Supporting Online Material for Single Molecule Measurement of Protein Folding Kinetics

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Materials and Methods

Protein

Figure S1 illustrates the dye-labeled cold shock protein (Csp) from *Thermotoga maritima*.

Microfluidic Mixer

A pattern of channels (Fig. 1) was cut into the surface of a silicon wafer using reactive ion etching. The channel depths varied from 8 μm to 50 μm, and the widths were between 5 μm and 50 μm. The observation channel, at one end of which the solutions were mixed, was

8 μm deep, 50 μm wide, and 10 mm long. Hash marks were etched adjacent to the observation channel at 50 μm intervals so that the distance from the mixing region could be accurately determined.

The concentration profiles shown in Figure 1 were modeled using pressure-determined steady-state laminar flow and sample and denaturant diffusion constants of 10^{-6} cm^2/s (= 0.1 μm^2/ms) and 10^{-5} cm^2/s (= 1 μm^2/ms), respectively. Computations were done using CFD-ACE+ software (CFDRC, Huntsville, AL).

Fluid entry to and exit from the mixer took place through four pyramidal holes, etched (using KOH) through the silicon wafer at the ends of the three inlet channels and the outlet channel. The mixer chip was clamped by its edges to a fixture that provided face-type static O-ring seals to the holes via the bottom side of the chip. For folding, a 50 mM pH 7 phosphate buffer solution was introduced in the two outer inlet channels, and a solution containing 75 pM labeled Csp, 4 M guanidinium chloride (GdmCl, Pierce Sequanal Grade), 0.01% polyoxyethylene sorbitan monolaurate (Pierce Surfact-Amps 20, "Tween 20") by volume (to prevent protein adhesion to the mixer walls), and 50 mM pH 7 phosphate buffer was placed in the center inlet channel. The pressure of the three inlets was held at 6.9 kPa above that of the outlet using a precision mechanical regulator. This resulted in a flow rate at the center of the observation channel of approximately 1 mm/s. The three inlet channels terminate at the upstream end of the observation channel (Fig. 1), where the solutions are mixed.

A 170 μm-thick 7740 Pyrex coverslip was anodically bonded to the top of the silicon device in order to seal the channels and provide a transparent window for laser excitation and fluorescence collection. Although Pyrex is well-suited for bonding directly to silicon, it produces roughly an order of magnitude more fluorescence background than an equal thickness of fused silica. This introduces additional uncertainty in the absolute determination of transfer efficiencies. A mixer fabricated from fused silica would therefore be very desirable for future

work of this type.

Mixer function was confirmed by tests that measured unfolding of labeled Csp (Fig. S2).

Data Analysis

Comparisons of the low-count tails of many raw signal histograms with Poisson distributions were used to determine the ratio of the background level to the average signal level. This number (≈ 0.75 in the present experiments) was found not to vary substantially from measurement to measurement or between the two channels. The ratio was then used to estimate the background level in each channel for a given data set. Background levels obtained in this manner were consistent with those found during manually identified intervals when no molecule was present in the focal region. Although this method is only a first-order approximation, it gives more accurate results than would a measurement of blank control solution since much of the background is caused by out-of-focus sample molecules.

After background subtraction, the sums of donor counts (n_{d}) and acceptor counts (n_{a}) for each single molecule event were used to compute measured values of the energy transfer efficiency $E_{\mathrm{m}} = \frac{n_{\mathrm{a}}}{n_{\mathrm{a}}+\gamma n_{\mathrm{d}}}$, where γ is a factor that accounts for differences in the fluorophore quantum yields and the detection efficiencies of the two channels. Based on calibration measurements (*1*), we set $\gamma = 1$. The actual transfer efficiency E (as opposed to the measured value E_{m}, which is broadened by many sources of uncertainty), can be related to the distance r between the fluorophores using Förster's theory (*2*): $E = \frac{1}{1+(r/R_0)^6}$

Ensemble Measurements

Ensemble folding kinetics were measured in a DX.18MV sequential mixing stopped-flow spectrometer (Applied Photophysics, Leatherhead, UK) with an R6094 photomultiplier tube (Hamamatsu, Bridgewater, NJ) by following the change in donor fluorescence beyond 495 nm upon

excitation at 450 nm. To initiate refolding, a 1 μM solution of labeled protein unfolded in 4 M GdmCl was diluted 11-fold with aqueous buffer or GdmCl solutions of varying concentrations to give the desired final GdmCl concentrations. Five to fifteen kinetic traces were averaged and fit to single exponentials.

Relative populations measured in the single molecule experiments can be distorted, for example by the smaller diffusion constant of protein in the unfolded state, which leads to a disproportionately large number of events in the unfolded data peak. A comparison of the ensemble equilibrium unfolding transition with single molecule equilibrium FRET efficiency histograms was used to obtain the actual fraction of native protein at each time during the folding experiment. Ensemble donor and acceptor fluorescence were measured as functions of equilibrium denaturant concentrations. As denaturant concentration increases, donor fluorescence increases and acceptor fluorescence decreases. The midpoints of the donor and acceptor transitions coincide, and measured intensities were fit with a two state model (*1*) in order to find the folded and unfolded populations at given denaturant concentrations. Equilibrium single-molecule transfer efficiency distributions were then examined to see what peak height ratio corresponded to the given population ratio from the fit.

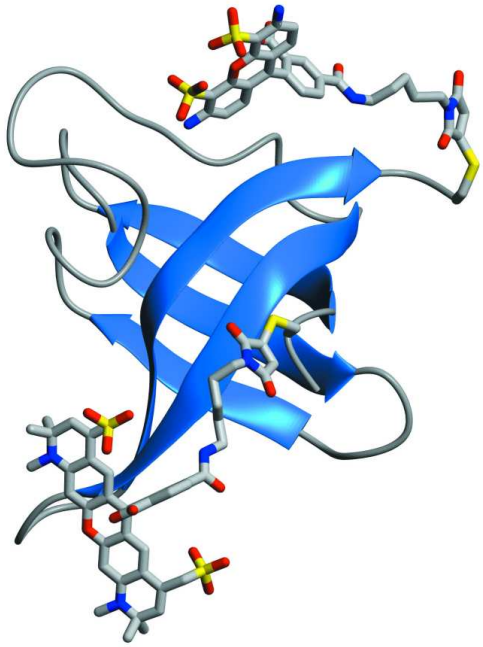

Figure S1: Representation of Csp based on the solution NMR structure (*3*, *4*). The protein is labeled with fluorescent donor and acceptor dyes at the sulfhydryl groups of the terminal Cys residues. Alexa Fluor 488 (donor, top) and Alexa Fluor 594 (acceptor, bottom) are shown here in arbitrary orientations. In the native state, the termini of the protein are separated by 1 nm.

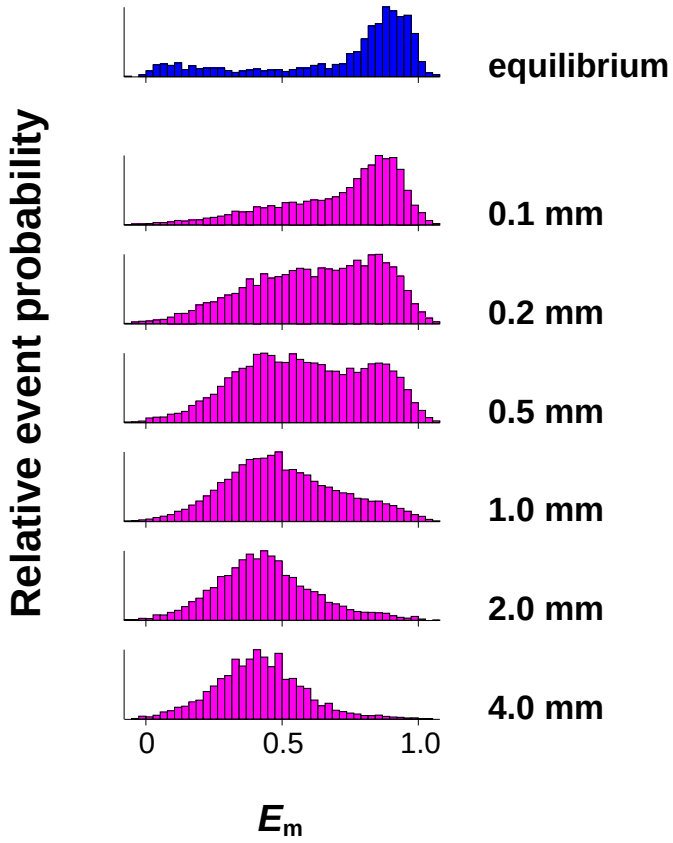

Figure S2: Histograms of measured FRET efficiency E_{m} during unfolding. A solution of labeled Csp in phosphate buffer (center inlet channel) was mixed with 8 M GdmCl (two outer inlet channels), and observations were made at the indicated distances from the mixing region. Unfolding is clearly evinced by the increase in event probability at lower transfer efficiency (greater dye-dye separation) as the reaction progresses.

References and Notes

1. B. Schuler, E. A. Lipman, W. A. Eaton, *Nature* **419**, 743 (2002).

2. B. W. VanDerMeer, G. Coker, S. Y. S. Chen, *Resonance Energy Transfer: Theory and Data* (Wiley, 1994).

3. W. Kremer, *et al.*, *Europ. J. Biochem.* **268**, 2527 (2001).

4. R. Koradi, M. Billeter, K. Wüthrich, *J. Mol. Graphics* **14**, 51 (1996).