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Everett A. Lipman et al.
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which suggests that different R proteins are activated by different mechanisms or that intermediate signaling steps are still to be identified that would lead to a similar mode of activation.

A key common point in these models is that pathogen virulence proteins are recognized as a consequence of their virulence function, rather than by direct interaction with a plant R protein. Such indirect recognition would be expected to significantly constrain the coevolution of pathogens and their plant hosts, as evasion of detection would require modification of virulence functions. Furthermore, by recognizing pathogen virulence proteins based on their enzymatic activity rather than their shape, plants could likely detect a large number of pathogen effectors with a limited number of R proteins. Although the extent of this mechanism is unknown [only three R genes are known to have dual specificity (24–26)], this seems an attractive strategy for the plant to maximize its surveillance capacity against the multitude of potential pathogen effectors. This may be a critical aspect of the plant immune system, as, unlike vertebrates, plants cannot generate a diversity of antibodies via somatic recombination.

AvrPphB belongs to a family of cysteine proteases found in both animal and plant pathogens (10), at least one of which, AvrPpiC2, is also known to induce resistance responses in specific plant genotypes (27). Several other bacterial Avr proteins display structural similarity to a second family of cysteine proteases (the YopJ family), and mutations in the putative catalytic residues of one of these, AvrBSt, abolish avirulence activity (28). Similarly, the fungal Avr protein AvrPita is homologous to known metalloproteases, and mutations in its putative catalytic residues also abolish avirulence activity (29).

In light of our data, these observations suggest that proteolysis of host target proteins may be a common trigger for many plant R gene pathways.

References and Notes

12. Materials and methods are available as supporting online material.

Single-Molecule Measurement of Protein Folding Kinetics

Everett A. Lipman,1,2† Benjamin Schuler,1,2§ Olgica Bakajin,3 William A. Eaton1,2†

In order to investigate the behavior of single molecules under conditions far from equilibrium, we have coupled a microfabricated laminar-flow mixer to a confocal optical system. This combination enables time-resolved measurement of Förster resonance energy transfer after an abrupt change in solution conditions. Observations of a small protein show the evolution of the intramolecular distance distribution as folding progresses. This technique can expose subpopulations, such as unfolded protein under conditions favoring the native structure, that would be obscured in equilibrium experiments.

Fluorescence detection of Förster resonance energy transfer (FRET), in which the probability of excitation energy transfer between two chromophores is used to determine their separation, is a powerful tool for investigating the behavior of single biological macromolecules (1, 2). Two variants of the technique are used. In the first, a dye-labeled molecule is immobilized on a surface, and its fluorescence emission is recorded continuously until one of the dyes has bleached. This allows uninterrupted observation and has been applied to both RNA and protein folding (3–6). Alternatively, fluorescence can be observed as molecules diffuse freely through the focus of a laser beam (7). This approach has the advantage of preventing interactions between the sample and a surface.

Free-diffusion experiments have yielded direct enumeration of thermodynamic states and information about the sizes and dynamical properties of unfolded molecules when both folded and unfolded molecules are present, information that is difficult or impossible to obtain from bulk samples (8–10). A limitation of the free-diffusion experiments performed to date is that they could only be used to examine states that are substantially populated at equilibrium. In order to study the properties of single molecules under nonequilibrium conditions, we have coupled a microfabricated laminar-flow mixer to a confocal optical system. This has allowed us to measure the FRET efficiencies of individual dye-labeled protein molecules as they flow through the illuminated volume at selected times subsequent to the triggering of folding by an abrupt change in denaturant concentration.

The microfabricated device and the sample flow in the mixing region are illustrated in Fig. 1. The mixer (11) is similar to that
designed by Kauffmann et al. (12) for ensemble infrared measurements. The Reynolds numbers (about 10^{-2}) in these experiments are small enough to guarantee laminar flow, and the microscopic dimensions of the mixer channels ensure that rapid mixing can occur by diffusion alone. Under these conditions, displacements due to diffusion and flow are comparable over a period of a few milliseconds, and the solutions are mixed by the time (about 50 ms) they have reached a point 50 μm beyond the center inlet.

![Microfluidic mixer](image)

**Fig. 1.** Microfluidic mixer. (A) Channel pattern and photograph of mixing region as seen through the microscope objective and bonded coverslip. Solutions containing protein, denaturant, and buffer were driven through the channels with compressed air (11). Arrows indicate the direction of flow. (B) View of the mixing region. Computed denaturant concentration (11) is indicated by color. The laser beam (light blue) and collected fluorescence (yellow) are shown 100 μm from the center inlet. (C) Cross section of the mixing region. The 1/e intensity contour of the laser beam is illustrated along with the cone of fluorescence emission collected by the microscope objective. Actual measurements were made at distances ≥100 μm from the center inlet channel. Because the flow velocity at the vertical center of the observation channel is about 1 μm ms^{-1}, this corresponds to times ≥0.1 s.

A 488-nm laser was used to excite sample molecules in the mixer (11), and the resulting fluorescence was collected with an optical system identical to that used by Schuler et al. (9). The beam was positioned downstream of the mixing region at distances chosen to correspond, via the flow rate, to the desired delays. Counts from the detectors were collected in 1-ms intervals as individual molecules passed through the focal volume.

The molecule studied here is the cold shock protein (Csp) from the bacterium Thermotoga maritima (11), a small β-barrel protein that exhibits two-state thermodynamics and kinetics (13, 14). Csp was labeled with a green fluorescent donor dye (Alexa Fluor 488, Molecular Probes, Eugene, Oregon) and a red fluorescent acceptor (Alexa Fluor 594) via amino- and carboxyl-terminal cysteine residues (9, 11).

Representative FRET efficiency histograms measured at various times after dilution of denaturant to trigger folding are shown in Fig. 2A. The maximum at high transfer efficiency corresponds to folded molecules. For this subpopulation, excitation of the donor dye results in rapid energy transfer to the acceptor because the termini of the protein are separated by only 1 nm in the native structure (15). Most of the fluorescence photons are therefore emitted by the acceptor. A second maximum occurs at a transfer efficiency of about 0.5. This is produced by unfolded protein, for which the average distance between the dyes is greater. Consequently, the energy transfer rate is decreased, and the fraction of photons emitted by the acceptor is reduced.

After mixing, a redistribution of the populations is observed. As the number of un-

![Comparison of single-molecule and ensemble folding kinetics](chart)

**Fig. 2.** (A) Histograms of measured FRET efficiency, $E_m$, during folding. (Top) The $E_m$ distribution at equilibrium before mixing. The vertical red line indicates the mean value for $E_m$ in the unfolded state after mixing. Measurements of $E_m$ were taken (typically for 30 minutes) at various distances from the mixing region and thus at various times after the change in GdmCl concentration. Data collection and analysis were carried out as previously described (9, 11). (B) Comparison of single-molecule and ensemble folding kinetics (11). The decrease in donor fluorescence from stopped-flow experiments is shown in green. Single-molecule data are represented by filled circles, with a corresponding single exponential fit (black). The fit to the single-molecule data has a rate constant of $6.6 \pm 0.8$ s^{-1}, in agreement with the result from ensemble measurements ($5.7 \pm 0.4$ s^{-1}). Measurements of Csp unfolding are shown in fig. S2.

![Dependence of mean values](chart)

**Fig. 3.** Dependence of (A) the mean values ($\langle E_m \rangle$) and (B) peak widths (σ) of $E_m$ histograms for unfolded Csp as a function of GdmCl concentration. Peak widths are standard deviations of Gaussian fits. Data were obtained from single-molecule measurements in the microfluidic mixer. The dotted lines show a third-degree polynomial fit to the data (A) or indicate their mean value (B). The colored region indicates the range of denaturant concentrations where reliable data cannot be obtained from corresponding equilibrium experiments.
folded molecules decreases, there is a corresponding increase in the number of folded molecules (Fig. 2A), and eventually the distribution converges to its equilibrium form at the final concentration of denaturant. The positions of the peaks, however, remain constant, indicating that the average end-to-end distances of the molecules in the subpopulations do not change significantly during the course of the reaction. This is the behavior expected for a two-state system, as inferred for this protein from ensemble methods (13, 14). The fidelity of the technique for measuring accurate kinetics is demonstrated in Fig. 2B, which shows that nearly identical folding rates are obtained from single-molecule data and ensemble-stopped-flow measurements.

Whereas the peak positions are constant during the folding reaction, the mean FRET efficiency of unfolded molecules is shifted from 0.51 in the presence of 4 M guanidinium chloride (GdmCl) to 0.64 after mixing is complete and solution conditions are conducive to folding (Fig. 2A). The lower denaturant concentration after dilution results in complete and solution conditions are conducive to folding and ensemble-stopped-flow measurements.

Exonuclease Reveal Base Dependence and Dynamic Disorder

Antoine M. van Oijen,1 Paul C. Blainey,1 Donald J. Crampton,2 Charles C. Richardson,2 Tom Ellenberger,2 X. Sunney Xie1*  

We used a multiplexed approach based on flow-stretched DNA to monitor the enzymatic digestion of λ-phage DNA by individual bacteriophage λ exonuclease molecules. Statistical analyses of multiple single-molecule trajectories observed simultaneously reveal that the catalytic rate is dependent on the local base content of the substrate DNA. By relating single-molecule kinetics to the free energies of hydrogen bonding and base stacking, we establish that the melting of a base from the DNA is the rate-limiting step in the catalytic cycle. The catalytic rate also exhibits large fluctuations independent of sequence, which we attribute to conformational changes of the enzyme-DNA complex.

Recent advances in single-molecule enzymatic assays have profoundly changed how biochemical reactions are studied (1–7). With the removal of ensemble averaging, distributions and fluctuations of molecular properties can be characterized, transient intermediates identified, and catalytic mechanisms elucidated. Here, we report a single and multiplexed single-molecule assay for studying the mechanisms and dynamics of nucleic acid enzymes.

Individual DNA molecules are attached at one end to a glass surface by a biotin-streptavidin linkage and at the opposite end to polystyrene beads by a digoxigenin-antidigoxigenin linkage (8). When a laminar