Supporting Information

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SI Methods

Protein Purity and Concentrations Used. The purity of the proteins as assessed by SDS/PAGE was >95%. The mass of each protein was confirmed by mass spectrometry using a Micromass Q-TOF Ultima instrument. Protein concentration was determined by the measurement of the absorbance at 280 nm. The protein concentration used was 20–25 μM for the kinetic studies in which CD, FRET, and ANS were used as the probes. For the fluorescence-monitored kinetic studies, the protein concentration used was 5–10 μM. In all of the equilibrium measurements, the protein concentration used was 5–10 μM. All of the buffers used were of the highest purity grade obtained from Sigma. The stock urea concentration was determined by measurement of the refractive index on an Abbe refractometer.

Equilibrium Unfolding Experiments. The sample was excited at 295 nm, and the intrinsic Trp fluorescence emission was collected at 320 nm, or at 380 nm in the case of FRET-monitored experiments (1). The excitation and the emission bandwidths were 0.3 nm and 10 nm, respectively. The far-UV CD-monitored equilibrium unfolding transition at 222 nm was acquired on the SFM-4 module with a polarizer accessory (see below). The data were analyzed by using a two-state N⇌U model (2).

Stopped-Flow Kinetic Refolding Experiments. For far-UV CD-monitored kinetic experiments, a polarizer assembly coupled to a photoelastic modulator unit (Hinds Instruments) was attached to the SFM-4 device. The data were collected by using a PMS 400 unit. Far-UV CD was measured at 222 nm with a $\lambda/4$ retardation set on the photoelastic modulator. All of the fluorescence-monitored kinetic experiments were done with a FC-08 cuvette (dead time = 1.8 ms). For the FRET- and CD-monitored kinetic experiments, FC-15 and FC-20 cuvettes were used, with dead times of 6.2 ms and 9.0 ms, respectively. In all measurements of fluorescence, the sample was excited at 295 nm. Measurements of fluorescence in the absence of FRET were made at 320 nm using a bandpass filter (Oriel). In the FRET experiment, the quenching of the fluorescence from the donor Trp was monitored by using a 387-nm bandpass filter (Semrock). In the case of ANS fluorescence-monitored kinetic experiments, the fluorescence emission from ANS was acquired by using a 450-nm bandpass filter.

Continuous-Flow Kinetic Refolding Experiments. ***ANS-fluorescence-monitored kinetics.*** Both the native buffer and the unfolding buffers contained the same concentration of ANS. The ANS concentration used was 1 mM for the experiments done with 20 μM protein. The sample was excited at 295 nm, and the change in ANS fluorescence due to FRET from the Trp residue was monitored by using a 450-nm bandpass filter.

The relative intensity profile was obtained with respect to the fluorescence intensity profile of a 25 μM solution of NATA by using the following equation: $S_{rel} = (S_x - S_{buf})/(S_{NATA} - S_{buf})$, where S_{NATA} is the fluorescence of the NATA solution. The intensity profile so obtained was fit to a single exponential equation. The millisecond data shown in Fig. 2 were acquired under identical conditions on a SFM-4 device. For a direct comparison of the stopped-flow and the continuous-flow data, both of the data sets were normalized by using the fluorescence signal of a 25 μM NATA solution under identical conditions.

FRET-monitored kinetics. A relative intensity profile was used for the final analysis and was obtained as follows: $S_{rel} = (S_x - S_{buf})/(S_u - S_{ub})$. Here S_x is the signal intensity from a given pixel, S_{buf} is the corresponding value of the signal intensity of the buffer only, S_u is the signal of the unfolded protein from the same pixel, and S_{ub} is the corresponding signal of the unfolding buffer. The sample was excited at 295 nm, and the emission was collected by using a 387-nm bandpass filter (Semrock). The obtained intensity profile was fit to an exponential equation. The millisecond data shown in Fig. 2 were acquired under identical conditions on a SFM-4 device.

Construction and Operation of the Continuous-Flow Mixer. To directly follow the kinetics of the initial folding reaction in the sub-ms time domain, a continuous-flow microsecond mixing device was constructed. Fig. S1 illustrates an outline of the basic design of the microsecond mixer assembly. The design principle of the mixer is based on the generation of turbulence by flowing two liquids through a narrow orifice (3, 4). At the heart of the mixer is a 150-μm-wide and 50-μm-deep microchannel etched on a flat surface of a stainless steel block (Fig. S1*a*). The two liquids to be mixed are pumped from either end of the microchannel to meet at its center, from where the mixed solution enters into a flow cell. The reaction ages as the mixed solution flows down the 2-cm length of the flow cell. The fluorescence image of the flow cell is acquired by using a CCD camera (2,048 × 512 pixels). Of 512 pixels along the *y* axis of the CCD chip, the fluorescence signals from only the central 5–15 pixels are integrated, which ensures that fluorescence emission only from the middle 70–200 μm of the flow cell is recorded.

To determine the dead time of the continuous flow mixer, the kinetics of the quenching of *N*-acetyl tryptophanamide (NATA) fluorescence by *N*-bromo succinamide (NBS) were measured. This was carried out under pseudo-first-order conditions where the concentration of NBS was kept in >10-fold excess over that of the NATA (Fig. S1*b*). The bimolecular rate constant obtained from the slope of the pseudo-first-order rate constant versus [NBS] plot (Fig. S1*c*) was determined to be 5.2×10^5 $M^{-1}s^{-1}$, which is in good agreement with the value reported earlier (5). The mixing dead time corresponds to the time difference between the position of the pixel corresponding to the starting point of the reaction (taken as the pixel number where the fits through all of the kinetic traces extrapolate to the fluorescence signal of the unquenched NATA) and the pixel where the first reliable artifact-free data are obtained (Fig. S1*b*). The dead time at a flow rate of 1.16 ml/s is 100 ± 10 μs, and that at 1 ml/s is 120 ± 10 μs.

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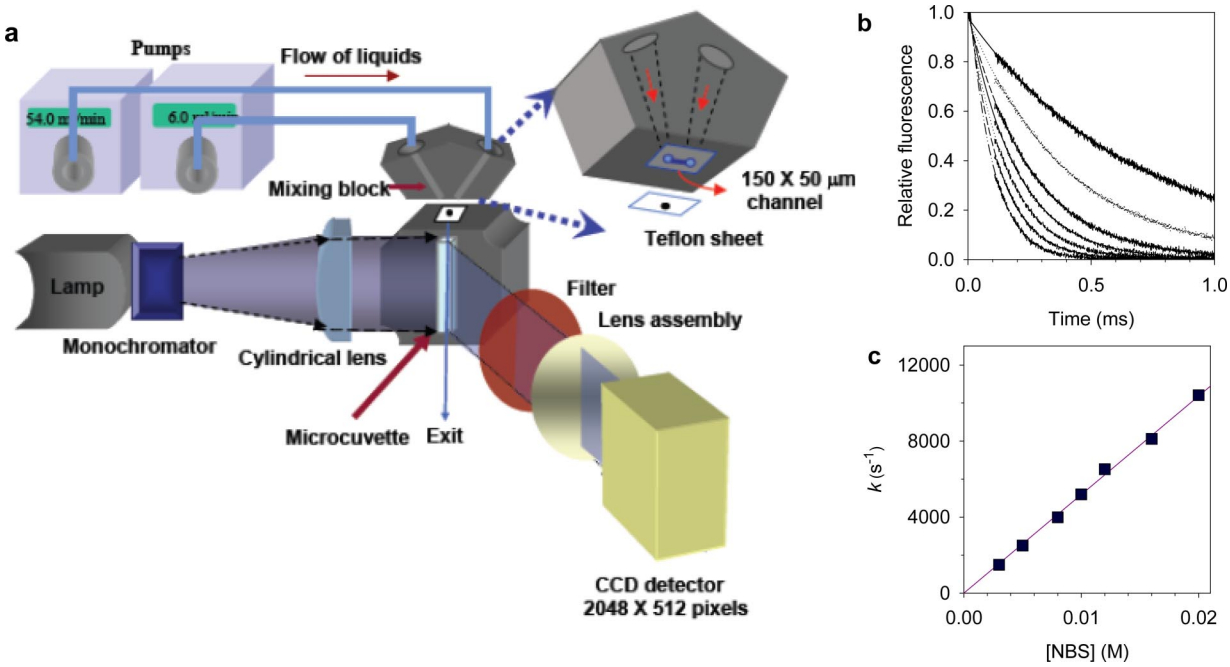

Fig. S1. The continuous-flow microsecond mixer. (*a*) Design of the microsecond mixer. (*b*) Determination of the mixing dead time. The reaction kinetics of the quenching of the fluorescence of a 50 μM NATA solution were measured in different NBS concentrations ranging from 3 mM (top trace) to 20 mM (bottom trace). (*c*) The pseudo-first-order rate constants (■) obtained from fitting the kinetic traces to a single exponential equation are plotted against the corresponding NBS concentrations. The slope of this plot yields a value of 5.2×10^5 $M^{-1}s^{-1}$ for the bimolecular rate constant for the quenching reaction of NATA fluorescence by NBS.