Direct single-molecule observation of a protein living in two opposed native structures

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Biological activity in proteins requires them to share the energy landscape for folding and global conformational motions, 2 key determinants of function. Although most structural studies to date have focused on fluctuations around a single structural basin, we directly observe the coexistence of 2 symmetrically opposed conformations for a mutant of the Rop-homodimer (Repressor of Primer) in single-molecule fluorescence resonance energy transfer (smFRET) measurements. We find that mild denaturing conditions can affect the sensitive balance between the conformations, generating an equilibrium ensemble consisting of 2 equally occupied structural basins. Despite the need for large-scale conformational rearrangement, both native structures are dynamically and reversibly adopted for the same paired molecules without separation of the constituent monomers. Such an ability of some proteins or protein complexes to switch between conformations by thermal fluctuations and/or minor environmental changes could be central to their ability to control biological function.

Results and Discussion

Probing syn and anti Rop Structures Using Single-Molecule FRET. Single-molecule techniques are powerful tools to investigate structure, dynamics and function of biomolecules while minimizing complications from ensemble averaging (26–33). Single-molecule FRET (smFRET) involves the nonradiative transfer of energy between a donor and an acceptor dye, and its strong distance dependence (with FRET efficiency given by $E_{\text{FRET}} = \frac{1}{1 + (R/R_0)^6}$ (26, 34)) provides a molecular ruler for measuring distances in the 30–80 Å range. This long-range distance measurement capability makes it well-suited to directly observe the occupations of different structural basins and monitor the large global changes in geometry between the syn and anti Rop structures.

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To prepare proteins for FRET measurements, for each Rop variant (WT, A2L2, and A2I2), samples were separately labeled with either Alexa Fluor 488 (donor) or Alexa Fluor 647 (acceptor) dyes at the C-terminal cysteines. Labeling was performed under folding conditions, i.e., on dimeric Rop, to prevent dye access to internal cysteines in WT Rop (Figs. S1 and S2). After unfolding and dimer dissociation in guanidinium chloride (GdmCl), donor and acceptor-labeled monomers were combined and refolded to form the FRET dimer. This procedure yields a mixture of donor–donor-, donor–acceptor-, and acceptor–acceptor-labeled dimers; as a result, ensemble FRET values would be difficult to interpret and unlikely to show conclusive structural evidence.

We thus performed single-molecule FRET experiments to examine structural distributions, while focusing solely on the donor–acceptor FRET pairs formed by Rop dimers. First, Rop dimers were dispersed in native buffer at a low concentration (100 pM) enabling observation of individual dimers by smFRET, in a “freely-diffusing” format (35, 36). In these experiments, laser excitation of the donor dye and simultaneous detection of donor and acceptor fluorescence were performed using a high numerical aperture objective and confocal detection. When molecules diffuse through the focal volume, emitted bursts of fluorescence from the donor and acceptor dyes are separately and simultaneously recorded by high-efficiency avalanche photodiodes. The series of such bursts in a time-trajectory were then analyzed to produce histograms of FRET efficiency ($E_{\text{FRET}}$), which contain information about distance, structural populations and distributions (26, 37, 38) (see SI Text). Examination of the WT Rop smFRET histograms showed a single FRET peak centered at $E_{\text{FRET}}$ ~ 0.45 (Fig. 3A). We then performed similar experiments with the A2I2 mutant and obtained histograms with a FRET peak at ~0.75 (Fig. 3C).

These results are consistent with the expected anti and syn structures respectively, which place the donor/acceptor dyes further or closer to each other (Fig. 1). We note that the major source of the observed peak-broadening is experimental shot-noise, in addition to other contributions (35, 36). Having documented the smFRET signatures for the anti and syn Rop geometries, using the well-characterized WT and A2I2 variants as references, we next examined the A2L2 mutant.

**Fig. 1.** Monomer structure and heptad repeat of the Rop 4-helix bundle. The only differences between WT-Rop and its mutants Ala2Leu2-6 (A2L2) and Ala2Ile2-6 (A2I2) are the inner residues composing the hydrophobic core of the coil-coil. (A) As shown for A2L2, alanine (yellow) and leucine (red) residues are aligned in 6 layers. (B and C) The “a” and “d” residues of the heptad repeats are packed together in the dimer core. (D) Sequences of the Rop variants used in this work. The AV-mutant is used as a control for the WT to eliminate possible artifacts from labeling buried cysteine residues (see SI Text and Figs. S1 and S2).

**Fig. 2.** Schematic of anti (Left) and syn (Right) dimer assembly for the A2L2 mutant, leading to an active (anti) or inactive (syn) Rop-dimer. (A) For the repacked Rop-variants, the mutations have created a near symmetry of the molecule. This symmetry opens the possibility of 2 opposite geometries: The 2 monomers can bind in a syn or in an anti conformation (16, 17). The FRET-dyes (indicated as green and red large spheres) are attached to the C termini and have significantly different interdye spatial distances in the 2 states. Thus, smFRET experiments can readily distinguish between the 2 conformations. Both conformations lead to a very similar packing of the alanine and leucine/isoleucine residues within the hydrophobic core, as indicated by the red and yellow surfaces representing the innermost 2 layers. (B) However, the structural rearrangement has a direct impact on Rop-dimer functionality: The RNA-binding interface (yellow) is formed on the combined surface of each monomer’s first helix, only when the 2 monomers are in the anti geometry (18, 19).
Surprisingly, when we carried out smFRET experiments on this variant, we discovered that the histogram observed closely matches the one obtained for the A2L2 mutant, with a peak at high-$E_{\text{FRET}}$ (Fig. 3B; see Fig. S3 for overlay). Based on the previous peak assignments, this result clearly demonstrates that the A2L2 adopts a syn arrangement in native buffer. To further investigate the energetic balance between the 2 structures, we next explored the denaturation behavior of the Rop variants.

Earlier studies using simulations (16, 17) showed that the syn and anti geometries lead to dissimilar kinetic and folding behavior. One might therefore envision the possibility that the 2 structures are differentially affected by denaturant. To probe the stability of the Rop dimers, using smFRET, we performed a titration with the denaturant guanidinium chloride (GdmCl). WT and A2L2 both maintain their single FRET peaks up to GdmCl concentrations of 5 and 4 M respectively, where complete loss of the FRET peak suggests rapid dimer-dissociation. In contrast, A2L2 has strikingly different behavior: A second peak appears at $E_{\text{FRET}} \approx 0.35$ in addition to the original peak at $E_{\text{FRET}} \approx 0.7$ in the FRET histograms at slightly denaturing conditions (Fig. 3E). Additionally, dissociation at single molecule concentrations occurs at a much lower concentration of denaturant (1 M GdmCl). The 2 peaks closely match the ones observed for the reference WT and A2L2 (Fig. 3D and F).

Indeed, a very similar histogram could be obtained when WT and A2L2 were mixed together (see Fig. S4). Hence, the balance between the syn and anti structures can be tuned by the denaturant concentration, and an equal population was reached $\approx 0.6$ M GdmCl. These data demonstrate that although A2L2 folds predominantly into the syn geometry under native conditions, the population balance can be dramatically shifted with mild perturbations (Fig. 5B). Computer simulations show that rmsd-fluctuations of these 3 mutants around the syn and anti conformations differ (16). For the WT, the anti conformation fluctuates less; for A2L2, syn and for A2L2 both conformations fluctuated comparably. This indicates that details of packing are likely responsible for the dissimilar behavior of the mutants.

Two peaks can be distinguished in the histograms obtained for A2L2, which reveals that the structural interconversions between syn and anti structures occur at timescales significantly greater than the approximately millisecond observation time.

**Do Transitions Between syn and anti Structures Occur Intermolecularly or Intramolecularly?** Given these intriguing observations, we still need to answer the following question: Does A2L2 switch predominantly between the syn and anti basins by initial dissociation of syn dimer followed by recombination of the monomers, or does this switch occur directly in each individual dimer without the need for dissociation? To distinguish between these possibilities, we performed 3-color smFRET experiments as depicted in Fig. 4.

We started with a mixture of A2L2-monomers in native buffer labeled with either Alexa Fluor 488 or Alexa Fluor 647 and formed stable dimers in the syn structure. We then diluted the proteins rapidly within a slightly denaturing solution of 0.45 M GdmCl (conditions resulting in a mixture of syn and anti states), containing a large excess (up to 300-fold) of monomers labeled with a third dye, Alexa Fluor 594. If the syn Rop-dimer needs to dissociate into monomers to form the anti conformation, the Alexa Fluor 647-labeled monomers should be replaced by the excess of competing Alexa Fluor 594-labeled monomer. Using the same experimental setup, because FRET between the Alexa Fluor 488-Alexa Fluor 594 dye-pair is higher for both syn and anti states (see Fig. S5), we should observe in that case a transfer toward a higher-FRET peak. This was indeed observed in the control experiment shown in Fig. 4C. Instead, the resulting histograms in Fig. 4F are very similar to those obtained in the 2-color smFRET experiment, clearly demonstrating that there is no substantial exchange of
monomers during the syn-anti conformational transition and no higher-order multimers are adopted. We conclude that despite the large-scale conformational change required, the Rop dimer can switch between the 2 structural basins without monomer exchange or change in oligomeric state (Fig. 4).

Overall, a key result from this work is the striking observation that native A2L2 adopts the syn conformation, which does not possess the RNA-binding interface, even though A2L2 has an in vitro RNA-binding affinity comparable to the WT (22, 25), and an in vivo screen that links Rop function to GFP expression demonstrates that A2L2 is functional in E. coli, whereas A2I2 is not (39) (Fig. 5). The low stability of A2L2 together with its tunable ability to interconvert between syn and anti conformations provide new insight into these apparently conflicting observations of a predominantly inactive syn conformation with the ability to bind RNA. The results suggest a possible mechanism in which RNA binds to the molecules in the alternative anti structural basin, shifting the dimer equilibrium toward Rop’s active conformation, thereby creating the functional population observed in vivo and in vitro. Although the full Rop-dimer—RNA kissing loop quaternary complex has micromolar dissociation constants making detailed single-molecule observation more involved, ongoing developments in fast dilution techniques combined with multicolor smFRET will soon permit direct monitoring of the complete landscape for the coupled Rop-RNA binding and folding.

Our data are also in accord with computational predictions about the properties of core-repacked Rop variants, which suggested that slow folding kinetics might be the result of topological homogeneity, whereas faster kinetics result from structural heterogeneity (16, 17). We confirmed that the unfolding rate of A2L2 is much faster than WT [estimated to be 30,000× faster (25)].

Herein, we have used the strength of single molecule detection (26, 33, 40) to directly evaluate the distribution of molecular states in the Rop dimer system, and discover an interconversion between anti (active) and syn (inactive) native structures. More generally, other proteins might also possess conformations on the verge of structural heterogeneity and express a behavior similar to Rop (41). The balance between multiple competing structural basins would be affected by amino acid mutations, and could even be dynamically altered by changing environmental conditions such as concentration of particular ions or other small molecules, and changes in temperature or pH. It seems quite plausible that living systems exploit such a conformational competition as a regulatory mechanism, for example by modulating binding to specific partners or by tuning protein activity during cellular processes.

Fig. 4. 3-color FRET-measurements test separation of monomers during conformational transitions. (A) Initially, the A2L2 dimers labeled with Alexa Fluor 488 (green) and Alexa Fluor 647 (red) are formed in native buffer (0 M GdmCl), favoring the syn conformation. We add a 100- to 300-fold higher concentration of unfolded A2L2 monomers labeled with Alexa Fluor 594 (purple) and simultaneously change the GdmCl concentration of the mixture to 0.45 M. This triggers conformational transitions from the syn to the anti structure. The 2 hypotheses (“separation of monomers” or “rearrangement within dimer”) would lead to different mixtures of donor–acceptor pairs, fluorescent and nonfluorescent species. As described in Fig. S5, using the same 2 donor and acceptor detection channels, these smFRET measurements can easily detect if Alexa Fluor 488 labeled monomers have separated and reassembled with Alexa Fluor 594 monomers. (C) For complete separation of monomers, a shifted peak ~0.85 should be observed, as obtained in the control experiment where A488, A594, and A647 monomers are mixed in 6 M GdmCl before refolding to similar conditions (0.45 M GdmCl). (B) Because the FRET populations obtained after mixing match the original data, we conclude that the syn-to-anti transition occurs intramolecularly without A2L2 dimer dissociation.
sum of signals from the 2 channels) was then used to separate fluorescence signals from background, and FRET efficiencies were calculated for each accepted event and plotted in the form of a histogram.

The FRET-efficiency histograms were fitted with Gaussian functions, using Origin (OriginLab) and IGOR (WaveMetrics) softwares, and the peak positions and areas obtained from the fitting parameters. At least 8 measurements were made for each sample to construct a FRET histogram. FRET efficiencies are defined on the basis of the corrected donor ($I_D$) and acceptor ($I_A$) fluorescence intensities as

$$E_{FRET} = \frac{I_A}{I_A + \gamma I_D}$$

where $\gamma$ is a correction factor dependent on the donor ($\phi_D$) and acceptor ($\phi_A$) quantum yields, and donor channel ($\eta_D$) and acceptor channel ($\eta_A$) detection efficiencies as follows:

$$\gamma = \frac{\eta_A \Phi_A}{\eta_D \Phi_D}$$

$\gamma$ is known from previous measurements to be close to 1 (42, 43) and is assumed to be constant at 1 for the purpose of this article. This is a reasonable assumption because we do not use absolute distances to make conclusions in our article, and the dye labels on the floppy C termini are in very similar environments for syn and anti conformations (hence, dye quantum yields in the 2 states are expected to be the same).

Rather than use absolute distance measurements, we use standards to assign states corresponding to the FRET peaks for the A$_2$L$_2$. For each condition, we compare the histograms obtained for A$_2$L$_2$ to the one obtained for WT-Rop and A$_2$I$_2$. Because the crystal structure have been determined for these 2 dimers respectively in the anti and syn conformations, and the relative distance changes for the FRET peaks are consistent with these 2 structures, we use them as references to determine the conformation(s) adopted by the A$_2$L$_2$ variant (see Figs. S3 and S4).

**Materials and Methods**

**Preparation and Dual-Labeling of Rop Dimers.** Expression and purification of WT and mutant Rop proteins were carried out following procedures described in refs. 20–22 and 25. For protein labeling, Rop C-terminal cysteine mutants were reacted with Alexa Fluor 488 maleimide (donor), Alexa Fluor 594 maleimide (acceptor) 1 or Alexa Fluor 647 maleimide (acceptor 2) dyes (Molecular Probes) in 6 M guanidine hydrochloride (GdmCl), 100 mM Tris, pH 7.2, 4 °C, overnight and in the dark. The mono-labeled proteins were subsequently purified from the unlaabeled dyes, using NAP columns (GE Healthcare) or Microcon Centrifugal devices (Millipore); the identity and purity of the reaction products were verified by ESI-MS mass spectrometry (Scripps Center for Mass Spectrometry).

For WT Rop at the C-terminal cysteine, the protein was labeled separately under unfolding conditions (200 mM NaCl, 100 mM Tris, pH 7.2) to protect the internal cysteines from the reactive dyes (see Figs. S1 and S2). The AV-Rop mutant, with internal cysteines removed by mutation, produces the same FRET histograms as the WT labeled under unfolding conditions. This verifies that $<5\%$ of the measured WT dimers would present mislabeling of the internal cysteines. The WT is the most stable structure of the constructs investigated here, because its unfolding occurs in no less than a day in 6 M GdmCl. In comparison, the labeling reaction itself is complete on a much shorter timescale (1 h), consistent with the nondetectable internal labeling. Moreover, our study shows that the formation and stability of the Rop-dimers is extremely sensitive to packing effects within the hydrophobic core. If any dye was present on the hydrophobic surface of a WT monomer, it would perturb deeply the binding interface and likely prevent the formation of the dimer. As a result, these mislabeled WT proteins would not create FRET pairs and would not be detected in our experiments. We thus conclude that the FRET events detected in our single-molecule experiments correspond to WT-Rop where the exposed terminal cysteines were predominantly labeled by our protocol.

**Single-Molecule FRET Measurements and Analysis.** Single-molecule FRET measurements were performed as described in refs. 26, 34, 35, and 42 (see additional details in SI Text). Briefly, the FRET efficiency histograms described in this article were generated by using a 2-channel data collection mode to simultaneously record donor and acceptor signals as a function of time, with a binning time of 500 μs. The donor–acceptor solutions used were $100 \, \text{pM}$ in fluorophore concentration, ensuring that virtually all of the detected signals were due to single molecules.

The background counts, leakage of donor into the acceptor channel and direct excitation of acceptor were estimated in separate experiments. For changing denaturant concentration, the free-energy landscape changes and the 2 states (syn, S) and (anti, A) get populated accordingly (left to right: 0, 0.6, 1, and 2 M GdmCl). When no denaturant is present, the Syn and Anti basins are respectively strongly and weakly populated, whereas the unfolded ensemble is not assembled. For 0.6 M GdmCl, Syn and Anti are equally populated and transitions between the 2 states occur directly without disassociation of the monomers composing the dimer. Under unfolding concentrations $>1.5$ M GdmCl, Syn and Anti are energetically disfavored and the separated monomers (U) becomes the dominant part of the energy landscape.

**Fig. 5.** Rop activity and schematic free-energy landscape of A$_2$L$_2$. (A) A well-developed in vivo GFP screen for Rop activity shows that the A$_2$L$_2$ repacked variant is active whereas the A$_2$I$_2$ variant is inactive as compared with the positive and negative controls. The screen monitors the plasmid level of a ColE1 vector with a reporter GFP. In this case, the format of the screen is such that high levels of fluorescence indicate low levels of the ColE1 reporter plasmid and thus an active Rop variant. The link negative control is the empty p15A plasmid (pACT7lac-Cm) on which we have cloned the other Rop variants. This screen requires that the cells be grown at 42 °C to attain runaway plasmid replication of the ColE1 plasmid (for a negative phenotype). (B) Schematics of the free-energy landscape of A$_2$L$_2$ for different denaturant concentrations. For changing denaturant concentration, the free-energy landscape changes and the 3 states Anti (A), Syn (S), and Unfolded (U) get populated accordingly (left to right: 0, 0.6, 1, and 2 M GdmCl). When no denaturant is present, the Syn and Anti basins are respectively strongly and weakly populated, whereas the unfolded ensemble is not assembled. For 0.6 M GdmCl, Syn and Anti are equally populated and transitions between the 2 states occur directly without disassociation of the monomers composing the dimer. Under unfolding concentrations $>1.5$ M GdmCl, Syn and Anti are energetically disfavored and the separated monomers (U) becomes the dominant part of the energy landscape.

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Supporting Information

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SI Materials and Methods: Single-Molecule FRET Experiments

Single molecule FRET experiments were carried out on a home built laser confocal microscope system, using an Axiovert 200 microscope (Zeiss). Excitation was achieved by focusing the 488 nm line of a 543-AP-A01 tunable argon-ion laser (Melles Griot) inside the sample solution, 30 μm from the glass cover-slip surface, using a water immersion objective (1.2 NA, 63×; Zeiss). The fluorescence emission was collected using the same objective, separated from the excitation light, using a dichroic mirror (Q495LP; Chroma Tech), spatially filtered using a 100 μm pinhole, then separated into donor and acceptor components using a second dichroic mirror (560 DCXR; Chroma). The donor and acceptor signals ($I_D$ and $I_A$) were further filtered using an HQ 525/50M band-pass filter (donor; Chroma) and a 590 LPV2 long-pass filter (acceptor; Chroma), then detected using SPCM-AQR-14 avalanche photodiode (APD) photon counting modules (Perkin-Elmer Optoelectronics). Photon counts were recorded using a photon counting card (PCI 6602, National Instruments) interfaced with a computer. Data analysis was performed as described in Materials and Methods.
Fig. S1. Schematic of the WT and mutant dual-labeling strategies. The A2L2, A2L2, or AV mutants can be labeled in denaturant, whereas the WT-Rop is labeled under folding conditions. See main text for details.
Fig. S2. Protection of internal cysteines of Rop WT by dimerization. (A) Schematic of the WT Rop monomer, displaying the two internal cysteines that are buried in the hydrophobic core when the dimer forms. (B and C) Comparison of the histograms obtained for WT Rop and the AV mutant in native buffer confirms that the exposed WT cysteines are predominantly labeled using our protocol. (D) Sequences of the WT Rop and of the AV mutant.
Fig. S3. Conformational balance for A2L2 probed by single-molecule FRET. In native buffer, the histogram obtained for A2L2 matches closely the one obtained for the reference A2I2. A2L2 and A2I2 have the same syn conformation in native buffer. Data measured for A2L2 at 0 M GdmCl (A) to the histogram obtained in the same conditions for A2I2 (B). (C) Overlay showing the similarity of the two histograms (A2L2, black line; A2I2, gray bars). The histogram obtained for A2L2 in slightly denaturing conditions (0.6 M GdmCl) displays two FRET peaks (Fig. S4B), each of which correlates well with the WT and A2I2 reference peaks (Fig. S4A,C). We therefore conclude that A2L2 is able to adopt both anti and syn conformations. Furthermore, to test that we indeed observe a mixture of syn and anti conformations for A2L2 at 0.6M, we mixed 50% WT and 50% A2I2 and observed a very similar histogram, showing a very similar occupation of syn and anti structures in the two cases (Fig. S4D).
Fig. S4. Experimental control. A2L2 populates both anti and syn conformations under mild denaturation. (A–C) smFRET histograms obtained at 0.6 M GdmCl for WT, A2L2 and A2I2. (D) Overlay of the data observed for A2L2 (black line) and of a mixture of 50 pM WT + 50 pM A2I2 (gray bars). The two dimers were preformed in native buffer, measured separately then mixed in 0.6 M GdmCl, leading to a histogram that matches the one obtained for A2L2.
Fig. S5. Competition by monomers labeled with a second acceptor tests for dimer dissociation during conformational transitions. The 3-color experiments conducted in this work exploit the differences of distance-dependence of the FRET efficiencies between various FRET pairs. Especially, the A488-A647 and A488-A594 dye pairs have specific and well-separated Förster distances ($R_0$), which for a given donor-acceptor pair is the inter-dye distance for which $E_{\text{FRET}} = 0.5$. The red and purple lines in Fig. S5 shows the FRET efficiency as a function of inter-dye distance $r$,

$$E_{\text{FRET}} = \frac{1}{1 + \left(\frac{r}{R_0}\right)^6}$$

for the A488-A647 and A488-A594 dye pairs, calculated with $R_0$ values of 40 Å and 55 Å respectively. As described in A, the slightly denaturing conditions trigger interconversion between syn and anti folding states for the A$_2$L$_2$ mutant. These two conformations can be separated thanks to the adequate $R_0$ value of the A488-A647 FRET pair. If monomers labeled with A488 were to separate and refold with the A594-monomers present in excess in the solution, novel FRET species would appear (B). As the Förster distance $R_0$ is greater for the A488-A594 pair, both the syn and anti states would have higher FRET efficiencies. Using the same two detection channels, these two conformations cannot be distinguished (as observed in Fig. 4C), but the resulting FRET peak with $E_{\text{FRET}} = 0.85$ is clearly separated from the original peaks obtained with the A488-A647 pair. Our experiment demonstrates simply that little exchange of monomers occur during the structural switch between syn and anti structures.