the ligand from the external environment and deliver it to the membrane transporter for import into the cell. The structure of FeuA has a characteristic SBP fold (17) consisting of two subdomains connected by a hinge region, with ligand binding occurring between the subdomain interface (Fig. 1 B; (21)). Crystallography studies suggest that FeuA undergoes conformational changes involving a domain reorientation to engulf the ligand, leading to opening and closing of the protein (21). This apparently simple binary conformational switch, which is involved in molecular recognition, is investigated in this work to obtain insights into the coupling between ligand interactions and protein conformational changes.

**MATERIALS AND METHODS**

**Gene isolation, protein expression, and purification**

The *feuA* gene (Uniprot: P40409) was isolated by PCR from the genome of *B. subtilis* subsp. *subtilis* str. 168. The primers (Table S1) were designed to exclude the signal peptide (amino acids 1–19), and cytochrome 20 (which is probably post-translationally lipidated) with NdeI/HindIII restriction sites. The generated PCR fragment was A-tailed and ligated into the PGEM-T Easy Vector System (Promega, Madison, WI) (22). After removing the NdeI restriction site internal to the *feuA* gene by a silent mutation, the gene was subcloned in the pET20b vector (Merck, Darmstadt, Germany) using the NdeI/HindIII sites. Protein derivatives, including the cytochrome and the silent mutation, were constructed using QuickChange mutagenesis (23). All sequences were checked for correctness by sequencing.

Cells harboring plasmids expressing the FeuAHis, wild-type and derivatives were grown at 37°C until an optical density of 0.5 was reached. Protein expression was then induced by addition of 0.25 mM isopropyl β-D-1-thiogalactopyranoside. After 3 h of induction, cells were harvested. DNase 500 µg/mL (Merck) was added and passed twice through a French pressure cell at 1500 psi. 2 mM phenylmethylsulfonyl fluoride was added to inhibit proteases. The soluble supernatant was isolated by centrifugation at 50,000 × g for 30 min at 4°C. The soluble material was purified and loaded on Ni²⁺-Sepharose resin (GE Healthcare, Chicago, IL) in 50 mM Tris-HCl (pH 8.0), 1 M KCl, 10 mM glycerol, 10 mM imidazole, 1 mM dithiothreitol (DTT). The immobilized proteins were washed (50 mM Tris-HCl (pH 8.0), 50 mM KCl, 10% glycerol, 10 mM imidazole, 1 mM DTT and subsequently with 50 mM Tris-HCl (pH 8.0), 1 M KCl, 10% glycerol, 50 mM imidazole, 1 mM DTT) and eluted (50 mM Tris-HCl (pH 8.0), 50 mM KCl, 10% glycerol, 300 mM imidazole, 1 mM DTT). Protein fractions were pooled (supplemented with 5 mM EDTA and 10 mM DTT), concentrated (10,000 MWCO Amicon; Merck), dialyzed against 100–1000 volumes of buffer (50 mM Tris-HCl, pH 8.0, 50 mM KCl, 50% glycerol, 10 mM DTT), aliquoted, and stored at −20°C.
Protein labeling

Labeling was performed with the maleimide dyes Alexa555 and Alexa647 (Thermo Fisher Scientific, Waltham, MA). The purified proteins were treated with 10 mM DTT for 30 min at 4 °C to reduce oxidized cysteines. The protein sample was diluted to 1 mM DTT, immobilized on a Ni²⁺-Sepharose resin, and washed with 10 column volumes of buffer A (50 mM Tris-HCl (pH 7.4) and 50 mM KCl). The resin was incubated 2–8 h at 4 °C with the dyes dissolved in buffer A. The molar dye concentration was 20 times higher than the protein concentration. Unbound dyes were removed by washing the column with 20 column volumes of buffer A and eluted with 400 mM imidazole. The labeled proteins were further purified by size-exclusion chromatography (Superdex 200; GE Healthcare) using buffer A. The sample composition was assessed by absorbance measurement at 280 nm (protein), 559 nm (Alexa555), and 645 nm (Alexa647) to determine labeling efficiency. For all samples, the labeling efficiency was >90%.

Ensemble fluorescence measurements

The fluorescence spectra were recorded on a scanning spectrofluorometer (Jasco FP-8300; Jasco, Tokyo, Japan). Emission spectra were recorded by excitation at 635 nm (5 nm bandwidth) in steps of 2 nm (2 nm emission bandwidth and 8 s integration time). Fluorescence anisotropy values were determined as described before (4). Fluorescence measurements were performed in buffer A at a concentration of 100–250 nM of labeled proteins and free fluorophores at room temperature.

Solution-based smFRET and ALEX

Solution-based single-molecule Förster resonance energy transfer (smFRET) and alternating laser excitation (ALEX) (24) experiments were performed using a home-built confocal microscope as described previously (4). In brief, two laser diodes (Coherent Obis; Coherent, Santa Clara, CA) with emission wavelengths of 532 and 637 nm were modulated in alternating periods of 50 μs. Average laser powers were 30 μW at 532 nm (~30 kW/cm²) and 15 μW at 637 nm (~15 kW/cm²). The fluorescence was collected by excitation at a depth of 20 μm and detected by two single-photon avalanche diodes (TAU-SPADs; Picoquant, Berlin, Germany). Measurements were carried out at 25–100 pM of labeled protein at room temperature in buffer A in the absence or presence of FeBB (EMC Biochemicals, Tübingen, Germany). Microscope no. 1.5H precision cover slides (VWR Marienfeld, Germany) were coated with 1 mg/mL bovine serum albumin to reduce oxidized cysteines. For all samples, the labeling efficiency was >90%.

Analysis of solution-based smFRET data

Photons were binned in 1 ms intervals, and only bins with a total of >200 photons considering all detection channels were analyzed. Three photon count rates were measured: N_{DA} (acceptor emission upon donor excitation), N_{DD} (donor emission upon donor excitation), and N_{AA} (acceptor emission upon acceptor excitation) (24). The background counts were estimated by excluding all time bins containing more than 20 counts and calculating the mean count rate over all remaining time bins. The leakage and direct excitation contributions were determined from the donor- and acceptor-only-labeled molecules as described by Lee et al. (25). Cross talk and background-correcting N_{XY} yields N_{XX}. The proximity Förster resonance energy transfer (FRET) efficiency E_{PR} is

$$ E_{PR} = \frac{N_{DA}}{N_{DA} + N_{DD}}, $$

and the stoichiometry S is

$$ S = \frac{N_{DA} + N_{DD}}{N_{DA} + N_{DD} + N_{AA}}. $$

The donor- and acceptor-labeled protein (sub)populations within the E_{PR} and S data set were clustered using a Gaussian mixture model, with one (apo) or two (holo) multivariate normal distributions. Molecules were assigned to the component yielding the highest posterior probability and are within 98% of the probability mass. For each cluster, the average N_{DA}/N_{DD}, N_{DP}/N_{DA}, and E_{PR} were calculated. All postprocessing steps were programmed in MATLAB (MathWorks, Natick, MA).

Theory of interprobe distance ratio estimation

The distance between the donor and acceptor fluorophore r is related to the FRET efficiency E via

$$ E = \frac{R_0^6}{R_0^6 + r^6} = \frac{n_{DA}}{n_{DA} + \gamma n_{DD}}, $$

where R_{0} is the Förster radius and n_{DA} and n_{DD} are the background- and spectral-cross-talk-corrected donor and acceptor emission count rates when the donor is excited, respectively. γ = φ_{DA}/φ_{A}φ_{D} depends on the donor and acceptor quantum yields—φ_{D} and φ_{A}, respectively—and the detection efficiencies of the donor and acceptor emission detection channels—n_{D} and n_{A}, respectively (25). Equation 3 can be rewritten as

$$ \left( \frac{r}{R_0} \right)^6 = \frac{\gamma}{\frac{n_{DD}}{n_{DA}}}. $$

Let r_{1} and r_{2} denote the (average) donor and acceptor fluorophore distance of two states, here denoted by state 1 and state 2. By using the definition of γ and noting that R_{0} is proportional to φ_{D}, we find that the ratio between r_{1} and r_{2} satisfies

$$ \left( \frac{r_1}{r_2} \right)^6 = \frac{\phi_{1 \cdot A}}{\phi_{2 \cdot A}} \frac{N_{1 \cdot DD}}{N_{2 \cdot DD}}, $$

where n_{1 \cdot DA} and n_{1 \cdot DD} are the count rates n_{DA} and n_{DD} of state i (i = 1, 2), respectively, and φ_{1 \cdot A} and φ_{2 \cdot A} are the acceptor quantum yields of states 1 and 2, respectively. Let us now consider how the distance ratio (r_{1}/r_{2})^{6} can be estimated from the data. We use the following notation: N_{i \cdot XY} represents the measured count rate of n_{i \cdot XY} that is, the background- and spectral-cross-talk-corrected count rate of Y emission (donor, acceptor) upon X excitation (donor, acceptor) when being in state i (i = 1, 2); and R_{i} and r_{i} are the measured and true interprobe distances of state i (i = 1, 2), respectively. When we can assume that the relaxation times of the excited states of the fluorophores are short compared to the time between two consecutively detected photons, then there is no correlation between consecutive photons, and the distribution of N_{i \cdot XY} can be approximated by a Poisson distribution (26). Then,

$$ \left( \frac{R_1}{R_2} \right)^6 = \frac{N_{1 \cdot AA}}{N_{2 \cdot AA}} \frac{N_{1 \cdot DD}}{N_{1 \cdot DA}} \frac{N_{1 \cdot DA}}{N_{2 \cdot DD}} $$

with

$$ \frac{N_{1 \cdot AA}}{N_{2 \cdot AA}} = \frac{1}{k} \sum_{k} N_{1 \cdot AA} / N_{2 \cdot AA}. $$


where \( k, p, \) and \( w \) denote the number of observations, is an unbiased and consistent estimator for \((r_1/r_2)^6\). The sum in Eq. 7 extends over all observations, i.e., the total number of traces or time bins. When the acceptor quantum yield of both states is similar, we have \( \phi_1 \cdot A = \phi_2 \cdot A \) so that Eq. 5 becomes

\[
\frac{\langle r_1 \rangle^6}{\langle r_2 \rangle^6} = \frac{\langle N_{1, DD} \rangle}{\langle N_{1, DA} \rangle} \cdot \frac{\langle N_{2, DA} \rangle}{\langle N_{2, DD} \rangle}.
\]

An unbiased and consistent estimator for Eq. 8 is

\[
\frac{\langle R_1 \rangle^6}{\langle R_2 \rangle^6} = \frac{\langle N_{1, DD} \rangle}{\langle N_{1, DA} \rangle} \cdot \frac{\langle N_{2, DA} \rangle}{\langle N_{2, DD} \rangle}.
\]

In the presence of fluorophore quenching by the ligand, we use Eq. 6, and when no quenching occurs, we use Eq. 9 to infer the interprobe distance ratio. Details and a full derivation are provided in Appendix S1 in the Supporting Materials and Methods.

### Scanning confocal microscopy

Confocal scanning microscopy was performed using a home-built confocal scanning microscope as described before (27,28). In brief, scanning was performed using a XYZ-piezo stage (P-517-3CD with E-725.3CD; Physik Instrumente, Karlsruhe, Germany). The detector signal was registered using a HydrelHarpp 400 ps event timer and a module for time-correlated single-photon counting (both Picoquant). Data were recorded with constant 532 nm excitation at intensities between 0.1 and 5 \( \mu W \) (25–1250 W/cm\(^2\)) for smFRET measurements and 0.1 \( \mu W \) (<25 W/cm\(^2\)) when the protein was only labeled with Alexa647 (640 nm excitation) or Alexa555 (532 nm excitation). Scanning images of 10 \( \times \) 10 \( \mu m \) were recorded with 50 nm step size and 1 ms integration time at each pixel. After each surface scan, the positions of labeled proteins were identified manually. Surface immobilization and a flow-cell arrangement was prepared as done previously (28,29). Measurements were done at room temperature in buffer A with 1 mM 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid and 10 mM cysteine.

### Analysis of fluorescence trajectories

Fluorescence trajectories were recorded in time bins of varying length as stated in the text or figure captions. We use the following notation: \( \langle N_{1, DD} \rangle \) is the uncorrected count rate, \( \langle N_{1, DA} \rangle \) is the background-corrected count rate, and \( \langle N_{2, DD} \rangle \) is the background- and spectral-cross-talk-corrected count rate of \( Y \) emission (donor, acceptor) upon donor excitation. The apparent FRET efficiency is \( \langle N_{1, DD} \rangle/\langle N_{1, DA} \rangle \). Only traces lasting longer than 20 time bins, having on average more than 10 photons per time bin that showed clear bleaching steps, were used for further analysis.

Equation 9 was used to estimate the interprobe distance ratio, with \( N_{DA}/N_{DD} = (\langle N_{DA} \rangle - (1 + d\beta)\langle N_{DD} \rangle)/(1 + dB) \) and \( N_{DA}/N_{DD} = (1 + dB) \) for the derivation. Background was determined as the average count rate per channel when the fluorophores have bleached. The correction factors were determined using solution-based ALEX (25). In brief, the \( I \) and \( d \) factors were determined from the donor- and acceptor-only labeled FeuA molecules and the \( \beta \) and \( \gamma \) factors using the protein MalE (4) as reference standard. All correction factors were determined on the same microscope also used for the surface-based measurements (details of setup in Gouridis et al. (28)).

The state trajectories were modeled by a hidden Markov model (HMM) (30). For this, an implementation of HMM was programmed in MATLAB (MathWorks) as described previously (4). We assume that the FRET and acceptor-only and donor-only fluorescence trajectory can be considered as an HMM with only two states, having a one-dimensional Gaussian- or a Poisson-output distribution, respectively. The Gaussian distribution of state \( i (i = 1, 2) \) is defined by the average and variance. The Poisson distribution of state \( i (i = 1, 2) \) is defined by the average intensity of the acceptor or donor in state \( i \). The likelihood function was maximized using the Baum-Welch algorithm (31). The most probable state trajectory was found using the Viterbi algorithm (32).

### RESULTS

## Direct observation of ligand binding and unbinding events

To investigate ligand binding by FeuA at the single-molecule level, we labeled FeuA with the Alexa647 fluorophore in one of its subdomains by introducing a single cysteine residue at a non-conserved position, which is solvent-exposed and distant from the binding pocket (Q112C; Fig. 1 B). First, we determined the emission spectra of FeuA-Alexa647 and free Alexa647 in the presence or absence of the ligand FeBB. We observed that the fluorescence intensity of FeuA-Alexa647 was quenched in the presence of 5 \( \mu M \) FeBB (Fig. 1 C). Because no quenching was observed for free Alexa647 (Fig. 1 C), we attribute this to binding of FeBB by FeuA.

To directly observe the binding and unbinding of ligand, the fluorophore emission of individual surface-tethered labeled proteins was measured over time by confocal scanning microscopy (Fig. 1 D). Representative fluorescence intensity trajectories of FeuA in the presence of 40 nM FeBB are shown in Fig. 1 E. All analyzed fluorescence trajectories show a single bleaching step, indicating that single molecules are examined (Fig. 1 E). Only in the presence of FeBB we observed stochastic switching between two intensity levels (Fig. 1 E), caused by fluorescence quenching of Alexa647 by FeBB. Thus, the intensity fluctuations can be interpreted as individual binding and unbinding events of FeBB to FeuA. To substantiate this claim, we determined the relative population of the lower intensity level to estimate the dissociation constant (\( K_d \)) of FeBB binding by FeuA. From the analysis of 50 traces in the presence of 40 nM FeBB, we find that 66% of the time, FeuA is
complexed with ligand. By using the Hill-Langmuir equation $P = L/(L + K_D)$, where $P$ is the relative population of the ligand-bound state and $L$ the ligand concentration, we obtain an estimated $K_D$ of $20 \pm 3$ nM (SD from bootstrapping; see also Fig. S1). This is in good agreement with the value obtained from intrinsic tryptophan fluorescence ($K_D = 27 \pm 1$ nM) (33). In summary, our assay can be used to directly probe the presence or absence of the ligand FeBB in FeuA. However, how the binding and unbinding events are coupled to the conformational changes in FeuA remains unclear.

Conformational states of FeuA

We used FRET to investigate whether ligand binding alters the FeuA conformation in solution and at room temperature. In our assay, each of the two subdomains was stochastically labeled with either a donor (Alexa555) or an acceptor fluorophore (Alexa647). Surface-exposed and non-conserved residues, showing large distance changes according to the crystal structures of the open and closed states (21), were chosen as cysteine positions for fluorophore labeling (Q112C/I255C; Fig. 1B). The relationship between FRET efficiency and interprobe distance requires free fluorophore rotation, which was verified by steady-state anisotropy measurements (Table S2).

We used confocal microscopy with ALEX (24) to explore the conformational states of individual, freely diffusing proteins (Fig. 2A). During the diffusional transit through the excitation volume of a confocal microscope, the labeled protein generates a short fluorescent burst, allowing for the determination of the apparent FRET efficiency and the stoichiometry $S$ (see Materials and Methods for details). To retrieve interprobe distances, the apparent FRET efficiency was corrected for background and spectral cross talk to obtain the proximity ratio $E_{PR}$. In our assays, changes in the apparent FRET efficiency and $E_{PR}$ can originate from interprobe distance changes, but also because of fluorophore quenching caused by FeBB binding (Fig. 2B). Finally, $S$ relates the total fluorescence recorded after donor excitation in the green and red detection channels to the total fluorescence after direct donor and acceptor excitation in each detection channel.

The $E_{PR}$ and $S$ values of many individual proteins were acquired in the absence and presence of saturating concentrations of FeBB (100 µM) (Fig. 2, C and D). By separating donor-acceptor-labeled proteins from the donor- and acceptor-only-labeled proteins based on the $S$ range, an $E_{PR}$ histogram was constructed (Fig. 2E). The $E_{PR}$ histogram of ligand-free FeuA is unimodal and well fitted by a single Gaussian distribution. In the presence of 100 µM FeBB, two populations of donor-acceptor-labeled proteins are observed and are centered around different $E_{PR}$ and $S$ values (Fig. 2, D and E). FRET analysis of surface-tethered proteins in the presence of 100 µM FeBB (83 traces) reveals that FeuA does not switch between these FRET states, i.e., fluorescence trajectories are obtained in either FRET state, with no switching between them (Fig. 2F). The cysteine positions in the crystal structure have distinct distances to the ligand-binding site. Therefore, the two

![FIGURE 2](image-url) Conformational changes of FeuA in solution. (A) A cartoon view of labeled proteins diffusing through the excitation volume of the confocal microscope is shown. (B) Quenching and FRET depend on the protein conformation and the ligand FeBB. (C and D) $E_{PR}$ and $S$ of FeuA(Q112C/I255C) in the absence (C) and in the presence of 100 µM FeBB (D) are shown. The two donor- and acceptor-labeled protein populations in (D) are indicated in red and orange and the single population in (C) in pink. (E) $E_{PR}$ histogram of the selected molecules in (C) and (D). Color coding in (E) corresponds to the selected molecules in (C) and (D) with the same color. Solid line is a Gaussian fit. A 95% confidence interval for the average $E_{PR}$ and count rate ratios are shown. (F) Representative fluorescence trajectories of surface-immobilized FeuA(Q112C/I255C) in the presence of 100 µM FeBB are shown. Top panel shows calculated apparent FRET efficiency (blue) from the donor (green) and acceptor (red) photon counts as shown in the bottom panels. Black line indicates the average efficiency. The number of analyzed molecules is provided in Table S3. To see this figure in color, go online.
FRET states most likely arise because of the different donor-and acceptor-labeling positions. This is expected from stochastic labeling of two different cysteine positions, causing differences in fluorophore quenching by FeBB and thus differences in $E_{PR}$ and $S$ values. Indeed, analysis of individual acceptor- and donor-only-labeled proteins shows that quenching is position- and fluorophore-dependent (Fig. 3; Table S3).

To correct the FRET efficiencies for fluorophore quenching, we related the populations in Fig. 2 $D$ to their corresponding labeling positions. The low $S$ value of the high FRET population in Fig. 2 $D$ (orange population) implies that the quenching of the donor is more prominent than that of the acceptor. To quantify the quenching, we constructed and studied all four single-cysteine FeuA variants that were used in our FRET assays (Fig. 3). The quenching behavior of the high FRET population in Fig. 2 $D$ (orange population) was observed to occur when Q112C was labeled with a donor and I255C with an acceptor (Fig. 3, $A$ and $B$). The largely unaltered $S$ value of the low FRET population in Fig. 2 $D$ (red population) suggests that the donor and acceptor quenching is similar and was observed to occur when the labeling position is reversed, i.e., Q112C is labeled with an acceptor and I255C with a donor (Fig. 3, $C$ and $D$).

To evaluate whether ligand binding causes conformational changes in FeuA, we developed an analysis scheme that 1) takes into account the influence of donor and acceptor quenching and 2) considers FRET between the donor and acceptor fluorophores. We show in the Materials and Methods and Appendix S1 in the Supporting Materials and Methods that

$$\left(\frac{R_B}{R_F}\right)^6 = \frac{\langle N_{B, AA} \rangle}{\langle N_{F, AA} \rangle} \frac{\langle N_{B, DD} \rangle}{\langle N_{F, DA} \rangle} \frac{\langle N_{F, DA} \rangle}{\langle N_{F, DD} \rangle}$$

(10)

is an unbiased and consistent estimator for $(r_B/r_F)^6$, where $r_B$ and $r_F$ are the true and unknown interprobe distances of the ligand-bound ($B$) and ligand-free ($F$) proteins, respectively, and $R_B$ and $R_F$ are the measured distances of $r_B$ and $r_F$, respectively. In Eq. 10, $N_{i, XY}$ denotes the measured count rate of $Y$ emission (donor, acceptor) upon $X$ excitation (donor, acceptor) when being in state $i$ (bound, free), and $\langle \rangle$ denotes the average. Noteworthy, the distance ratio is independent of donor quenching, i.e., of $N_{B \cdot DA}/N_{F \cdot DD}$.

The average ratios $N_{B \cdot DD}/N_{B \cdot DA}$ and $N_{F \cdot DA}/N_{F \cdot DD}$ were obtained from the selected molecules in Fig. 2, $C$ and $D$ and noting that $E_{PR} = N_{i, DA}(N_{i, DA} + N_{i, DD})$ (Fig. 2 $E$). The average ratio $N_{B \cdot AA}/N_{F \cdot AA}$ was obtained.

![Figure 3](https://example.com/figure3.png)

**Figure 3** Fluorescence quenching in FeuA depends on the labeling position and the fluorophore. ($A$–$D$) Fluorescence trajectories (left) of single-fluorophore-labeled FeuA in the presence of 25 nM FeBB ($A$ and $C$) or 40 nM ($B$ and $D$) and corresponding histogram of the count rate ratios $N_{B \cdot DD}/N_{F \cdot DD}$ or $N_{B \cdot DA}/N_{F \cdot AA}$ of all molecules (right) are shown. In the fluorescence trajectories, donor (green) and acceptor (red) count rate with the most probable state trajectory of the HMM (black) are shown. In the histogram, bars are the data and solid line a Gaussian fit. A 95% confidence interval for the average of the brightness ratios is indicated. The number of analyzed molecules is provided in Table S3. To see this figure in color, go online.
from individual acceptor-only-labeled proteins and by
determining the ratio between the acceptor fluorescence
intensity of the free or ligand-bound states (Fig. 3, B and D).
All values used for the interprobe distance ratio estimation
are summarized in Table 1. Finally, by using Eq. 10, we
find that the interprobe distance ratio $r_B/r_F$ when Q112C is
labeled with the donor and I255C with the acceptor is esti-
ated to be $0.90 \pm 0.01$ (95% confidence interval) and re-
mains unaltered ($0.91 \pm 0.01$) when the labeling position is
reversed (Table 1). This is in agreement with the value ob-
tained from the crystal structures (21), because the Cα-Cα
distance between residues Q112 and I255 in the ligand-
bound structure is reduced to 0.86 of the distance in the
ligand-free structure.

In summary, the analysis reveals that FeBB induces a
conformational change in FeuA in solution and at room
temperature. Moreover, the reduced interprobe distance in
the presence of ligand is consistent with the view that the
conformational transition from the open to the closed state
in FeuA and related SBPs (4) is triggered by ligand interac-
tions. However, the precise mechanism of ligand binding
and whether there are short-lived intermediate states or
fast conformational transitions cannot be concluded from
these measurements.

**Rare intrinsic conformational transitions in FeuA**

To directly observe how binding and unbinding of the
ligand FeBB are coupled to conformational changes in
FeuA and to obtain insights into conformational dynamics,
individual proteins were studied for extended times
by investigating surface-tethered FeuA molecules with
smFRET. It is worthwhile to note that in our surface-based
smFRET assays we do not determine absolute distances,
but only monitor relative interprobe distance changes by
determining the instrument-dependent apparent FRET
efficiency.

With this, we investigated the dynamics of ligand-free
FeuA and addressed whether the protein can also close
intrinsically, that is, when the ligand is absent. Compared
to the solution-based smFRET experiments, examining indi-
vidual surface-tethered proteins greatly increases the sensi-
tivity to detect rare events. To investigate a truly ligand-free
protein, a high concentration of unlabeled FeuA protein
($\sim 20 \mu M$) was added to scavenge any potential ligand
contamination that could otherwise cause ligand-induced
closing events.

Consistent with the solution-based smFRET measure-
ments (Fig. 2 C), FeuA is in a single FRET state (the open
conformation) in the majority of the fluorescence-trajec-
tories (437 traces out of 459; Fig. 4 A). In these traces,
FeuA shows no detectable changes in the apparent FRET ef-
iciency (or fluorescence intensity) related to conforma-
tional changes. However, in a small number of traces (22
traces out of 459), we observed rare transitions to a high
FRET state, suggesting that FeuA can intrinsically close
(Fig. 4 B). Indeed, by using Eq. 9 (see Materials and
Methods), the average interprobe distance ratio between
this high and low FRET state is $0.88 \pm 0.02$ (95% confi-
dence interval). This shows that the interprobe distance is
reduced and inferred to represent closure of the protein.
Importantly, the absence of additional quenching effects
provides direct evidence that the conformational change oc-
curs independently of FeBB. Despite the low number of
closing transitions that could be detected in the 459 traces
(22 events), approximate estimates for the kinetics can be
made. The ligand-free closed state has an average lifetime
of $42 \pm 7$ ms (mean $\pm$ SEM; Fig. 4 C), and from the total
observation time of $\sim 15$ min, we estimate that this state is
formed on average only once every $\sim 40$ s (15 min/22 tran-
sitions). In summary, ligand-free FeuA is predominantly in
an open conformation and can extremely rarely close, lead-
ing to the formation of a short-lived closed conformation.

**Ligand-bound FeuA is in the closed conformation**

We then investigated the conformational dynamics of the
ligand-bound protein. In our assay, the total fluorescence in-
tensity reports on the presence of the ligand, whereas addi-
tional apparent FRET efficiency changes are indicative of
protein conformational changes. However, in the 173 fluores-
cence-trajectories that were recorded in the presence of $\sim K_D$
concentrations of FeBB, we could not observe any FRET
changes within the period a ligand was bound by FeuA
(Fig. 5 A; Fig. S1 A). The average apparent FRET efficiency
of the initial and final 200 ms of the ligand-binding events
were not significantly different from the period in between
that ($p = 0.28$, one-way analysis of variance; Fig. 5 B). This
suggests that once the ligand is bound, FeuA remains closed,
and other conformational transitions, such as the formation
of an open-liganded state, do not occur (or occur on timescales
faster than the time resolution of 200 ms; see below).

With this in mind, we determined the lifetime of the
ligand-bound state at varying FeBB concentrations (173

| Assumed labeling position | $\langle N_{B-A} \rangle / N_{F-A} \rangle$ | $\langle N_{B-GO} \rangle / N_{F-GO} \rangle$ | $\langle N_{F-DA} \rangle / N_{F-GO} \rangle$ | $R_B^6$ | $R_B^6$ |
|--------------------------|---------------------------------|---------------------------------|---------------------------------|--------|--------|
| Q112C donor and I255C acceptor | 0.733 ± 0.010 | 0.671 ± 0.021 | 1.113 ± 0.016 | 0.547 ± 0.020 | 0.904 ± 0.006 |
| Q112C acceptor and I255C donor | 0.432 ± 0.008 | 1.168 ± 0.045 | 1.113 ± 0.016 | 0.562 ± 0.025 | 0.908 ± 0.007 |

Error denotes a 95% confidence interval.
traces total). We observed that the lifetime of this state was largely concentration independent ($p = 0.80$, one-way analysis of variance) having an average lifetime of $10.0 \pm 0.6$ s (mean $\pm$ SEM; Fig. 5, C and D). Interestingly, the ligand-bound closed conformation is 250-fold longer-lived than the intrinsic closed state ($10.0 \pm 0.6$ s vs. $42 \pm 7$ ms).

The ligand is bound via the induced-fit mechanism

Next, we investigated which conformational state binds the ligand (intrinsically closed or the open state), and thus, whether the ligand binds via a conformational selection or induced-fit mechanism (Fig. 1 A). We expect that when the intrinsic closed conformation binds the ligand, the ligand-binding frequency would be limited by the intrinsic closing frequency ($\approx 1.5$ min$^{-1}$). So, we determined the lifetimes of the ligand-free states at varying FeBB concentrations (Fig. 5, C and D; Fig. S1 A). We observed that the binding frequency increases with FeBB concentration and is already $6.5 \pm 0.6$ min$^{-1}$ (mean $\pm$ SEM) for the lowest concentration used (10 nM FeBB) (Fig. 5 D). Thus, ligand binding occurs at a faster rate than the intrinsic closing transitions. These data are consistent with an induced-fit mechanism. In addition, in 60 traces that were recorded with a higher time resolution of 5 ms, we observed that FeuA is in the open state before the ligand binds (Fig. 5 E). The average apparent FRET efficiency of the 10 ms period before the ligand binds ($0.511 \pm 0.014$, mean $\pm$ SEM) and the period before that ($0.516 \pm 0.001$, mean $\pm$ SEM), when the protein is in the open conformation, are not significantly different ($p = 0.70$, two-tailed unpaired t-test). This demonstrates that the open conformation binds the ligand and that ligand binding occurs via the induced-fit mechanism (Fig. 5 F).

The open-liganded state is extremely short-lived

An essential intermediate state of the induced-fit mechanism is the open-liganded state (Fig. 1 A). Based on our data, we can already conclude that the open-liganded state has to be shorter-lived than 200 ms (see Ligand-Bound FeuA Is in the Closed Conformation). To further investigate the lifetime of this state, we increased the excitation intensity to obtain a time resolution of 4 ms. To probe the open-liganded state, we used saturated concentrations of FeBB. Under these conditions, the ligand-free open conformation is expected to be absent, and any detectable open state would consequently correspond to the open-liganded form. However, by examining 94 individual FeuA molecules with a total observation time of 104 s, we could not detect any opening transitions (Fig. 6). All these traces show FRET fluctuations, but those could not be separated from noise or did not originate from a clear anticorrelated donor and acceptor fluorescence change, as expected for real changes in FRET efficiency. Although we could not directly observe the open-liganded state, we can provide an upper bound for its lifetime. Based on the time resolution of the measurement, we conclude that the open-liganded state should be shorter-lived than 4 ms. Thus, when FeuA is in complex with FeBB, closing is accelerated more than 10,000-fold.
FIGURE 5  FeuA binds ligand via an induced-fit mechanism. (A) The fluorescence trajectory of FeuA(Q112C/I255C) is shown. In all fluorescence trajectories presented in the figure, the top panel shows apparent FRET efficiency (blue) and donor (green) and acceptor (red) photon counts are shown in the bottom panel. Orange lines are the most probable state trajectory of the HMM. (B) An apparent FRET efficiency histogram of the first and last 200 ms of the binding event and the period between that is given. Mean ± SEM is indicated. (C) Cumulative distribution function of the time FeuA has FeBB bound (tbound; top) or is free (tunbound; bottom) is shown at different FeBB concentrations. (D) tbound (purple) and the rate of binding (1/tunbound; green) as a function of FeBB concentration are shown. Data are mean ± SEM and the solid line is a linear fit. From the fit, binding and unbinding rates of 1 Molar are obtained. (E) Fluorescence trajectories of FeuA showing a single binding event are given. (F) A schematic of the induced-fit mechanism is shown. A number of analyzed molecules is provided in Table S3. To see this figure in color, go online.

decompared to when the ligand is absent (from <4 ms to 40 s). This suggests a mechanism in which the ligand drastically accelerates the conformational change that is already present in the ligand-free protein.

The energy landscape of FeuA

So far, we focused on the dynamic aspects of the molecular recognition process, that ultimately originates from the precise architecture of the energy landscape of the protein. Next, we used our single-molecule results to determine the thermodynamic properties of FeuA (Fig. 7).

The binding process can most easily be treated within the context of Gibbs ensembles. The grand partition function Q(T, μ) of a single protein-ligand system, as shown in Fig. 7, is

\[ Q(T, \mu) = e^{-\beta G_0} + e^{-\beta G_c} + e^{-\beta (G_{CL} - \mu)} + e^{-\beta (G_{OL} - \mu)}, \]  

where \( \beta = (k_B T)^{-1} \), \( k_B \) is the Boltzmann constant, \( T \) is the absolute temperature, \( G_i \) is the free energy of state \( i \) (O, open-unliganded; C, closed-unliganded; CL, closed-ligated), and \( \mu \) is the chemical potential. We assume that the ligand solution can be treated as ideal, so \( \mu = \mu_0 + k_B T \ln L \), where \( L \) is the ligand concentration (relative to 1 Molar) and \( \mu_0 \) is the standard chemical potential (\( \mu = \mu_0 \) when \( L = 1 \)).

The probability that the protein is in the intrinsic closed conformation is

\[ P(C; L = 0) = \frac{e^{-\beta G_c}}{Q(T, \mu \rightarrow -\infty)} = \frac{1}{1 + e^{\Delta T}} \]  

where \( \Delta = G_C - G_O \) is the ligand-free protein conformational free energy. From the fraction of time spend in the intrinsic closed conformation in the absence of ligand (Fig. 4), we find that \( P(CL = 0) \) is 10\(^{-3}\), so \( \Delta = 7 k_B T \).

In the presence of ligand, the fraction of proteins occupied by a ligand is

\[ P((OL, CL); L) = \frac{e^{-\beta (G_{CL} - \mu)} + e^{-\beta (G_{OL} - \mu)}}{\Omega(T, \mu)}. \]  

By treating \( \mu \) as an ideal ligand solution (\( e^{\beta \mu} = e^{\beta \mu_0} L \)), we find that Eq. 13 is equal to

\[ P((OL, CL); L) = \frac{(e^{-\beta (G_{CL} - \mu_0)} + e^{-\beta (G_{OL} - \mu_0)}) L}{e^{-\beta G_c} + e^{-\beta G_O} + (e^{-\beta (G_{CL} - \mu_0)} + e^{-\beta (G_{OL} - \mu_0)}) L}. \]
Equation 14 can be expressed as the Hill-Langmuir equation,

\[ P(\{OL, CL\}; L) = \frac{L}{K_D + L}, \]

where \( K_D \) is the dissociation constant as determined in our study (Fig. S1 B). By making the approximation that \( e^{-\beta G_{CL}} \gg e^{-\beta G_{OL}} \) (see below), we find that \( K_D \) is equal to

\[ K_D = e^{\beta A} (1 + e^{\beta A}), \]

where \( A = (G_{CL} - \mu_0) - G_C \) is the protein-ligand interaction free energy of the closed conformation. We find that FeuA binds FeBB with a \( K_D \) of 15–20 nM (Fig. S1 B), so \( A = -25 \ k_BT \).

When the protein is saturated with ligand, the probability of being in the closed conformation is

\[ P(CL; L = \infty) = \frac{e^{-\beta G_{CL}}}{e^{-\beta G_{CL}} + e^{-\beta G_{OL}}} = \frac{1}{1 + e^{\theta}}, \]

where \( \theta = G_{CL} - G_{OL} \) is the ligand-bound protein conformational free energy and \( P(OL; L = \infty) = 1 - P(CL; L = \infty) \). We could not directly observe the \( OL \) state in the smFRET measurements (Fig. 6), but we can calculate an upper bound for \( P(OL; L = \infty) \).

An estimator for \( P(OL; L = \infty) \) is

\[ P(OL; L = \infty) = \frac{\tau_{OL}}{\tau_{OL} + \tau_{CL}}, \]

where \( \tau_{OL} \) and \( \tau_{CL} \) are the lifetimes of the \( OL \) and \( CL \) states, respectively. By using \( \tau_{OL} \leq 4 \ ms \) and \( \tau_{CL} = 10.0 \ s \), we find that \( P(OL; L = \infty) \leq 410^{-5} \) so that \( \theta < -8 \ k_BT \). Finally, we find that \( \sigma = (G_{OL} - \mu_0) - G_D = \Delta + \theta > -10 \ k_BT \), where \( \sigma \) is the protein-ligand interaction free energy of the open conformation.

Taken together, in the absence of ligand, the closed conformation is thermodynamically unstable and requires an energy input of \( \Delta = 7 \ k_BT \) for its formation. The situation is completely reversed when FeuA has a ligand bound: the open conformation is thermodynamically unstable and requires an energy input of more than \( -\theta = 8 \ k_BT \) for its formation. Furthermore, from the fact that \( \Delta = \sigma = (G_{CL} - G_D) - (G_{OL} - G_D) < -15 \ k_BT \), we conclude that the protein-ligand interactions are at least 15 \( k_BT \) stronger in the closed conformation compared to the open conformation.

**DISCUSSION**

Modulation of the protein conformational landscape by ligand interactions is fundamental to the function and regulation of proteins. Here, we used a single-molecule approach to investigate the ligand-binding process and to understand how this is coupled to the conformational dynamics of the protein. For this, we analyzed the protein conformation and changes thereof via FRET and visualized the presence or absence of a ligand molecule via fluorophore quenching.

From the analysis of FeuA, combined with recent work on other proteins (4,9,10,12,34), a picture emerges in which ligand-bound protein conformations are also part of the conformational ensemble of the unliganded protein. It appears that ligand binding only alters the free energies of the equilibrium states and the barriers between them. For FeuA, the equilibrium lies toward the open conformation in the absence of ligand, with the closed conformation being 7 \( k_BT \) higher in free energy. On the other hand, when FeuA has a ligand bound, the closed conformation is more than 8 \( k_BT \) lower in free energy than the open conformation. Because of these large free energy differences, it is clear that in FeuA, there is a strong, but not an absolute, coupling between ligand binding and protein conformational changes. This shows that nature has fine-tuned the conformational landscape to approximate the behavior of FeuA by a simple binary switch that is regulated by ligand interactions.

Two basic mechanisms that connect the open and closed conformations with the unliganded and liganded states are
the induced-fit and the conformational selection mechanism (Fig. 1A). In the induced-fit mechanism, the ligand binds to the open conformation and triggers closing, whereas in the conformational selection mechanism, the ligand is directly bound by the closed conformation (Fig. 1A). We demonstrate that ligand binding occurs via the induced-fit mechanism, by showing that the ligand binds to the open conformation and triggers closing (Fig. 5). We could argue that the conformational landscape provides the required directionality for the induced-fit mechanism. If ligand binding was to occur via the conformational selection mechanism, a substantial amount of thermal energy ($\Delta G = 7 k_BT$) would be required to form the ligand-competent, intrinsic closed conformation, making the process highly inefficient (Fig. 7). The induced-fit mechanism would be more efficient because no thermodynamically unfavorable intermediate states need to be formed during the binding process ($\sigma > -10 k_BT, \theta < -8 k_BT$; Fig. 7).

By using sensitive single-molecule methods, we observed that ligand-free FeuA can sample a temporally and thermodynamically unstable state that is different from the open conformation (Fig. 4). We provide direct evidence that the conformational change occurs independently of the ligand FeBB, because the FRET change occurs without any additional quenching effects that occurs when the ligand binds. Further investigations are required to characterize this conformation on a molecular level, but, based on the reduced interprobe distance relative to the open state, we infer that this rare protein state represents a closed conformation. We also note that we cannot exclude the existence of other conformations that exchange with the open conformation on the nanosecond-to-microsecond timescale. Such conformations could be other short-lived conformations such as a semi-closed state, as has been observed for the SBP maltose-binding protein (8). In this case, the apparent open state would be a temporal average of two states. For such scenarios, the ligand-binding mechanism could be more complex and might involve ligand binding to a dynamically unstable state that is different from the open conformation (Fig. 4). We provide direct evidence that the conformational changes in FeuA with transport in FeuBC. Ligand binding by FeuA and related SBPs via an induced-fit mechanism would allow the ABC transporter to discriminate between the ligand-free and ligand-bound states (39). The ligand-bound FeuA protein can be used to sense the presence of the correct ligand and initiate the transport process. Some SBPs have additional roles, as they are known to interact with chemoreceptors (40). Switching between two conformations allow the SBPs to transduce a signal, which is allosterically regulated by the ligand. Furthermore, we speculate that a wasteful conversion of chemical energy is prevented by the transient nature and high free energy of the intrinsic closed conformation, because any thermally driven mimic of the ligand-bound FeuA complex might be able to initiate the translocation cycle and consume the energy of ATP. In addition, a competition between the ligand-free and ligand-bound closed conformations to interact with the membrane-embedded transporter would inhibit substrate import (28).

As a final comment, we note that our data analysis approach to derive the interprobe distance ratio of two (conformational) states with altered quantum yield of donor and acceptor dyes could also be applied to situations in which FRET is changed because of protein-induced fluorescence enhancement (PIFE) (41,42). The approach suggested here is particularly attractive for PIFE because the interprobe distance ratio is independent of the donor quantum yield, and thus Cy3, which is the most popular dye for PIFE, could be used in a straightforward fashion without additional knowledge of its quantum yield (changes).

**CONCLUSIONS**

We designed a single-molecule assay and data analysis procedure to probe the FeuA conformational changes via FRET.
and the presence of the ligand FeBB via fluorophore quenching. We show that FeuA exists in an open and a closed conformation in solution and at room temperature. In the absence of ligand, FeuA is (predominately) in the open state, and ligand shifts the equilibrium toward the closed state. Ligand binding occurs via the induced-fit mechanism, that is, a ligand binds to the open state and subsequently triggers a rapid closing of FeuA in less than 4 ms. Unbinding of the ligand also occurs almost simultaneously with the opening of the protein. However, FeuA also rarely samples the closed conformation without the involvement of the ligand, and thus, ligand interactions are not necessary for closing. However, such interactions accelerate the closing transition 10,000-fold and decrease the opening rate 250-fold in FeuA.

**SUPPORTING MATERIAL**

Supporting Material can be found online at https://doi.org/10.1016/j.bpj.2019.08.005.

**AUTHOR CONTRIBUTIONS**

M.d.B., G.G., and T.C. conceived and designed the study. T.C. supervised the project. G.G. and Y.A.M. performed the molecular biology. M.d.B. performed the measurements, analyzed the data, developed the theoretical work, and wrote the initial manuscript. M.d.B. and T.C. finalized the manuscript with input of all authors. All authors contributed to the discussion and interpretation of the results.

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**SUPPORTING CITATIONS**

Reference (43) appears in the Supporting Material.

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