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 (Repessor 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.

energy landscape theory | protein folding | Rop dimer | single molecule FRET

During the last 2 decades, the advent of energy landscape theory combined with a new generation of experiments have demonstrated that small and intermediate-sized proteins fold in a robust way through an ensemble of converging pathways, a folding funnel, biased toward the native ensemble (1–5). Accordingly, evolutionary pressure forces proteins to have sufficiently reduced energetic frustration that the folding mechanism is dominantly controlled by native interactions, with non-native contacts being mostly neutral (6, 7). Under the same scenario, some larger and more interesting proteins and protein complexes may achieve more than one conformation while maintaining many native contacts. Examples are conformational substates required for protein function (8, 9), aggregation as in 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 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 $E_{\text{FRET}} = 0.75$ (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.

The Active State Is Not the Major Structure for A2L2 Rop Under Native Conditions, and the syn/anti Balance Is Altered by Mild Denaturation. Because the A2L2 mutant binds RNA with a similar affinity as the WT, it is believed to adopt the same anti structure (21).
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 A2I2 both maintain their single FRET peaks up to GdmCl concentrations of 5 and 4 M respectively, whereas 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 A2I2 (Fig. 3D and F).

Indeed, a very similar histogram could be obtained when WT and A2I2 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 A2I2 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. 4B are very similar to those obtained in the 2-color smFRET experiment, clearly demonstrating that there is no substantial exchange of

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Fig. 3. Single-molecule FRET histograms for Rop and its mutants obtained in native buffer (A–C) and in slightly denaturing conditions, 0.6 M GdmCl (D–F). The distance between the labeling sites is \( \approx 40\) Å in the anti and \( \approx 20\) Å in the syn conformation. (A and C) Because the FRET-efficiency is inversely linked to the distance between the donor and the acceptor, the peaks at \(E_{\text{FRET}} \approx 0.45\) (A) and \( \approx 0.75\) (C) are related to Rop being in the anti and syn conformations respectively. The gray bars give the data with red (anti) and blue (syn) showing Gaussian fits to them. (D) We conclude that the WT stays in anti, although the peak shifts slightly to lower FRET-efficiencies for higher concentrations of GdmCl. (E) This relative shift could be due to variations in dyes properties, or possibly small effects on local structures. The mutant A2I2 stays clearly in syn, because the FRET peaks remains at a stable value. (F) For the mutant A2L2 we observe an occupation of syn for 0 M denaturant (B) (see also Fig. S3) and a mixed ensemble of syn/anti states at 0.6 M GdmCl (E) (see also the control experiment Fig. S4).

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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.
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 unlabeled dyes, using NAP columns (Scripps Center for Mass Spectrometry) 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 folding 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 mutant, with internal cysteines removed by mutation, produces the same FRET histograms as the WT labeled under folding 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 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 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, and used to correct the signals before FRET analysis. A threshold of 50 counts (the sum of signals from 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 (Ia) and acceptor (Ia) fluorescence intensities as

\[
E_{\text{FRET}} = \frac{I_a}{I_a + \gamma D}
\]

where \(\gamma\) is a correction factor dependent on the donor (\(\Phi_D\)) and acceptor (\(\Phi_A\)) quantum yields, and donor channel (\(I_a\)) and acceptor channel (\(I_a\)) 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 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 A2L2. For each condition, we compare the histograms obtained for A2L2 to the one obtained for WT-Rop and A2L2. Because the crystal structures 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 A2L2 variant (see Figs. S3 and S4).

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